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**EDITING**  
Editorial Board of *World Journal of Gastroenterology*, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China  
Telephone: +86-351-4078656  
E-mail: wjg@wjgnet.com

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# State-of-the-art of irritable bowel syndrome and inflammatory bowel disease research in 2008

Lynne V McFarland

Lynne V McFarland, Department of Health Services Research and Development, VA Puget Sound Health Care System, Metropolitan Park West, 1100 Olive Way, Suite 1400, Seattle WA 98101, United States

Lynne V McFarland, Department of Medicinal Chemistry, School of Pharmacy, University of Washington, Seattle WA 98101, United States

Supported by Veterans' Affairs Health Services Research & Development

Correspondence to: Lynne V McFarland, PhD, Department of Health Services Research and Development, VA Puget Sound Health Care System, Metropolitan Park West, 1100 Olive Way, Suite #1400, Seattle WA 98101,

United States. [lynne.mcfarland@va.gov](mailto:lynne.mcfarland@va.gov)

Telephone: +1-206-2771095 Fax: +1-206-7642935

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## Abstract

Irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) are two of the leading causes of chronic intestinal conditions in the world. This issue of *World Journal of Gastroenterology (WJG)* presents a series of papers from world experts who discuss the current knowledge and opinions on these important conditions. Although great strides have been made in the diagnosis, treatment and pathology of IBS and IBD; much has yet to be explained. The etiologies and risk factors of these multifactorial conditions remain elusive. Specific diagnostic biomarkers need to be developed and safer treatments developed. The burden of IBS and IBD on the healthcare system is felt with repeated medical care visits and high costs. IBS and IBD patients can account for 30%-50% of office visits at gastroenterology services/clinics. Over one million people have IBD in the United States, with 30000 new cases being diagnosed every year. One-quarter million people in the UK are afflicted with IBD. The cost of medical care in the United States for IBD is estimated to be \$1.8 billion/year.

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**Key words:** Irritable bowel syndrome; Inflammatory bowel disease

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## IRRITABLE BOWEL SYNDROME (IBS)

### Incidence of IBS

IBS is a global problem and is more common in women than men. In developed countries, the prevalence of IBS ranges from 3%-25% of adults<sup>[1-4]</sup> and in the United States, IBS affects 15 million adults<sup>[5]</sup>. In the United Kingdom, IBS affects 10%-15% of the adult population<sup>[6]</sup>. The cost of direct and indirect medical care for IBS reached over \$200 billion dollars in the United States<sup>[5,7]</sup>.

### Diagnosis of IBS

As there are no biologic markers for IBS, the diagnosis is usually based on symptoms and exclusion of other known causes of intestinal distress<sup>[6]</sup>. Unlike IBD, IBS does not cause severe inflammation, ulcers or other structural damage that aids the diagnosis of IBD. IBS is a functional disorder characterized by abdominal bloating, flatulence, abdominal pain and bowel dysfunction. The varying nature of symptoms and lack of structural abnormalities presents a diagnostic challenge. There are three main types of disease phenotypes: diarrhea-predominant (IBS-D), constipation-predominant (IBS-C) or alternating diarrhea-constipation (IBS-A). The diarrhea-predominant type is more common (48%) in males, whereas constipation-predominant (39%) or alternating types (48%) are more common in women<sup>[1]</sup>. Several tools (for example, Rome III, Manning criteria) have been developed to standardize the diagnosis of IBS. Most of the historic research has been focused on the pathophysiology of diarrhea, but constipation has not been as well described. The review by McCrea and colleagues in this issue summarizes our current knowledge about the physiology and pathology of constipation<sup>[8]</sup>. The prevalence of constipation ranges from 15%-25% in the general population and is more common in women than men and in ages over 70 years old<sup>[9]</sup>. The typical definition of constipation (less than three stools/week) may not be a sensitive measure for this condition, as individuals vary widely in their own bowel habits. An interesting finding in this review is that physicians and patients define constipation differently. Neurotransmitters are important in the pathophysiology of IBS and this paper reviews how neurotransmitters are involved in the normal and abnormal

function, and anatomy of the gastrointestinal tract. Age-related anatomical changes seem to have minimal impact on normal colonic function, which might help to explain why increasing age is not a significant risk factor for IBS. McCrea *et al* then describe the association of dysmotility on the pathology of IBS-C. This review of constipation highlights the need to conduct further research into the etiologies and functional causes of constipation, especially as it relates to disease conditions such as IBS.

### Consequences of IBS

Patients with IBS report a significant reduction in quality of life and sexual function and have increased suicide ideation, absenteeism, have higher rates of depression and are heavy users of medical care<sup>[6,10]</sup>.

### Pathogenesis

IBS is a multifactorial condition and the pathophysiology may involve a triad of factors: altered intestinal motility, psychosocial factors and heightened sensory function. Risk factors include genetic factors, food allergies and microbial dysbiosis<sup>[11]</sup>. The low grade mucosal inflammation, altered motility and altered bowel microflora give rise to the characteristic clinical symptoms of IBS.

### Treatment for IBS

Current treatments for IBS target the patient's predominant symptoms at the time of the acute episode and the efficacy of these treatments vary widely<sup>[4]</sup>. Hammerle and Surawicz review the challenges of treating patients with IBS<sup>[12]</sup>. The effectiveness of conventional therapy for IBS varies due to the need to treat different types of symptoms (IBS-D, IBS-C or IBS-A) and the need to limit underlying etiologic triggers, which are commonly unknown. The authors point out the need for individualized treatment regimes, as symptoms and treatment responses vary widely. The chronic nature of IBS necessitates life-long treatments that may need adjustments reflective of shifting clinical presentations. Hammerle and Surawicz also review the role that serotonin has on the function of the intestinal tract and how decreased levels of neurotransmitters are involved in the pathology of IBS. Pharmacotherapy directed at modulating neural transmitters offer a promising class of treatments for IBS. 5-HT<sub>4</sub> antagonists may be an effective choice for IBS-D as they slow intestinal transit, increase stool firmness and reduce intestinal secretion. In contrast, 5-HT agonists (such as tegaserod) are more effective for IBS-C. Octreotide has been tested in human volunteers and slows diarrheal symptoms, but is only available intravenously, limiting its usefulness. Other types of antagonists are reviewed, but clearly more randomized clinical trials are needed. Other types of treatments (including antidepressants, antispasmodics, antibiotics, fiber, probiotics and dietary changes) are reviewed and may be effective in some patients, but more research is needed for these types of treatments as well.

As patients with IBS have been shown to have disrupted intestinal microflora and some episodes of IBS are triggered by gastroenteritis, a treatment strategy that involves microbial replacement is attractive<sup>[13]</sup>. Probiotics are beneficial microbes that are given to restore normal microflora and have

been shown to be effective for other types of diarrhea (antibiotic-associated diarrhea, *Clostridium difficile* disease, traveler's diarrhea and pediatric diarrhea)<sup>[14,15]</sup>. The meta-analysis by McFarland and Dublin explores the efficacy of various probiotics for the treatment of IBS<sup>[16]</sup>. The results from 20 randomized clinical trials with 23 different probiotic treatment arms were compared to controls. Generally, probiotics were found to significantly reduce IBS symptoms globally [pooled odds ratio (OR), 0.78; 95% confidence interval (95% CI), 0.62-0.94]. However, no one type of probiotic had sufficient numbers of confirmatory trials to conclude one type of probiotic was more effective than another probiotic. Probiotic trials in the past have been directed by other types of diarrheal disease, but probiotic treatment for IBD and IBS is receiving renewed attention. This review is a call to arms for researchers interested in this area. Larger clinical trials are needed in the future in order to have sufficient power to detect significant differences between the treatment groups, multiple confirmatory trials using the same strain of probiotic are needed and a consensus on a common outcome measure is needed. Many of the trials of IBS used different outcome measures, some measuring a global response (no relapses of disease), some measuring a reduction in symptom scores and some creating their own individual outcome measures. Obviously, this makes comparing different study results challenging. Despite these limitations, probiotics may offer a safe and effective strategy for patients with IBS. More clinical trials are needed.

## IBD

### Incidence

Several intestinal conditions are under the umbrella of "Inflammatory Bowel Disease (IBD)", including Crohn's disease, ulcerative colitis and pouchitis. IBD, once considered a disease of industrialized countries, is now reported globally. The highest incidences (8-66/100 000 population) of Crohn's are found in Wales, New Zealand, Canada, Scotland, France, the Netherlands and Scandinavia<sup>[17]</sup>. Other industrial countries such as in the United Kingdom, the United States and in Europe have intermediate rates ranging from 4-7/100 000<sup>[17,18]</sup>. Historically, IBD was infrequently reported in developing countries, but currently low incidence rates are reported (0.2-3/100 000) in such countries like Brazil, China, Korea, Greece, Japan, Malta and Slovakia<sup>[19]</sup>. The incidence in these countries has increased in recent years, whether it is due to an actual increase in the number of cases or better diagnostic and detection methods is not known<sup>[18]</sup>. Interestingly, there is a north-south gradient in Europe, with more severe disease in northern European countries<sup>[17]</sup>. Recent studies have found that Crohn's disease is more common in young patients. Most (74%) of in one study were under 30 years old and the typical age of onset is usually 15-30 years of age<sup>[18]</sup>. Gender differences are not consistent across the country. More men than women are diagnosed with Crohn's disease in China, in contrast to the United States, where more women than men have Crohn's<sup>[18]</sup>.

### Diagnosis

As there are no standard biomarkers for IBD, the diag-

nosis of Crohn's disease and UC are typically made based on clinical symptoms, endoscopic and histologic findings. Variances in symptom types and frequency and a lack of structural abnormalities observed upon endoscopic examination typically may delay the diagnosis of IBD for 6 mo to 1 year<sup>[18]</sup>. The clinical presentation of Crohn's disease is abdominal pain, diarrhea and weight loss, while patients with UC most often complain of abdominal pain, bloody diarrhea and stool mucus<sup>[18,20]</sup>. Crohn's disease results in inflammation, deep fistulas or abscesses anywhere along the gastrointestinal tract, but most commonly along the ileocolon. Colonoscopic examination of patients with ulcerative colitis shows pathology is limited to the large colon with surface inflammation and left-sided colitis, proctitis and proctosigmoiditis being most common<sup>[20]</sup>. Recent innovations in diagnostic techniques including noninvasive imaging techniques and more sensitive endoscopic equipment may improve the diagnosis of IBD.

### Consequences of IBD

Complications for Crohn's disease are frequent (40%) and include lower gastrointestinal bleeding, intestinal obstruction, perforation and the need for surgery<sup>[18]</sup>. Mortality in IBD patients is generally low (about 6%) with some studies finding IBD significantly increases the risk of mortality (OR, 1.4; 95% CI, 1.2-1.6) compared to non-IBD patients. The risk of mortality is higher for patients with Crohn's are compared with patients with UC<sup>[21]</sup>. In studies that have followed large numbers of UC patients for at least ten years, 70%-100% suffered at least one relapse of UC<sup>[22]</sup>. In patients enrolled in clinical trials and randomized to placebo, relapse rates for Crohn's disease ranged from 10%-60% and 11%-90% of UC patients relapsed<sup>[23]</sup>.

Another important consequence of UC may be a higher risk of colorectal cancer. Whether precancerous lesions are an etiologic factor for IBD or whether chronic intestinal inflammation increases the risk of colon cancer has been debated. Zisman and Rubin review the epidemiology of cancer and dysplasia in IBD patients<sup>[24]</sup>. Using historical data, the cumulative incidence of colorectal cancer increased by the duration of UC, with highest rates present after 30-40 years of UC. However, this increase may be an example of a period-cohort bias, as increased colonoscopy in the younger patients (with shorter durations) may have reduced cancer rates. The authors explore the possible risk factors for colorectal cancer in UC patients. The degree of inflammation seems to correlate with increased risk of colorectal cancer. Further research may be needed to prospectively document inflammatory biomarkers and then follow patients for the development of cancer. Other risk factors are discussed, including family history of colon cancer, primary sclerosing cholangitis and strictures, but the weight of evidence is weak for these factors. The link between colorectal cancer and Crohn's disease is less clear, as many have no colonic involvement. Despite this, the incidence and risk factors for Crohn's disease patients and colon cancer are remarkably similar to UC. The importance of colonoscopy surveillance is highlighted. Dysplasia, thought to be an intermediate step between chronic inflammation and carcinoma, is also reviewed by these authors. The unpredictable course of not

only dysplasia, but IBD itself, complicates the determination of the role of molecular markers and mutations. It becomes more paramount that methods for prevention are pressed into clinical use. Evidence that prophylactic chemotherapy is effective in reducing colorectal cancer is not conclusive. Colonoscopy remains the most recommended preventive method for preventing colorectal cancer and its use should be encouraged. Innovations in novel imaging technology may increase the detection of early stages of cancer in IBD patients. Despite the scarcity of research in some areas in this field, it seems likely that the chronic inflammation insult to the colon present in IBD patients may increase the risk of colorectal cancer.

### Pathogenesis

While research has uncovered some of the risk factors for Crohn's and UC, much about the etiology of these two conditions remains unknown. The pathogenesis of IBD may involve four major areas: it appears to be immunologically mediated, microbial dysbiosis is usually present, environmental factors trigger symptoms and genetic predispositions may play an important role<sup>[25]</sup>. Proinflammatory cytokines are produced during IBD episodes and altered immune response is common in both Crohn's disease and UC. Microbial dysbiosis has been documented in patients with IBD<sup>[26]</sup>. The 'hygiene hypothesis' postulates that decreased exposure to environmental microbes due to increased disinfectant use in some industrialized cultures may alter the development of the immune system early in life<sup>[27]</sup>. However, there is only indirect evidence for this correlation and this hypothesis remains unproven. Several bacterial candidates, including *Mycobacterium avium paratuberculosis* (MAP), have been investigated as potential etiologies for IBD, but the research has been inconclusive<sup>[28]</sup>. It is thought that inappropriate or exaggerated mucosal immune response to enteric infections may be involved in the initial etiology of some cases of IBD. In infants less than one year old who developed IBD, 50% had a prior bacterial infection requiring antibiotic therapy<sup>[29]</sup>. Although as intriguing as this finding is, more research is needed to determine if enteric infections cause IBD.

Environmental triggers for IBD may include smoking, diet, stress, gallstones, surgery and exposure to microbes. In one study, the risk of Crohn's disease was significantly elevated (OR, 35;  $P < 0.05$ ) if the patient smoked, had a sibling with Crohn's and carried at least two CARD15 genes<sup>[30]</sup>. In contrast, the average age of patients developing ulcerative colitis was older (mean  $44 \pm 15$  years) and there are no large differences by gender<sup>[20]</sup>. Other risk factors for UC include prior smoking history (but not current smoking), family history of UC and high body weight (elevated BMI)<sup>[25]</sup>.

The carriage of susceptibility genes such as CARD15/NOD2, IBD5, DLG5, IL23R and ATG16LI are associated with increased rates of Crohn's disease in developed countries, but interestingly are not associated with an increase in Crohn's in Asian countries<sup>[17,30,31]</sup>.

### Treatment for IBD

Without an exact reason for the etiologies of Crohn's disease and UC, finding effective treatments are challenging. As a hyper-immune response plays an important part in



IBD, immunomodulators and immune-suppressives have been used as a standard treatment for IBD. Corticosteroids and 5-aminosalicylates have been the traditional treatments for IBD, although many patients do not respond to these treatments or develop serious adverse effects during prolonged use, including increases in serious infections, reactivation of tuberculosis, development of lymphoma or demyelinating disease<sup>[20]</sup>. Currently, there are two main strategies for the treatment of Crohn's disease: the top-down or the step-up approach. Shergill and Terdiman review the controversies and suggest another approach to the treatment of Crohn's disease<sup>[32]</sup>. The top-down approach starts patients with newly diagnosed disease with the newer immunomodulators and biologic agents. The step-up approach begins with more conventional treatments (5-aminosalicylates, mesalamine) and then steps up to steroids if those fail. Immunomodulators are started only after the other treatments have failed. Symptom abatement with the least toxic drug is the guiding principal of the step-up approach. Shergill and Terdiman reassess this paradigm and conclude using a more aggressive therapy earlier in the disease may limit irreversible damage to the bowel, preventing future hospitalizations, surgeries and disabilities. Challenges inherent in determining the most effective treatment strategy include the varying nature of the symptoms, the subjective nature of many of the outcome measures and the lack of correlation between mucosal healing and the Crohn's disease activity index (CDAI), commonly used to determine treatment response. The authors point out treatments that heal the mucosa are often only started after irreversible damage has been done. Steroids are effective in rapidly suppressing flares, but have no benefit on the underlying damage to the mucosa. In contrast, immunomodulators take longer to reduce symptoms, but are able to induce mucosal healing and are able to maintain remissions for a longer time. Both steroids and immunomodulators have side effects which complicates treatment. These authors propose a new hybrid approach described as an "accelerated step-up" approach. Patients presenting with mild symptoms should be treated with mesalamine, antibiotics or budesonide to quickly reduce symptoms. If symptoms do not resolve or if the patient relapses, immunomodulators are then started. Since these agents take 2-4 mo to heal the mucosa, short-term steroids are added in the early months of therapy (unless contra-indicated by fistula or perforations). Once remission is achieved, immunomodulators could be tapered off. If none of these are effective, biologics such as anti-TNF drugs can be tried. The bottom line is that treatment of Crohn's disease is not easy and must be tailored to the individual patient and be constantly adjusted if recurrences happen. The review by Shergill and Terdiman provides a thoughtful assessment of different therapeutic choices and a balanced discussion of the benefits and risks of each treatment choice.

The development of antibiotic resistance presents an additional challenge in the treatment of patients with IBD. Beckler and co-authors describe a new PCR method to detect rifabutin and rifampicin resistance in *Mycobacterium avium paratuberculosis* (MAP) strains<sup>[33]</sup>. As mentioned earlier, MAP is thought to be one of the etiologic agents of Crohn's disease. Proponents of this hypothesis cite

the lack of antibiotic response when patients are treated. However, the poor response may be due to antibiotic-resistance rather than the lack of an association between MAP and Crohn's disease. Beckler *et al* found mutations associated with increased antibiotic resistance were located on the *rpoB* gene of MAP. They developed a PCR tool to detect this antibiotic resistance and this tool provides a rapid method to detect MAP infection in IBD patients. Classic microbiologic methods of culturing MAP are slow and insensitive. The addition of this innovative tool may help to gather sufficient evidence to finally determine if MAP is associated with Crohn's disease or not.

If IBD persists and standard treatments fail or if the patient develops serious complications, surgery is often the only available option for the patient. The lifetime risk for colectomy surgery is 70%-80% for Crohn's disease patients and 20%-30% of UC patients<sup>[2]</sup>. In UC patients with colectomy, as many as 50% will develop at least one episode of pouchitis post-operatively<sup>[34]</sup>. The rationale, types of procedures, benefits and risks of surgery are reviewed by Hwang and Varma<sup>[35]</sup>. The paper presents an extensive description of the available types of surgeries depending upon the site and type of disease. Colectomy is typically restorative, but has a high post-operative complication rate (23%-48%) of sepsis or pouchitis. Revision surgery is often required (19%-24%) and 21%-50% of patients suffer remissions even after surgery. Small bowel surgery for patients with Crohn's disease is usually needed due to repeated flares of disease or the development of extra-intestinal manifestations despite medical treatment. Indications for surgery of the colon include treatment failures, dysplasia or colorectal cancer and toxic colitis. Regardless of the site of surgery, recurrences are common (40%-70%). Surgery is unfortunately usually not curative and associated with significant morbidity. This highlights the need to focus research on effective treatments to manage IBD and IBS.

Other alternative therapies being tested include hypnotherapy, herbal medicines and probiotics<sup>[36]</sup>. As prior enteric infections have been associated with the development of IBD, therapies that target or ameliorate the intestinal disruption brought on by antibiotic therapy seem a logical choice. In a survey of 86 children with IBD in Scotland, 44% reported that they used probiotics to control their IBD symptoms<sup>[37]</sup>. The effectiveness of probiotics for IBD has shown mixed results, depending upon the type of probiotic and the condition treated. Evidence from clinical trials found *Saccharomyces cerevisiae boulardii* was effective for Crohn's disease, while *E. coli* Nissle, VSL#3 (a mixture of 8 bacterial strains) and *Lactobacillus rhamnosus* GG were not effective<sup>[23,38]</sup>. Effective probiotics for UC include a mix of *Bifidobacterium* and *Lactobacillus acidophilus*, while other studied strains were not effective. More promising results were reported in five clinical trials when VSL#3 was tested to prevent pouchitis after colectomy surgery<sup>[23]</sup>.

This series of articles presents the challenges that face healthcare providers and patients with IBS and IBD. These chronic conditions place a heavy burden on healthcare institutions and contribute to significant morbidity. Newer treatment strategies may help patients remain in remission longer, but our efforts should be focused on unraveling the etiologies of these diseases so that preventive measures

can be developed that will stop irreversible damage from occurring in the first place, lofty goals, but well worth our efforts.

## REFERENCES

- 1 Lee SY, Kim JH, Sung IK, Park HS, Jin CJ, Choe WH, Kwon SY, Lee CH, Choi KW. Irritable bowel syndrome is more common in women regardless of the menstrual phase: a Rome II-based survey. *J Korean Med Sci* 2007; **22**: 851-854
- 2 Roberts SE, Williams JG, Yeates D, Goldacre MJ. Mortality in patients with and without colectomy admitted to hospital for ulcerative colitis and Crohn's disease: record linkage studies. *BMJ* 2007; **335**: 1033
- 3 Cohen RD, Thomas T. Economics of the use of biologics in the treatment of inflammatory bowel disease. *Gastroenterol Clin North Am* 2006; **35**: 867-882
- 4 Cremonini F, Talley NJ. Irritable bowel syndrome: epidemiology, natural history, health care seeking and emerging risk factors. *Gastroenterol Clin North Am* 2005; **34**: 189-204
- 5 Sandler RS, Everhart JE, Donowitz M, Adams E, Cronin K, Goodman C, Gemmen E, Shah S, Avdic A, Rubin R. The burden of selected digestive diseases in the United States. *Gastroenterology* 2002; **122**: 1500-1511
- 6 Agrawal A, Whorwell PJ. Irritable bowel syndrome: diagnosis and management. *BMJ* 2006; **332**: 280-283
- 7 Foxx-Orenstein A. IBS--review and what's new. *MedGenMed* 2006; **8**: 20
- 8 McCrea GL, Miaskowski C, Stotts NA, Macera L, Varma MG. Pathophysiology of constipation in the older adult. *World J Gastroenterol* 2008; **14**: 2631-2638
- 9 Choung RS, Locke GR 3rd, Schleck CD, Zinsmeister AR, Talley NJ. Cumulative incidence of chronic constipation: a population-based study 1988-2003. *Aliment Pharmacol Ther* 2007; **26**: 1521-1528
- 10 Ladep NG, Okeke EN, Samaila AA, Agaba EI, Ugoya SO, Puepet FH, Malu AO. Irritable bowel syndrome among patients attending General Outpatients' clinics in Jos, Nigeria. *Eur J Gastroenterol Hepatol* 2007; **19**: 795-799
- 11 Barbara G, De Giorgio R, Stanghellini V, Cremon C, Corinaldesi R. A role for inflammation in irritable bowel syndrome? *Gut* 2002; **51** Suppl 1: i41-i44
- 12 Hammerle CW, Surawicz CM. Updates on treatment of irritable bowel syndrome. *World J Gastroenterol* 2008; **14**: 2639-2649
- 13 Barbara G, Stanghellini V, Cremon C, De Giorgio R, Corinaldesi R. Almost all irritable bowel syndromes are post-infectious and respond to probiotics: controversial issues. *Dig Dis* 2007; **25**: 245-248
- 14 McFarland LV. Meta-analysis of probiotics for the prevention of traveler's diarrhea. *Travel Med Infect Dis* 2007; **5**: 97-105
- 15 McFarland LV. Meta-analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of *Clostridium difficile* disease. *Am J Gastroenterol* 2006; **101**: 812-822
- 16 McFarland LV, Dublin S. Meta-analysis of probiotics for the treatment of irritable bowel syndrome. *World J Gastroenterol* 2008; **14**: 2650-2661
- 17 Economou M, Pappas G. New global map of Crohn's disease: Genetic, environmental, and socioeconomic correlations. *Inflamm Bowel Dis* 2007; **14**: 709-720
- 18 Lok KH, Hung HG, Ng CH, Li KK, Li KF, Szeto ML. The epidemiology and clinical characteristics of Crohn's disease in the Hong Kong Chinese population: experiences from a regional hospital. *Hong Kong Med J* 2007; **13**: 436-441
- 19 Economou M, Filis G, Tsianou Z, Alamanos J, Kogevinas A, Masalas K, Petrou A, Tsianos EV. Crohn's disease incidence evolution in North-western Greece is not associated with alteration of NOD2/CARD15 variants. *World J Gastroenterol* 2007; **13**: 5116-5120
- 20 Wang Y, Ouyang Q. Ulcerative colitis in China: retrospective analysis of 3100 hospitalized patients. *J Gastroenterol Hepatol* 2007; **22**: 1450-1455
- 21 Hutfless SM, Weng X, Liu L, Allison J, Herrinton LJ. Mortality by medication use among patients with inflammatory bowel disease, 1996-2003. *Gastroenterology* 2007; **133**: 1779-1786
- 22 Hoie O, Wolters FL, Riis L, Bernklev T, Aamodt G, Clofent J, Tsianos E, Beltrami M, Odes S, Munkholm P, Vatn M, Stockbrugger RW, Moum B. Low colectomy rates in ulcerative colitis in an unselected European cohort followed for 10 years. *Gastroenterology* 2007; **132**: 507-515
- 23 Elmer GW, McFarland LV, McFarland M. Inflammatory bowel disease, irritable bowel syndrome and digestive problems, Chapter 6. In: *The Power of Probiotics*. New York: Haworth Press, 2007: 111-130
- 24 Zisman TL, Rubin DT. Colorectal cancer and dysplasia in inflammatory bowel disease. *World J Gastroenterol* 2008; **14**: 2662-2669
- 25 Kugathasan S, Nebel J, Skelton JA, Markowitz J, Keljo D, Rosh J, LeLeiko N, Mack D, Griffiths A, Bousvaros A, Evans J, Mezzoff A, Moyer S, Oliva-Hemker M, Otley A, Pfefferkorn M, Crandall W, Wyllie R, Hyams J. Body mass index in children with newly diagnosed inflammatory bowel disease: observations from two multicenter North American inception cohorts. *J Pediatr* 2007; **151**: 523-527
- 26 Seksik P, Sokol H, Lepage P, Vasquez N, Manichanh C, Mangin I, Pochart P, Dore J, Marteau P. Review article: the role of bacteria in onset and perpetuation of inflammatory bowel disease. *Aliment Pharmacol Ther* 2006; **24** Suppl 3: 11-18
- 27 Koloski NA, Bret L, Radford-Smith G. Hygiene hypothesis in inflammatory bowel disease: a critical review of the literature. *World J Gastroenterol* 2008; **14**: 165-173
- 28 Feller M, Huwiler K, Stephan R, Altpeter E, Shang A, Furrer H, Pfyffer GE, Jemmi T, Baumgartner A, Egger M. *Mycobacterium avium* subspecies paratuberculosis and Crohn's disease: a systematic review and meta-analysis. *Lancet Infect Dis* 2007; **7**: 607-613
- 29 Ruemmele FM, El Khoury MG, Talbot C, Maura C, Mougenot JF, Schmitz J, Goulet O. Characteristics of inflammatory bowel disease with onset during the first year of life. *J Pediatr Gastroenterol Nutr* 2006; **43**: 603-609
- 30 Lewis CM, Whitwell SC, Forbes A, Sanderson J, Mathew CG, Marteau TM. Estimating risks of common complex diseases across genetic and environmental factors: the example of Crohn disease. *J Med Genet* 2007; **44**: 689-694
- 31 Kugathasan S, Fiocchi C. Progress in basic inflammatory bowel disease research. *Semin Pediatr Surg* 2007; **16**: 146-153
- 32 Shergill AK, Terdiman JP. Controversies in the treatment of Crohn's disease: The case for an accelerated step-up treatment approach. *World J Gastroenterol* 2008; **14**: 2670-2677
- 33 Beckler DR, Elwasila S, Ghobrial G, Valentine JF, Naser SA. Correlation between *rpoB* gene mutation in *Mycobacterium avium* subspecies paratuberculosis and clinical rifabutin and rifampicin resistance for treatment of Crohn's disease. *World J Gastroenterol* 2008; **14**: 2723-2730
- 34 Yu ED, Shao Z, Shen B. Pouchitis. *World J Gastroenterol* 2007; **13**: 5598-5604
- 35 Hwang JM, Varma MG. Surgery for inflammatory bowel disease. *World J Gastroenterol* 2008; **14**: 2678-2690
- 36 Mallon P, McKay D, Kirk S, Gardiner K. Probiotics for induction of remission in ulcerative colitis. *Cochrane Database Syst Rev* 2007: CD005573
- 37 Gerasimidis K, McGrogan P, Hassan K, Edwards CA. Dietary modifications, nutritional supplements and alternative medicine in paediatric patients with inflammatory bowel disease. *Aliment Pharmacol Ther* 2008; **27**: 155-165
- 38 Ewaschuk JB, Dieleman LA. Probiotics and prebiotics in chronic inflammatory bowel diseases. *World J Gastroenterol* 2006; **12**: 5941-5950





## TOPIC HIGHLIGHT

Lynne V McFarland, PhD, Series Editor

# Editorial statement

Lynne V McFarland

In the following eight articles we have provided an overview of the current research and epidemiology of two leading chronic gastrointestinal conditions that affect populations across the globe. The challenges of accurately diagnosing irritable bowel syndrome (IBS) are covered and the pathophysiology of constipation is thoroughly explored. Both standard and innovation strategies for the treatment of IBS are discussed, including 5-HT<sub>4</sub> antagonists, surgery and the use of probiotics. Inflammatory bowel disease (IBD) encompasses several multifactorial conditions including Crohn's disease, ulcerative colitis and pouchitis. An alarming link between IBD and colorectal cancer has been made, which heightens the importance of preventive colonoscopic screening. Treatment for IBD may involve immunomodulators, biologic agents and surgery. Technologic advances that increase the detection of etiologic agents for IBD are being developed and may help in the development of better therapeutic strategies for these complex conditions. I hope these articles will inspire clinicians and researchers to focus their efforts on preventing the development of these conditions before irreversible damage occurs and the difficult job of treating these patients is required.

- 2625    State-of-the-art of irritable bowel syndrome and inflammatory bowel disease research in 2008  
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- 2630    Editorial statement  
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Lynne V McFarland, PhD, Series Editor

## Pathophysiology of constipation in the older adult

G Lindsay McCrea, Christine Miaskowski, Nancy A Stotts, Liz Macera, Madhulika G Varma

G Lindsay McCrea, Christine Miaskowski, Nancy A Stotts, Liz Macera, Departments of Physiological Nursing, University of California, San Francisco 94143, United States

Madhulika G Varma, Department of Surgery, University of California, San Francisco 94143, United States

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**Correspondence to:** G Lindsay McCrea, RN, MS, Doctoral Student, Department of Physiological Nursing, University of California, 2 Koret Way-0610, San Francisco 94143, United States. [lindsay.mccrea@ucsf.edu](mailto:lindsay.mccrea@ucsf.edu)

**Telephone:** +1-925-4511858 **Fax:** +1-415-4768899

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### Abstract

This review provides information on the definition of constipation, normal continence and defecation and a description of the pathophysiologic mechanisms of constipation. In addition, changes in the anatomy and physiology of the lower gastrointestinal tract associated with aging that may contribute to constipation are described. MEDLINE (1966-2007) and CINAHL (1980-2007) were searched. The following MeSH terms were used: constipation/etiology OR constipation/physiology OR constipation/physiopathology) AND (age factors OR aged OR older OR 80 and over OR middle age). Constipation is not well defined in the literature. While self-reported constipation increases with age, findings from a limited number of clinical studies that utilized objective measures do not support this association. Dysmotility and pelvic floor dysfunction are important mechanisms associated with constipation. Changes in GI function associated with aging appear to be relatively subtle based on a limited amount of conflicting data. Additional research is warranted on the effects of aging on GI function, as well as on the timing of these changes.

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**Key words:** Constipation; Mechanisms; Functional constipation; Dysmotility; Older adults; Pelvic floor dysfunction; Gastrointestinal tract

**Peer reviewers:** Stefan Wirth, Professor, Dr, Children's Hospital, Heusenst. 40, Wuppertal 42349, Germany; Diego Garcia-

Compean, MD, Professor, Faculty of Medicine, University Hospital, Department of Gastroenterology, Autonomous University of Nuevo Leon, Ave Madero y Gonzalitos, 64700 Monterrey, NL, Mexico

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### INTRODUCTION

Constipation is a problem that affects all ages. However, it is a common problem in older adults and is often a concern to elders and clinicians. In older people, acute bouts of constipation can occur with acute illness or dietary alterations. In contrast, chronic constipation usually has an insidious onset of many years, often dating to childhood. The symptom experience can range from a mild, acute event that is remedied with a shift in fluid and dietary intake to a chronic condition that requires daily interventions with mixed results. Elders may falsely believe that constipation is a "natural" part of aging<sup>[1-4]</sup>.

The purposes of this review are to: define constipation; provide an overview of normal continence and defecation; and describe the pathophysiologic mechanisms of constipation. In addition, the changes in the anatomy and physiology of the lower GI tract associated with aging that may contribute to constipation are described.

### METHODS

To identify the relevant studies on the pathophysiology of constipation in the older adult, a number of strategies were employed. A literature search was conducted that included the following databases and time periods: MEDLINE (1966-2007) and CINAHL (1980-2007). The following MeSH terms were used: (constipation/etiology OR constipation/physiology OR constipation/physiopathology) AND (age factors OR aged OR older OR 80 and over OR middle age).

### DEFINITION OF CONSTIPATION

Constipation is not a disease entity, but a general term that is used to describe the difficulties that patients experience with moving their bowels. Clinical and research

literature documents that patients and clinicians use different definitions of constipation. Clinicians consider the frequency of defecation episodes, stool weight, colonic transit time, and anorectal manometry as proxy measures for constipation<sup>[5,6]</sup>. A commonly held belief amongst clinicians is that the problem of constipation is more imagined than real, as “the great majority of those complaining of constipation have a bowel motion (movement) more frequently than three times a week”<sup>[7]</sup>. The actual problem lies in the definition of constipation. The term itself holds different meanings depending on the individual. Individual perception of bowel function, whether or not the symptoms associated with constipation are endured, is quite distinct from how the medical dictionary defines the problem. Because of the subjective nature of the condition, no consensus exists on the definition of constipation.

Measurement of the frequency of stools has been used to define constipation. “Normal” frequency of stool evacuation comprises a broad or narrow range of time that has large intra- and inter-individual variability. The “usual” range is anywhere from one to three times per day to three times per week<sup>[8]</sup>. Less than three times per week may be considered normal if this does not represent a change from the usual frequency of baseline defecation events and is not associated with discomfort<sup>[9]</sup>. This self-report criterion does not describe the entire symptom experience of constipation.

Patients define constipation quite differently from clinicians. A study of 531 general practice patients found that 50% of them gave a different definition of constipation than their physicians<sup>[10]</sup>. Most patients define it by one or more of the following symptoms: hard stools, infrequent stools, the need for excessive straining, a sense of incomplete evacuation, and/or an excessive amount of time spent on the toilet or in unsuccessful evacuation<sup>[11]</sup>. Patients may perceive any or all of the following as constipation: straining to expel hard, dry stools; difficulty with initiating a bowel movement or an inability to defecate when desired; feelings of incomplete evacuation; and/or abdominal cramping and bloating. Subjective reports among middle aged and older individuals have identified the most common definition of constipation as being difficulty with defecation<sup>[12]</sup>.

Most patients with a complaint of constipation have a functional disorder that affects the colon and/or anorectum. “Functional” is used to describe symptoms or problems that have no underlying anatomic abnormalities. However, the normal function of an organ has changed. Functional bowel disorders are functional gastrointestinal disorders with symptoms that arise in the middle or lower gastrointestinal tract<sup>[4]</sup>. Functional constipation is defined as the reduced frequency of bowel movements and/or an altered act of evacuation<sup>[13]</sup>.

Functional bowel disorders, including functional constipation, are diagnosed primarily through patients’ reports of symptoms. As a result, a symptom based classification is needed for clinical diagnosis, evidence based management, and research. Since 1989, an international panel of experts has met four times and issued recommendations

**Table 1 Rome III criteria for defining functional constipation**

Loose stools rarely present with laxative use and insufficient criteria for IBS and
Two or more of the following (fulfilled for the last 3 mo with symptom onset at least 6 mo prior to diagnosis):
Fewer than three bowel movements per week
Straining <sup>1</sup>
Lumpy or hard stools <sup>1</sup>
Sensation of incomplete evacuation <sup>1</sup>
Sensation of anorectal obstruction or blockade <sup>1</sup>
Manual maneuvers (e.g., digital evacuation, support of the pelvic floor) To facilitate a bowel movement <sup>1</sup>

<sup>1</sup> ≥ 25% of defecations. Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders and functional abdominal pain. *Gastroenterology* 2006; 130: 1486.

on the diagnosis and management of Irritable Bowel Syndrome (IBS), as well as diagnostic criteria for other functional bowel disorders. Known as the Rome I, Rome II, and Rome III (Table 1) criteria, these recommendations have evolved to include functional anorectal disorders. All disorders are defined by specific symptoms. However, functional disorders of defecation include the results of diagnostic tests as part of the definition<sup>[14]</sup>.

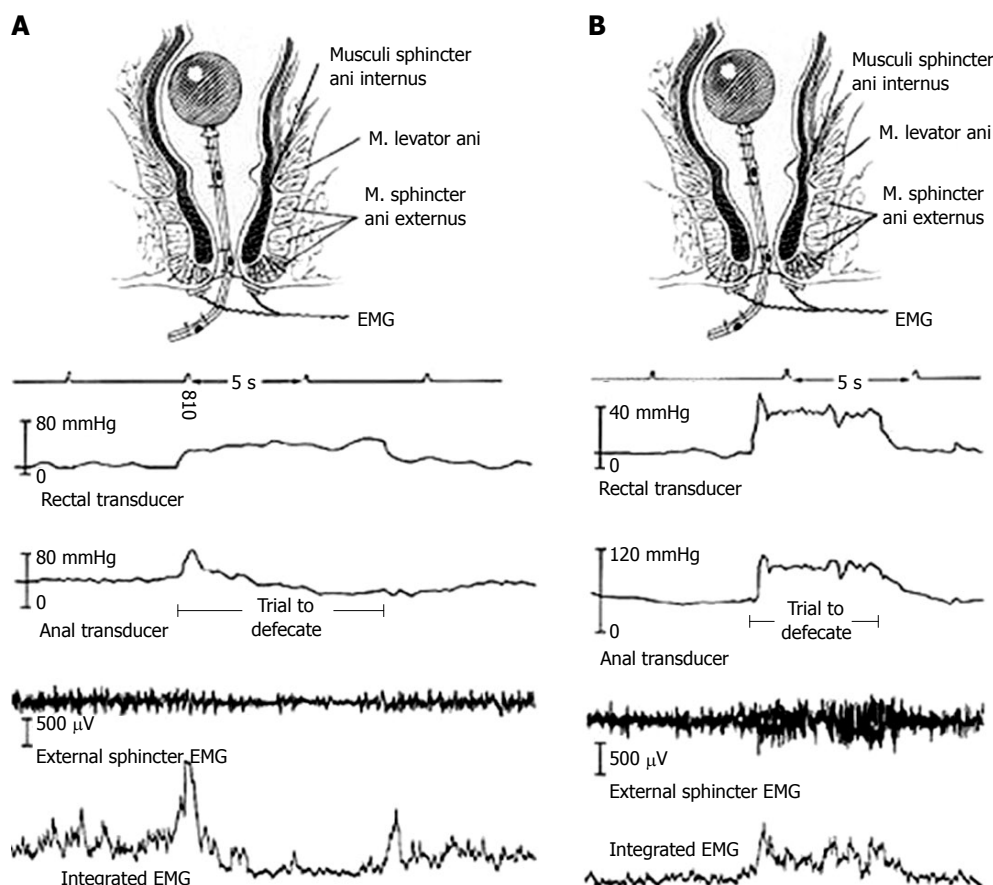
Rome III as a functional bowel disorder that presents as persistently difficult, infrequent, or seemingly incomplete defecation, which does not meet IBS criteria, defines functional constipation. Patients are diagnosed with functional constipation if they are devoid of organic alterations and present with at least two or more of the symptoms listed in Table 1, fulfilled for at least three months with symptom onset at least six months prior to diagnosis, and insufficient criteria for IBS<sup>[4]</sup>.

Several changes occurred in the development of the diagnostic criteria between Rome I and Rome III, in particular between Rome II and III. Studies using the Rome II criteria yielded lower prevalence rates for constipation than those using Rome I criteria. A factor that contributed to this decrease was a change in the Rome II criteria that did not allow for laxative-induced loose stools<sup>[15]</sup>. Rome III includes this criterion. In addition, the frequency of occurrence of the various criteria was modified from > 25% to ≥ 25%. This approach was taken to be consistent with the criteria for other functional bowel disorders<sup>[4]</sup>. Since Rome II, more consistent criteria for the diagnosis and management of constipation have appeared in the literature.

With this definition of constipation as background information, the next section of this paper reviews normal continence and defecation prior to a discussion of the pathophysiology of constipation and constipation in the older adult.

## NORMAL CONTINENCE AND DEFECATION

Normal anatomy and physiology of the gastrointestinal tract are well documented in the literature. Normal continence and defecation are complex processes that are altered in someone with constipation. Continence



**Figure 1** Manometric and electromyographic recordings seen with normal (A) and abnormal (B) defecation. EMG: Electromyography; Footnote: Wald A. Manometry. In: Schuster MM, Crowel MD, Koch KL, eds. *Atlas of Gastrointestinal Motility in Health and Disease* 2nd ed, Hamilton, and Ontario: BC Decker; 2002: 289-303.

is the ability to retain feces until an acceptable time for defecation. Defecation is the evacuation of fecal material from the colon. Both functions involve complex physiologic processes that are not completely understood. Voluntary regulation through the central nervous system (CNS) and involuntary intrinsic reflex mechanisms are involved in both of these functions. Fecal continence is maintained by anatomic factors, anorectal sensation, and rectal compliance<sup>[16]</sup>. Problems with continence and defecation can arise from an extrinsic disorder involving the central or peripheral nervous systems; from an intrinsic disorder of the colon, rectum, or anal sphincters; or from a combination of these mechanisms.

Normal stool output is about 200 mg daily. Activity in the proximal colon determines the consistency and volume of delivery of contents to the rectum. The rectum is a reservoir. As rectal filling gradually proceeds, anorectal sampling permits subconscious perception of the consistency of the content. An intact internal anal sphincter (IAS) ensures continence.

Autonomic neurons relay the anorectal sensation of the rectal contents. Activation of these afferents results in both conscious perception and activation of local reflexes, such as the rectoanal inhibitory reflex to begin the relaxation of the IAS. Reflex voluntary contraction of the external anal sphincter (EAS) maintains continence until voluntary defecation is possible. A similar reflex contraction occurs to maintain continence when a rise in abdominal pressure occurs, such as during a cough or with positional changes. Partial external contraction is

also observed during the passage of flatus, and coupled with an intact anorectal sensation this is the mechanism through which fecal continence is maintained during the passage of gas. Preservation of continence depends on the normal functioning of anorectal sensation, the appropriate perception of that sensory information, the integrity of lower and higher reflex arcs, and the action of the internal and external anal sphincters.

Defecation (Figure 1A) begins with rectal sensory awareness of a critical level of filling, which is relayed to the cerebral cortex as the perception of the need to evacuate the rectum. The actual volume that triggers the perception is dependent on the condition of the rectum (e.g. mucosal inflammation, rectal wall compliance) and the character of the contents (e.g. consistency, volume). When appropriate, the individual adopts a sitting or squatting position. This position results in a straightening of the anorectal angle that allows more effective propulsion of the rectal contents. The EAS and puborectalis muscles relax. The rectal contents provoke reflex relaxation of the IAS and the individual can then bear down. Abdominal pressure rises, abdominal wall muscles tense and relaxation of the pelvic floor allows some stool to enter the lower rectum. This movement of stool initiates a spontaneous rectosigmoid contraction, which pushes the stool through the relaxed anal canal<sup>[16]</sup>.

Large propulsive contractions of the rectum occur until the rectum is empty. Sensory input from the anus maintains the propulsive activity until the rectum is fully voided. This reflex appears to be mediated at the level of the spinal cord,

as even spinally injured patients can evacuate a complete stool from the rectum, once initiated<sup>[17]</sup>. Such patients tend to use digital rectal stimulation to initiate the propulsive contractions of the recto-sigmoid colon. As stool passes through the anal canal, it stretches the EAS and creates a traction force on it. After the last bolus of stool has passed, the closing reflex of the EAS is stimulated by the release of traction. Therefore, anal continence is maintained following defecation.

## CHANGES IN THE LOWER GASTROINTESTINAL TRACT ASSOCIATED WITH AGING

Age-related anatomic changes within the lower gastrointestinal tract may contribute to delayed transit time and decreased stool water content<sup>[18]</sup>. These changes can include intestinal wall atrophy, reduced blood supply, and intrinsic neuronal changes. However, no significant functional changes are readily apparent in the aging gastrointestinal tract. Secretion and absorption remain relatively constant. This constancy is thought to be due to the redundancy in each segment of the intestinal tract.

Gut transit time and colonic motility are similar in healthy older adults compared to younger participants<sup>[19]</sup>. In contrast, elderly people with chronic illness who report constipation have a prolonged total gut transit time of 4 to 9 d (normal < 3 d), with evacuation delayed through the lowest part of the large bowel and rectum. Nursing home residents have even more prolonged transit times of up to three weeks in those least mobile which makes them highly susceptible to slow transit constipation (STC) and overflow fecal incontinence<sup>[20]</sup>. Colonic function appears to be more influenced by factors associated with aging (e.g. chronic disease, immobility, and medications) than aging itself.

Age-related neurodegenerative changes in the enteric nervous system (ENS) may be key to functional changes observed with advanced age. In colons of people older than age 65, a 37% loss of enteric neurons was found when compared with younger people<sup>[21]</sup>. The number of nerve cells present in the myenteric plexus of the human large intestine was examined using laminar preparations of the muscularis externa in two groups of participants aged 20 to 35 and over 65 years. In addition, the collagen and elastic system related fibers in the myenteric ganglia were qualitatively evaluated. The total number of neurons was decreased in older individuals. Both collagen and elastic system fibers were more numerous in the ganglia from the older participants. The authors concluded that the decrease in neuron density with age is accompanied by an apparent increase in the fibrous components of the myenteric ganglia. These findings suggest that neurodegenerative changes may contribute to the disturbed colonic motility seen in the aging population.

Older people have age-related reductions in IAS pressure and pelvic muscle strength<sup>[22,23]</sup>, as well as changes in rectal sensitivity<sup>[24]</sup> and anal function. Women in particular, experience a larger decrease in squeeze pressures with aging especially after menopause, and due to injuries sustained during vaginal delivery<sup>[25,26]</sup>. These changes increase both the risk and the potential for constipation.

The interrelationship between aging and parity in the anorectal squeeze pressure in women is difficult to determine from currently available data. However, the authors suggested that menopausal effects might be relevant<sup>[3]</sup>. These age-related changes are not in and of them pathologic, but may contribute to the development of constipation in the elderly.

## PATHOPHYSIOLOGIC MECHANISMS OF CONSTIPATION

Two mechanisms explain the pathophysiology of constipation<sup>[27,28]</sup>. Colonic motility dysfunction, or dysmotility, is failure of coordinated motor activity to move stool through the colon. It is sometimes associated with: dietary factors, medications that can alter motility; or systemic disease (e.g. neurologic, metabolic, or endocrine disorders). Others exhibit abnormalities of the enteric nerves, such as decreased volume of interstitial cells of Cajal (ICC) and other neural elements<sup>[29]</sup>. The second mechanism involves pelvic floor dysfunction, or disorders of the anorectum and pelvic floor, which result in outlet dysfunction and an inability to adequately evacuate rectal contents. Functional constipation may occur as a result of disordered movement through the sigmoid colon and/or anorectum. Both mechanisms coexist in some patients<sup>[28]</sup>, making it difficult to determine the exact underlying mechanisms for constipation.

### Physiology of dysmotility

Dysmotility results in colonic delay (i.e. abnormally prolonged colonic transit time). Three types of colonic delay have been identified: right colonic (colonic inertia), left colonic, and rectosigmoid. Additionally, delay can occur in patients with no colonic dysmotility<sup>[30]</sup>. Mechanisms of delay include: dysfunction of the autonomic nervous system, disruption in the ENS<sup>[31]</sup>, disruptions in the neuroendocrine system<sup>[32,33]</sup>, and/or colonic myopathy<sup>[34,35]</sup>.

Impaired colonic propulsive activity may represent a major mechanism for colonic dysmotility. In patients with constipation ( $n = 45$ ), there were fewer mass movements segmental contractions<sup>[36]</sup>. No differences in post awakening values were found in patients with chronic constipation, which suggests that the brain-gut control of fundamental mechanisms governing colonic motility is preserved<sup>[37]</sup>.

A disorder of the ICC may have a role in the development of diminished or absent colonic motor activity<sup>[38]</sup>. In patients with STC, the number of ICC was significantly decreased in all layers of the colonic wall<sup>[29]</sup>, including the external muscle layer<sup>[39]</sup>. Thus, constipation in patients with colonic inertia is attributable to weak or absent electric activity.

When compared with healthy controls, patients with STC exhibit reduced daytime colonic pressure waves and a higher frequency of periodic rectal motor activity (PRMA) that were unrelated to proximal colonic activity. Their findings suggest that excessive and uncoordinated phasic rectal activity may further impede stool transport and contribute to STC<sup>[40]</sup>.



The gastrocolic response to ingestion of a meal in individuals with constipation is characterized by a shorter contractile activity in all three-colon segments and significantly fewer high amplitude propagated contractions (HAPCs)<sup>[41,42]</sup>. Changes in rectal wall contractility in response to feeding, as well as with the administration of a cholinergic agonist, and a smooth muscle relaxant, are decreased in constipated patients. This finding suggests an abnormality in rectal muscular wall contractility<sup>[43]</sup>.

The gastrocolic response after ingestion of a standardized liquid meal and the response to a local chemical stimulus were investigated in 10 healthy controls and 10 patients with STC. Increases in motility after a meal and bisacodyl were seen in healthy participants, but not in patients with STC. Timing of the high amplitude propagating contractions was prolonged and decreased in number in the patients with STC. In addition, symptom reports of a cramp felt at the time of a HAPC were significantly lower than in controls ( $P < 0.05$ )<sup>[44]</sup>.

The nerve fibers in the colonic circular muscle may be abnormal in patients with STC. A reduction in the density of excitatory nerve fibers with tachykinin and enkephalin immunoreactivity was found in the colonic circular muscle of patients with STC, whereas innervation of all the other layers was normal<sup>[45]</sup>. Small sensory fiber dysfunction has also been suggested in patients with STC<sup>[46]</sup>.

Abnormalities in neurotransmitters may contribute to dysmotility and the subsequent development of constipation. Changes in excitatory and inhibitory neurotransmitters have been evaluated with conflicting results. Levels of vasoactive intestinal peptide (VIP) were found to be unchanged<sup>[47]</sup> or increased<sup>[33]</sup> in patients with STC. The release of acetylcholine was found to be depressed in colonic tissue specimens from constipated patients<sup>[48]</sup>. Excessive nitric oxide was found in ICC preparations from the distal colon of patients with STC<sup>[49]</sup>. A decrease in serotonin immunoreactivity in the muscular mucosa and circular muscle was identified in patients who underwent subtotal colectomy for colonic inertia<sup>[50]</sup>. How changes in the release of various neurotransmitters contribute to the pathogenesis of STC has not been described in detail.

A number of gut hormones (i.e. cholecystokinin, peptide YY, somatostatin, enteroglucagon, pancreatic peptides) are thought to have potent effects on gastrointestinal motility. Plasma cholecystokinin and peptide YY have not been found to be altered in patients with STC<sup>[51]</sup>. However, specific abnormalities in circulating gut hormones have been identified in patients with STC including: higher levels of circulating somatostatin, lower levels of somatostatin integrated with an incremental meal response, and decreased levels of enteroglucagon 30-60 min after a meal<sup>[52]</sup>. Significantly fewer enteroglucagon and serotonin immunoreactive cells were found in patients with STC<sup>[33]</sup>. However, how changes in these hormones contribute to the pathogenesis of STC has not been described.

### **Changes in physiology associated with disease states**

Disease states that alter slowly wave patterns or spike responses will alter contraction and motility<sup>[53]</sup>. Abnormalities in colonic motility seen in diabetic patients

with constipation are due in part to altered autonomic neural control manifested as an abnormal gastrocolonic response. Slow wave patterns appear unaltered in healthy participants compared to patients with constipation and diabetes. Minimal spike potential activity is seen in both healthy and diabetic patients during fasting. Following a meal, spike potential activity quickly increases during the first 10 min and is sustained for 30 min in healthy participants. This activity is inhibited by the pre-administration of an anticholinergic drug, which suggests that the postprandial response is mediated through the cholinergic nervous system. In diabetic patients without constipation, the response to a meal is the same as in controls. In chronic insulin dependent diabetic patients with constipation, the normal postprandial increase in spike potential is not present. The lack of spike potential leads to abnormal postprandial motor activity in the colon, which results in constipation<sup>[54,55]</sup>.

### **Pelvic floor dysfunction**

The second major mechanism for constipation is pelvic floor dysfunction, which results in disordered defecation. It is most commonly due to dysfunction of the pelvic floor muscles or anal sphincters<sup>[15]</sup>. Different terms that are used to describe these disorders include anismus, pelvic-floor dyssynergia, paradoxical pelvic floor contraction, obstructed defecation, functional rectosigmoid obstruction, and functional fecal retention in childhood<sup>[56]</sup>. The pathophysiology of these disorders is not completely understood.

### **Physiology of pelvic floor dysfunction**

When constipation is accompanied by an immobile perineum, patients have impaired balloon expulsion, impaired and delayed artificial stool expulsion, decreased straightening of the anorectal angle, decreased descent of the pelvic floor with defecation, and prolonged rectosigmoid transit times. All are thought to be signs of pelvic floor dysfunction rather than delayed transit time<sup>[57]</sup>.

When compared to healthy controls, patients with obstructed defecation demonstrate lower intrarectal pressure and defecation indices and higher anal residual pressures on anorectal manometry recordings during straining (Figure 1B). Impaired rectal contraction, paradoxical anal contraction, or inadequate anal relaxation seen in patients with obstructed defecation suggests that rectoanal coordination is impaired<sup>[58]</sup>.

The overall frequency of propagating sequences in the colon does not differ between patients with obstructed defecation and healthy controls. In fact, patients with obstructed defecation were found to have a significant increase in the frequency of retrograde and antegrade propagating sequences ( $P < 0.05$ ) in the left colon and a significant reduction in the amplitude of propagating pressure waves throughout the entire colon ( $P < 0.03$ ). In the 15 min before defecation, controls showed a highly significant increase in frequency ( $P = 0.001$ ) and amplitude ( $P = 0.01$ ) of propagating sequences. In contrast, patients did not demonstrate this or the typical spatiotemporal organization of propagating sequences normally observed before expulsion of stool<sup>[59]</sup>.

### Neural influences on pelvic floor dysfunction

Parasympathetic afferent nerves are stimulated by both slow or cumulative and fast or intermittent distention of the rectum, whereas sympathetic afferent nerves are only stimulated by fast distention. In a study that examined the role of sympathetic afferent nerves in the mediation of rectal filling sensations, women with obstructed defecation were found to have either blunted or absent rectal sensory perception<sup>[60]</sup>. Participants experienced a nonspecific sensation in the pelvis or lower abdomen with fast distention, which suggested that sympathetic efferents were deficient. In spite of this, rectal wall compliance was normal in the patients with obstructed defecation<sup>[61]</sup>.

The gastrocolic reflex has been evaluated in patients with obstructed defecation. It was found to be absent or prolonged in patients with obstructive defecation in whom transit time is prolonged. The gastrocolic reflex was found to be intact if slow transit was absent<sup>[62]</sup>.

Patients with STC can also have anorectal motility disturbances. The minimum relaxation volume, the rectal defecatory threshold, the rectal maximal tolerable volume, and the rectal compliance are significantly higher in patients with STC than in healthy controls ( $P < 0.01$  or  $P < 0.05$ )<sup>[63]</sup>. The anorectal reflex is active in puborectalis paradoxical syndrome, but the rectoanal reflex is not, indicating a possible myogenic defect in the puborectalis muscle<sup>[64]</sup>.

How people develop defecation disorders is unclear. In two-thirds of patients, dyssynergic or obstructed defecation appears to be an acquired behavioral disorder of defecation and in the rest the process of defecation may not have been learned since childhood<sup>[65]</sup>.

## PATHOPHYSIOLOGY OF CONSTIPATION IN THE OLDER ADULT

Constipation is often considered a natural part of aging but it is a disorder that is not caused by aging itself. Although changes in the gastrointestinal tract associated with aging may predispose one to develop constipation, the disorder usually has a multifactorial etiology and may be a lifetime disorder.

As shown in Table 2, numerous factors may contribute to the development of constipation<sup>[6-9,66,67]</sup>. Though bowel transit time and frequency of bowel movements do not change with aging, a number of comorbid conditions may contribute to the development of constipation<sup>[66]</sup>. Some data suggest that older adults perceive constipation as straining during defecation rather than decreased bowel frequency<sup>[68,69]</sup>. Another study of elderly individuals who reported constipation demonstrated that straining and hard bowel movements were the most frequent complaints<sup>[69]</sup>. A determination of the most likely etiology for constipation requires identification of the primary complaint<sup>[66]</sup>.

Aging is associated with changes in the structure and function of the colon and defecatory mechanisms. Regional differences in colonic properties and in neurotransmitter functions have implications for normal function and dysfunction<sup>[70]</sup>.

Rectal sensation plays a critical role in normal defecation and may change with aging. In one study elderly patients

Table 2 Etiology of constipation in the older adult

Endocrine and metabolic disease
Diabetes mellitus
Hypothyroidism
Neurologic disease
Autonomic neuropathy
Cerebrovascular disease
Multiple sclerosis
Parkinson's disease
Spinal cord injury
Psychological conditions
Anxiety
Depression
Structural abnormalities
Anorectal conditions: fissures, hemorrhoids, rectal prolapse or rectocele
Obstructive colonic lesions
Lifestyle
Dehydration
Low calorie diet
Low fiber diet
Immobility
Iatrogenic
Medications

with constipation and a history of fecal impaction had impaired rectal and perineal sensation and required significantly larger volumes of rectal distention to stimulate the normal urge to defecate<sup>[24]</sup>. A second report described impaired rectal perception of stool in elderly patients with constipation<sup>[71]</sup>, while sensation appeared to remain intact in those patients without constipation.

Disordered defecation can occur as a result of injury to the pudendal nerve. The incidence of increased pudendal nerve terminal motor latency, an indicator of pudendal nerve dysfunction, is increased in elderly females<sup>[3]</sup>. Injury to the pudendal nerves can lead to abnormal perineal descent, which can impact rectal emptying by causing partial prolapse of the anal canal by the anterior rectal mucosa<sup>[72]</sup>.

Several types of anorectal abnormalities occur in older people with constipation including dyschezia and pelvic dyssynergia. Dyschezia is characterized by reduced tone, increased compliance, and impaired sensation such that a greater degree of rectal distention is required to induce the defecatory mechanism<sup>[24]</sup>. Seen most commonly in frail elders, these individuals have recurrent rectal impactions, a frequent consequence of which is fecal soiling. Fecal soiling affects 28% of older people. However, it is a problem that is not assessed by doctors or nurses<sup>[73]</sup>. Pelvic dyssynergia, also termed anismus, involves a failure to relax the pelvic floor and external anal sphincter muscles during defecation.

## IMPLICATIONS FOR FUTURE RESEARCH

Constipation is not a straightforward problem. However, recent research on the mechanisms and effects of constipation on the elderly is extremely sparse. Until the mechanisms of constipation are completely understood, it is likely that treatment will be at best, minimally successful. Dysmotility and pelvic floor dysfunction



are clearly important mechanisms associated with constipation, but further work is needed to understand the anatomic, physiologic, and lifestyle factors that affect these mechanisms. In addition, longitudinal data on gastrointestinal motility are needed to determine the effects of aging on normal lower GI and pelvic floor anatomy and function. Studies are needed that evaluate changes in the physiology of the pelvic floor in women over the menopause transition and whether these changes contribute to functional outcomes seen at that time and on into old age.

Changes in GI function associated with aging appear to be relatively subtle based on a limited amount of conflicting data. Additional research is warranted on the effects of aging on GI function, as well as on the timing of these changes. A deeper understanding of the basic mechanisms of dysfunction, changes in the colonic wall, and pelvic floor dysfunction in the older adult could provide new directions for the assessment and management of constipation in this vulnerable group.

While significant progress has occurred in the development of consensus criteria for the assessment of constipation the definition is not standardized. The Rome criteria constitute a self-reported, complaint-based diagnostic system with a significant overlap between the criteria for dysmotility and pelvic dysfunction disorders. Studies are needed to clarify the discrete differences between colonic motor dysfunction and functional defecation disorders.

## REFERENCES

- 1 Chan AO, Cheng C, Hui WM, Hu WH, Wong NY, Lam KF, Wong WM, Lai KC, Lam SK, Wong BC. Differing coping mechanisms, stress level and anorectal physiology in patients with functional constipation. *World J Gastroenterol* 2005; **11**: 5362-5366
- 2 Chang L, Toner BB, Fukudo S, Guthrie E, Locke GR, Norton NJ, Sperber AD. Gender, age, society, culture, and the patient's perspective in the functional gastrointestinal disorders. *Gastroenterology* 2006; **130**: 1435-1446
- 3 Laurberg S, Swash M. Effects of aging on the anorectal sphincters and their innervation. *Dis Colon Rectum* 1989; **32**: 737-742
- 4 Longstreth GF, Thompson WG, Chey WD, Houghton LA, Mearin F, Spiller RC. Functional bowel disorders. *Gastroenterology* 2006; **130**: 1480-1491
- 5 Ashraf W, Park F, Lof J, Quigley EM. An examination of the reliability of reported stool frequency in the diagnosis of idiopathic constipation. *Am J Gastroenterol* 1996; **91**: 26-32
- 6 Koch T, Hudson S. Older people and laxative use: literature review and pilot study report. *J Clin Nurs* 2000; **9**: 516-525
- 7 Campbell AJ, Busby WJ, Horwath CC. Factors associated with constipation in a community based sample of people aged 70 years and over. *J Epidemiol Community Health* 1993; **47**: 23-26
- 8 Schaefer DC, Cheskin LJ. Constipation in the elderly. *Am Fam Physician* 1998; **58**: 907-914
- 9 Abyad A, Mourad F. Constipation: common-sense care of the older patient. *Geriatrics* 1996; **51**: 28-34, 36
- 10 Herz MJ, Kahan E, Zalevski S, Aframian R, Kuznitz D, Reichman S. Constipation: a different entity for patients and doctors. *Fam Pract* 1996; **13**: 156-159
- 11 Koch A, Voderholzer WA, Klauser AG, Muller-Lissner S. Symptoms in chronic constipation. *Dis Colon Rectum* 1997; **40**: 902-906
- 12 Ross DG. Subjective data related to altered bowel elimination patterns among hospitalized elder and middle-aged persons. *Orthop Nurs* 1993; **12**: 25-32
- 13 Drossman DA. The functional gastrointestinal disorders and the Rome II process. *Gut* 1999; **45** Suppl 2: II1-II5
- 14 Bharucha AE, Wald A, Enck P, Rao S. Functional anorectal disorders. *Gastroenterology* 2006; **130**: 1510-1518
- 15 Thompson WG, Irvine EJ, Pare P, Ferrazzi S, Rance L. Functional gastrointestinal disorders in Canada: first population-based survey using Rome II criteria with suggestions for improving the questionnaire. *Dig Dis Sci* 2002; **47**: 225-235
- 16 Bharucha AE. Pelvic floor: anatomy and function. *Neurogastroenterol Motil* 2006; **18**: 507-519
- 17 Read NW. Feedback regulation and sensation. *Dig Dis Sci* 1994; **39**: 375-405
- 18 Lynch AC, Anthony A, Dobbs BR, Frizelle FA. Anorectal physiology following spinal cord injury. *Spinal Cord* 2000; **38**: 573-580
- 19 Hanani M, Fellig Y, Udassin R, Freund HR. Age-related changes in the morphology of the myenteric plexus of the human colon. *Auton Neurosci* 2004; **113**: 71-78
- 20 Loening-Baucke V, Anuras S. Sigmoidal and rectal motility in healthy elderly. *J Am Geriatr Soc* 1984; **32**: 887-891
- 21 Brocklehurst JC, Kirkland JL, Martin J, Ashford J. Constipation in long-stay elderly patients: its treatment and prevention by lactulose, poloxalkol-dihydroxyanthroquinolone and phosphate enemas. *Gerontology* 1983; **29**: 181-184
- 22 Gomes OA, de Souza RR, Liberti EA. A preliminary investigation of the effects of aging on the nerve cell number in the myenteric ganglia of the human colon. *Gerontology* 1997; **43**: 210-217
- 23 McHugh SM, Diamant NE. Anal canal pressure profile: a re-appraisal as determined by rapid pullthrough technique. *Gut* 1987; **28**: 1234-1241
- 24 McHugh SM, Diamant NE. Effect of age, gender, and parity on anal canal pressures. Contribution of impaired anal sphincter function to fecal incontinence. *Dig Dis Sci* 1987; **32**: 726-736
- 25 Read NW, Abouzekry L, Read MG, Howell P, Ottewell D, Donnelly TC. Anorectal function in elderly patients with fecal impaction. *Gastroenterology* 1985; **89**: 959-966
- 26 Ryhammer AM, Laurberg S, Sorensen FH. Effects of age on anal function in normal women. *Int J Colorectal Dis* 1997; **12**: 225-229
- 27 Sultan AH, Kamm MA, Hudson CN. Pudendal nerve damage during labour: prospective study before and after childbirth. *Br J Obstet Gynaecol* 1994; **101**: 22-28
- 28 Cheung O, Wald A. Review article: the management of pelvic floor disorders. *Aliment Pharmacol Ther* 2004; **19**: 481-495
- 29 Sagar PM, Pemberton JH. Anorectal and pelvic floor function. Relevance of continence, incontinence, and constipation. *Gastroenterol Clin North Am* 1996; **25**: 163-182
- 30 Wedel T, Spiegler J, Soellner S, Roblick UJ, Schiedeck TH, Bruch HP, Krammer HJ. Enteric nerves and interstitial cells of Cajal are altered in patients with slow-transit constipation and megacolon. *Gastroenterology* 2002; **123**: 1459-1467
- 31 Mertz H, Naliboff B, Mayer EA. Symptoms and physiology in severe chronic constipation. *Am J Gastroenterol* 1999; **94**: 131-138
- 32 Bassotti G, Villanacci V. Slow transit constipation: a functional disorder becomes an enteric neuropathy. *World J Gastroenterol* 2006; **12**: 4609-4613
- 33 El-Salhy M, Norrgard O, Spinnell S. Abnormal colonic endocrine cells in patients with chronic idiopathic slow-transit constipation. *Scand J Gastroenterol* 1999; **34**: 1007-1011
- 34 Sjolund K, Fasth S, Ekman R, Hulten L, Jiborn H, Nordgren S, Sundler F. Neuropeptides in idiopathic chronic constipation (slow transit constipation). *Neurogastroenterol Motil* 1997; **9**: 143-150
- 35 Knowles CH, Nickols CD, Scott SM, Bennett NI, de Oliveira RB, Chimelli L, Feakins R, Williams NS, Martin JE. Smooth muscle inclusion bodies in slow transit constipation. *J Pathol* 2001; **193**: 390-397
- 36 Knowles CH, Scott SM, Lunniss PJ. Slow transit constipation: a disorder of pelvic autonomic nerves? *Dig Dis Sci* 2001; **46**: 389-401
- 37 Bassotti G, Chistolini F, Nzepa FS, Morelli A. Colonic

- propulsive impairment in intractable slow-transit constipation. *Arch Surg* 2003; **138**: 1302-1304
- 38 **Bassotti G**, Germani U, Fiorella S, Roselli P, Brunori P, Whitehead WE. Intact colonic motor response to sudden awakening from sleep in patients with chronic idiopathic (slow-transit) constipation. *Dis Colon Rectum* 1998; **41**: 1550-1555; discussion 1555-1556
  - 39 **Shafik A**, Shafik AA, El-Sibai O, Mostafa RM. Electric activity of the colon in subjects with constipation due to total colonic inertia: an electrophysiologic study. *Arch Surg* 2003; **138**: 1007-1011; discussion 1011
  - 40 **Tong WD**, Liu BH, Zhang LY, Zhang SB, Lei Y. Decreased interstitial cells of Cajal in the sigmoid colon of patients with slow transit constipation. *Int J Colorectal Dis* 2004; **19**: 467-473
  - 41 **Rao SS**, Sadeghi P, Batterson K, Beaty J. Altered periodic rectal motor activity: a mechanism for slow transit constipation. *Neurogastroenterol Motil* 2001; **13**: 591-598
  - 42 **Bassotti G**, Imbimbo BP, Betti C, Dozzini G, Morelli A. Impaired colonic motor response to eating in patients with slow-transit constipation. *Am J Gastroenterol* 1992; **87**: 504-508
  - 43 **Grotz RL**, Pemberton JH, Levin KE, Bell AM, Hanson RB. Rectal wall contractility in healthy subjects and in patients with chronic severe constipation. *Ann Surg* 1993; **218**: 761-768
  - 44 **De Schryver AM**, Samsom M, Smout AI. Effects of a meal and bisacodyl on colonic motility in healthy volunteers and patients with slow-transit constipation. *Dig Dis Sci* 2003; **48**: 1206-1212
  - 45 **Porter AJ**, Wattchow DA, Hunter A, Costa M. Abnormalities of nerve fibers in the circular muscle of patients with slow transit constipation. *Int J Colorectal Dis* 1998; **13**: 208-216
  - 46 **Knowles CH**, Scott SM, Wellmer A, Misra VP, Pilot MA, Williams NS, Anand P. Sensory and autonomic neuropathy in patients with idiopathic slow-transit constipation. *Br J Surg* 1999; **86**: 54-60
  - 47 **Tzavella K**, Riepl RL, Klauser AG, Voderholzer WA, Schindlbeck NE, Muller-Lissner SA. Decreased substance P levels in rectal biopsies from patients with slow transit constipation. *Eur J Gastroenterol Hepatol* 1996; **8**: 1207-1211
  - 48 **Mitolo-Chieppa D**, Mansi G, Rinaldi R, Montagnani M, Potenza MA, Genuardo M, Serio M, Mitolo CI, Rinaldi M, Altomare DF, Memeo V. Cholinergic stimulation and nonadrenergic, noncholinergic relaxation of human colonic circular muscle in idiopathic chronic constipation. *Dig Dis Sci* 1998; **43**: 2719-2726
  - 49 **Zhao RH**, Baig MK, Thaler KJ, Mack J, Abramson S, Woodhouse S, Tamir H, Wexner SD. Reduced expression of serotonin receptor(s) in the left colon of patients with colonic inertia. *Dis Colon Rectum* 2003; **46**: 81-86
  - 50 **Mollen RM**, Hopman WP, Kuipers HH, Jansen JB. Plasma cholecystokinin, plasma peptide YY and gallbladder motility in patients with slow transit constipation: effect of intestinal stimulation. *Digestion* 2000; **62**: 185-193
  - 51 **van der Sijp JR**, Kamm MA, Nightingale JM, Akkermans LM, Ghatei MA, Bloom SR, Jansen JB, Lennard-Jones JE. Circulating gastrointestinal hormone abnormalities in patients with severe idiopathic constipation. *Am J Gastroenterol* 1998; **93**: 1351-1356
  - 52 **Battle WM**, Snape WJ Jr, Alavi A, Cohen S, Braunstein S. Colonic dysfunction in diabetes mellitus. *Gastroenterology* 1980; **79**: 1217-1221
  - 53 **Meunier P**, Rochas A, Lambert R. Motor activity of the sigmoid colon in chronic constipation: comparative study with normal subjects. *Gut* 1979; **20**: 1095-1101
  - 54 **Schang JC**, Devroede G. Fasting and postprandial myoelectric spiking activity in the human sigmoid colon. *Gastroenterology* 1983; **85**: 1048-1053
  - 55 **Lembo A**, Camilleri M. Chronic constipation. *N Engl J Med* 2003; **349**: 1360-1368
  - 56 **Pezim ME**, Pemberton JH, Levin KE, Litchy WJ, Phillips SF. Parameters of anorectal and colonic motility in health and in severe constipation. *Dis Colon Rectum* 1993; **36**: 484-491
  - 57 **Rao SS**, Welcher KD, Leistikow JS. Obstructive defecation: a failure of rectoanal coordination. *Am J Gastroenterol* 1998; **93**: 1042-1050
  - 58 **Dinning PG**, Bampton PA, Andre J, Kennedy ML, Lubowski DZ, King DW, Cook IJ. Abnormal predefecatory colonic motor patterns define constipation in obstructed defecation. *Gastroenterology* 2004; **127**: 49-56
  - 59 **Gosselink MJ**, Schouten WR. Rectal sensory perception in females with obstructed defecation. *Dis Colon Rectum* 2001; **44**: 1337-1344
  - 60 **Gosselink MJ**, Hop WC, Schouten WR. Rectal compliance in females with obstructed defecation. *Dis Colon Rectum* 2001; **44**: 971-977
  - 61 **Gosselink MJ**, Schouten WR. The gastrorectal reflex in women with obstructed defecation. *Int J Colorectal Dis* 2001; **16**: 112-118
  - 62 **Liu S**, Zou K, Song J. A study of anorectal manometry in patients with chronic idiopathic constipation. *J Tongji Med Univ* 2000; **20**: 351-352
  - 63 **Shafik A**, Shafik AA, El-Sibai O, Ahmed I. Study of the role of the second defecation reflex: anorectal excitatory reflex in the pathogenesis of constipation. *J Am Coll Surg* 2003; **196**: 729-734
  - 64 **Rao SS**, Tuteja AK, Vellema T, Kempf J, Stessman M. Dyssynergic defecation: demographics, symptoms, stool patterns, and quality of life. *J Clin Gastroenterol* 2004; **38**: 680-685
  - 65 **De Lillo AR**, Rose S. Functional bowel disorders in the geriatric patient: constipation, fecal impaction, and fecal incontinence. *Am J Gastroenterol* 2000; **95**: 901-905
  - 66 **Donald IP**, Smith RG, Cruikshank JG, Elton RA, Stoddart ME. A study of constipation in the elderly living at home. *Gerontology* 1985; **31**: 112-118
  - 67 **Whitehead WE**, Drinkwater D, Cheskin LJ, Heller BR, Schuster MM. Constipation in the elderly living at home. Definition, prevalence, and relationship to lifestyle and health status. *J Am Geriatr Soc* 1989; **37**: 423-429
  - 68 **Harari D**, Gurwitz JH, Avorn J, Bohn R, Minaker KL. How do older persons define constipation? Implications for therapeutic management. *J Gen Intern Med* 1997; **12**: 63-66
  - 69 **Talley NJ**, O'Keefe EA, Zinsmeister AR, Melton LJ 3rd. Prevalence of gastrointestinal symptoms in the elderly: a population-based study. *Gastroenterology* 1992; **102**: 895-901
  - 70 **Camilleri M**, Lee JS, Viramontes B, Bharucha AE, Tangalos EG. Insights into the pathophysiology and mechanisms of constipation, irritable bowel syndrome, and diverticulosis in older people. *J Am Geriatr Soc* 2000; **48**: 1142-1150
  - 71 **Bannister JJ**, Abouzekry L, Read NW. Effect of aging on anorectal function. *Gut* 1987; **28**: 353-357
  - 72 **Engel AF**, Kamm MA. The acute effect of straining on pelvic floor neurological function. *Int J Colorectal Dis* 1994; **9**: 8-12
  - 73 **O'Keefe EA**, Talley NJ, Zinsmeister AR, Jacobsen SJ. Bowel disorders impair functional status and quality of life in the elderly: a population-based study. *J Gerontol A Biol Sci Med Sci* 1995; **50**: M184-M189

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## Updates on treatment of irritable bowel syndrome

Christopher W Hammerle, Christina M Surawicz

Christopher W Hammerle, Department of Medicine, University of Colorado Health Sciences Center, Denver 80011, United States  
Christina M Surawicz, Harborview Medical Center, University of Washington, BOX 359773 HMC, 325, 9th Avenue, Seattle, WA 98104, United States

**Author contribution:** Hammerle CW and Surawicz CM contributed equally to this work; Hammerle CW and Surawicz CM wrote the paper.

**Correspondence to:** Christina M Surawicz, MD, Professor of Medicine, Harborview Medical Center, University of Washington, BOX 359773 HMC, 325, 9th Avenue, Seattle, WA 98104, United States. [surawicz@u.washington.edu](mailto:surawicz@u.washington.edu)

Telephone: +1-206-3414634 Fax: +1-206-7318698

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### Abstract

Irritable bowel syndrome (IBS) is a highly prevalent gastrointestinal disorder characterized by abdominal pain and discomfort in association with altered bowel habits. It is estimated to affect 10%-15% of the Western population, and has a large impact on quality of life and (in)direct healthcare costs. IBS is a multifactorial disorder involving dysregulation within the brain-gut axis, and it is frequently associated with gastrointestinal motor and sensory dysfunction, enteric and central nervous system irregularities, neuroimmune dysregulation, and post-infectious inflammation. As with other functional medical disorders, the treatment for IBS can be challenging. Conventional therapy for those with moderate to severe symptoms is largely unsatisfactory, and the development of new and effective drugs is made difficult by the complex pathogenesis, variety of symptoms, and lack of objective clinical findings that are the hallmark of this disorder. Fortunately, research advances over the past several decades have provided insight into potential mechanisms responsible for the pathogenesis of IBS, and have led to the development of several promising pharmaceutical agents. In recent years there has been much publicity over several of these new IBS medications (alosetron and tegaserod) because of their reported association with ischemic colitis and cardiovascular disease. While these agents remain available for use under restricted prescribing programs, this highlights the need for continued development of safe and effective medication for IBS. This article provides a physiologically-based overview of recently developed and frequently employed pharmaceutical agents used to treat IBS, and discusses some non-pharmaceutical options that may be beneficial in this disorder.

### INTRODUCTION

Irritable bowel syndrome (IBS) is one of the most common functional gastrointestinal disorders affecting Western countries, with rates estimated as high as 10%-15% in the general population<sup>[1-3]</sup>. IBS is a heterogeneous condition broadly characterized by recurrent abdominal pain and discomfort with altered bowel habits and no detectable structural abnormalities<sup>[1]</sup>. In addition to constipation and/or diarrhea, frequently reported symptoms include abdominal pain and cramps, flatulence, fecal urgency, straining, a sense of incomplete evacuation and relief of pain or discomfort upon defecation.

IBS can be classified according to the predominant bowel symptoms: IBS with constipation predominant features (IBS-C), IBS with diarrhea predominant features (IBS-D), and IBS with alternating symptoms of diarrhea and constipation (IBS-A). While the exact pathophysiology of IBS is unclear, dysregulation within the brain-gut axis and interactions between genetics<sup>[4,5]</sup>, psychosocial factors<sup>[6,7]</sup>, post-inflammatory changes<sup>[8-10]</sup>, and motor<sup>[11]</sup> and sensory dysfunction<sup>[12]</sup> all likely play a role.

While only a fraction of IBS patients seek medical care<sup>[13]</sup>, this condition accounts for up to 20% of all referrals to gastroenterologists<sup>[14]</sup>. IBS has widespread economic ramifications in terms of both healthcare utilization and indirect costs incurred as a result of absenteeism from work<sup>[15,16]</sup>. Moreover, IBS is a cause of substantial morbidity and is associated with a lower health care-related quality of life<sup>[17]</sup>. It is clear that effective drugs for IBS are greatly needed.

### GENERAL TREATMENT APPROACH

IBS is a chronic, recurring condition with a wide range of symptoms. Therefore, the general goal of treatment

is to alleviate the symptoms of abdominal pain, altered bowel transit (diarrhea or constipation) and any associated symptoms such as bloating and fecal incontinence. The treatment approach should be individualized, and will depend on the intensity of symptoms and the degree of other comorbid conditions<sup>[18]</sup>. As with other functional medical disorders, the cornerstone of successful management revolves around establishing an effective patient-physician relationship<sup>[18,19]</sup>. The physician should be non-judgmental, listen actively to determine the patient's needs and concerns, and encourage the patient to participate in their medical care<sup>[20]</sup>. Well-established physician relationships and positive interactions with health care providers have been shown to be associated with fewer IBS-related follow-up visits and a lower utilization of health care resources<sup>[21]</sup>.

The majority of IBS patients are successfully managed in the primary care setting. These patients typically have mild symptoms and respond well to dietary and lifestyle modifications, education, and reassurance about their disease. Gut-directed medical therapy (anticholinergics, antispasmodics and newer IBS-specific agents) is used more frequently in patients with moderate to severe symptoms and is occasionally accompanied by the use of low dose tricyclic antidepressants (TCAs) and/or other psychiatric medications. The most severe, and smallest percentage of IBS cases, are often refractory to standard treatment and are likely to be seen at tertiary specialty centers. These patients will often require mental health providers, psychotropic medications, and may need frequent appointments with primary care providers to offer ongoing support throughout treatment<sup>[19,22]</sup>.

In all cases of IBS, it is important to establish realistic and consistent treatment goals<sup>[18]</sup>. Patients should be aware that a single drug is not likely to eradicate all symptoms, and that time, patience, and "trial and error" use of medications will be required. Additionally, physicians should educate patients about their diagnosis and provide reassurance that though this condition is a real medical disorder, it is a benign process that portends a normal life expectancy. In a long-term prognostic study of IBS, Owens and colleagues found that less than 10% of IBS patients developed an organic gastrointestinal disease, and that patients with IBS had survival rates not different from expected<sup>[21]</sup>. Misconceptions about the causes, diagnosis and treatment of IBS are common among patients. In a recent questionnaire-based study of 636 IBS patients, 80% believed their condition developed as a result of anxiety, nearly two-thirds believed diet was responsible for IBS, and one in seven patients believed that IBS leads to cancer<sup>[23]</sup>. Education about the possible mechanisms of IBS may dispel some of these misconceptions and it will help lay a foundation for the use of pharmacologic interventions if needed.

Physicians should be aware of other comorbidities that may worsen symptoms, and as with all chronic conditions, a detailed history about environmental stressors, social or emotional disturbances, impaired daily functioning, and underlying psychiatric conditions should be collected. This may help determine why the patient is seeking medical care and it could help uncover any potential hidden agendas.

## PHARMACEUTICAL THERAPIES

### Serotonin axis

Serotonin (5-hydroxytryptophan) is the most important neurotransmitter (NT) in the pathogenesis of IBS. It is a paracrine signaling molecule found extensively throughout the gastrointestinal tract (approximately 90% of all body stores) that modulates key functions such as motility, sensation, blood flow, and secretion<sup>[24-27]</sup>. Serotonin is stored primarily in enterochromaffin cells (90%) and in neurons of the enteric motor system (10%). Its release is triggered by luminal distension and chemical signals, and it binds to receptors located on enteric motor neurons, peripheral afferents, and within central nervous system domains that control appetite, mood and sexual function. The majority of serotonin receptors are G protein-coupled, with the exception of the 5-HT<sub>3</sub> receptor, which is a ligand-gated ion channel. 5-HT<sub>3</sub> and 5-HT<sub>4</sub> appear to be the most important NTs in IBS<sup>[28]</sup>. 5-HT<sub>3</sub> receptors modulate visceral pain, aid in peristalsis, and its receptors within the CNS appear to influence the emotional component of visceral stimulation<sup>[28-31]</sup>. 5-HT<sub>4</sub> is important in gastric emptying, colonic secretions, facilitating the peristaltic reflex, and it contracts or relaxes smooth muscle depending upon its location within the alimentary tract<sup>[26,32,33]</sup>. Serotonin levels and its activity on receptors are in part regulated by the serotonin reuptake transporter (SERT). Coates *et al* recently demonstrated that patients with IBS have decreased levels of SERT mRNA and protein expression in intestinal epithelial cells when compared to healthy volunteers<sup>[34]</sup>. Furthermore, polymorphisms within the SERT promoter region have been shown to be associated with the IBS-D phenotype<sup>[35]</sup>, and findings from a recent study suggest that their existence may influence the response to a 5-HT<sub>3</sub> antagonist in female IBS-D patients<sup>[36]</sup>. While the exact role of serotonin signaling in the pathophysiology of IBS remains to be fully elucidated, pharmacotherapy directed at modulating its activity has proven to be an effective way of treating many IBS symptoms.

### 5-HT<sub>3</sub> antagonists

Antagonism of the 5-HT<sub>3</sub> receptor results in slower small bowel and colonic transit times, a reduction in intestinal secretion, increased stool firmness, and an increase in colonic compliance<sup>[29,37]</sup>. There is also data suggesting that antagonism of this receptor in the amygdala, dorsal pons and ventral striatum may be responsible for perceived improvement in visceral pain, though the exact mechanism is unclear<sup>[38,39]</sup>.

Alosetron is a selective 5-HT<sub>3</sub> receptor antagonist initially approved by the Food and Drug Administration (FDA) in 2000 for use in female patients with IBS-D who fail in conservative treatment. Approval of this medication was based on several large randomized controlled-trials that found alosetron (1 mg *po* twice daily) was more effective than placebo in controlling abdominal pain and discomfort in IBS-D<sup>[40-43]</sup>. Alosetron was associated with a statistically significant decrease in the percentage of days with urgency, and it led to firmer stools and a decrease in stool frequency. Clinical improvement was noted throughout the treatment



period and usually occurred within 1-4 wk of therapy; symptoms returned to baseline following discontinuation of the medication. The most frequently reported adverse event was mild to moderate constipation which was self-limited and responded well to cessation of therapy. A meta-analysis by Cremonini in 2003 examined six randomized controlled-trials of alosetron and calculated a combined odds ratio of 1.8 for the adequate relief of pain [95% confidence interval (95% CI), 1.57-2.10] and the NNT of 7 patients in order to compare its relieving effect with placebo (95% CI, 5.74-9.43)<sup>[44]</sup>.

In late 2000, alosetron was withdrawn from the U.S. market over concerns regarding side-effects of severe constipation (approximately 70 cases), ischemic colitis (approximately 50 cases), and bowel perforation<sup>[45,46]</sup>. Following substantial public pressure, it was re-introduced into the U.S. market in 2002 under a restricted prescribing program and further post-marketing studies are currently underway. In a recent placebo-controlled study, Krause and colleagues examined the safety and efficacy of alosetron at varying doses, and found that one of 250 patients developed self-limited ischemic colitis (0.5 mg daily)<sup>[47]</sup>. In a systematic review of post-marketing surveillance data, Chang and colleagues estimated the rate of ischemic colitis to be 1.1 per 1000 patients. All cases of documented ischemic colitis were reversible and no long-term sequelae were noted<sup>[45]</sup>. These data suggest that the rate of adverse events is relatively low and that alosetron is a well-tolerated, effective medication that should be considered in the treatment of IBS-D. There are ongoing studies of alosetron in men.

Cilansetron is another 5-HT<sub>3</sub> antagonist that has been used for treatment for men and women with IBS-D. Its mechanism of action is similar to alosetron and it has comparable bioavailability and metabolism. Two large phase III trials have shown that cilansetron is similar in efficacy to alosetron, but concerns over ischemic colitis and severe constipation led to the FDA denying approval in 2005<sup>[48-51]</sup>.

Ramosetron is a potent 5-HT<sub>3</sub> receptor antagonist that is effective in relieving chemotherapy-induced nausea and vomiting, and is currently being developed for IBS-D<sup>[52,53]</sup>. Preliminary pharmacokinetic data suggest that it has a greater affinity, slower dissociation, and stronger antagonism at the 5-HT<sub>3</sub> receptor than either alosetron or cilansetron<sup>[54]</sup>. It also appears to be superior to both of these medications in inhibiting stress-induced defecation and stress-induced changes in colonic transit rates in rats<sup>[54,55]</sup>. Data in human use are not available but can be anticipated in the near future.

### 5-HT<sub>4</sub> antagonists

5-HT<sub>4</sub> receptors mediate the release of the excitatory NTs acetylcholine, substance P and calcitonin gene-related peptide which modulate peristalsis<sup>[32,33]</sup>. Selective antagonism at this receptor has been postulated to provide symptomatic relief in patients suffering from IBS-D. In a study of 52 healthy subjects, Piboserod (SB207266), a selective 5-HT<sub>4</sub> antagonist, was shown to block the effects of cisapride (5-HT<sub>4</sub> agonist) and it tended to delay colonic transit times

( $P = 0.06$ )<sup>[56]</sup>. In a placebo-controlled study of 15 patients with IBS-D, Houghton and colleagues reported that 20 mg of SB207266 significantly improved orocecal transit times and demonstrated a non-statistical trend toward improved rectal sensitivity<sup>[57]</sup>. Further studies with this class of medication are needed to confirm these results.

### 5-HT<sub>4</sub> agonists

Activation of 5-HT<sub>4</sub> receptors results in the release of excitatory neurotransmitters from enteric cholinergic neurons that function to modulate smooth muscle tone, electrolyte secretion and the peristaltic reflex<sup>[26,32,33]</sup>. Tegaserod is a 5-HT<sub>4</sub> agonist that was initially approved by the FDA for use in female patients with IBS-C, and in men and women under the age of 65 with chronic idiopathic constipation. It stimulates intestinal secretion of water and chloride and decreases the nociceptive response to rectal distension<sup>[58]</sup>. Several large, randomized-trials have shown that tegaserod has a significant impact on a patient's overall assessment of global relief, and that it statistically improves abdominal pain and bloating<sup>[59-62]</sup>. A 2004 Cochrane meta-analysis supported these results by concluding that tegaserod was associated with improvement in global relief of GI symptoms [relative risk (RR), 1.17; 95% CI, 1.08-1.27] and that it improved bowel consistency and frequency<sup>[63]</sup>. The primary side-effect of tegaserod is diarrhea (NNH of 20 patients), but it is usually transient and resolves with ongoing treatment<sup>[63,64]</sup>. Long-term use of tegaserod appears to be efficacious and re-treatment response rates appear to be similar to initial treatment<sup>[65,66]</sup>. Recent data regarding the effect of tegaserod in men have shown that it accelerates colonic transit time, but improvement in bowel symptoms failed to reach statistical significance<sup>[67]</sup>. In March 2007, Novartis voluntarily removed tegaserod from the U.S. and Canadian market as an FDA safety analysis of pooled data from 12 clinical trials demonstrated a statistical increase in the incidence of myocardial infarction, stroke and unstable angina. In July 2007, tegaserod was re-introduced to the U.S. market, but under a restricted investigational new drug (IND) protocol which limits its use to treatment of IBS-C and chronic idiopathic constipation in women under 55 years of age who meet specific guidelines. It continues to remain off market for general use.

Prucalopride is a new agent in a class of medications known as benzofurans. It is an agonist at the 5-HT<sub>4</sub> receptor, and data have suggested that it enhances GI transit in patients with functional constipation compared to placebo<sup>[68]</sup>. However, the clinical development of this drug remains unclear at this time based on the reported cases of intestinal carcinogenicity in animals<sup>[69]</sup>.

### Mixed 5-HT<sub>4</sub> agonist/5-HT<sub>3</sub> antagonist

Renzapride is a mixed agent that has shown promise for patients with IBS-C and with IBS-A. It is a full agonist of the 5-HT<sub>4</sub> receptor and an antagonist of the 5-HT<sub>3</sub> receptor. In a dose-ranging efficacy trial by Camilleri and colleagues, a statistically significant linear dose response to renzapride was observed for colonic transit and ascending colonic emptying time, but not for gastric emptying or small bowel transit time<sup>[70]</sup>. No clinical or laboratory adverse

events occurred during this study. In a dose-escalating pilot study of 17 patients, renzapride (2 mg twice daily) reduced overall gastrointestinal transit time and abdominal pain, increased the number of pain-free days and improved stool consistency<sup>[71]</sup>. Adverse events were similar in drug and placebo groups. Further studies are underway.

Mosapride is a benzamide compound with prokinetic properties that possesses both 5-HT<sub>4</sub> receptor agonist and 5-HT<sub>3</sub> receptor antagonist properties. In a study of Parkinson's patients, mosapride (15 mg/d) decreased colonic transit times and was associated with subjective improvements in bowel frequency<sup>[72]</sup>. Additional data in animals and humans have shown that mosapride accelerates gastric emptying and decreases overall colonic transit<sup>[73,74]</sup>, but does not appear to affect small bowel transit times<sup>[75]</sup>. The primary side effect is mild diarrhea, but unlike cisapride, it does not seem to cause the electrophysiological abnormalities that could lead to Torsades de Pointes<sup>[76]</sup>.

### ATI-7505

Another drug for the treatment of IBS-C is ATI-7505, a potent agonist of the 5HT<sub>4</sub> receptor that is similar in chemical structure to the prokinetic agent, cisapride. It has been engineered to reduce the cardiovascular toxicity of cisapride and does not have P450 dependent clearance at therapeutic levels<sup>[77]</sup>. Preliminary data from Camilleri and colleagues show that ATI-7505 accelerates overall colonic transit (10 mg three times daily), accelerates gastric emptying (20 mg three times daily), and loosens stool consistency. No safety issues were identified in this study<sup>[77]</sup>.

### Adrenergic modulators

Clonidine, an  $\alpha_2$  agonist initially developed as an antihypertensive agent, has been found to increase colonic compliance, delay small bowel transit, and reduce colonic tone and sensitivity in response to distension<sup>[78-81]</sup>. In a small study of 44 IBS-D patients, clonidine was associated with a significant reduction in bowel dysfunction and an improvement in IBS symptoms (67% *vs* 46% of placebo), but it did not alter gastrointestinal transit times or gastric volumes<sup>[82]</sup>. Relief was sustained throughout the 4-wk treatment protocol, and drowsiness, dizziness and dry mouth were the most frequently reported adverse events. Side-effects typically occurred when doses exceeded greater than 0.1 mg twice daily. This initial study suggests that  $\alpha_2$  agonists may play a beneficial role in IBS-D, though unwanted hypotension may be a side-effect that precludes its chronic use. Further studies are warranted.

### Somatostatin

Octreotide, a somatostatin analogue, works by activating somatostatin type-2 receptors and has been shown to reduce visceral sensitivity in response to rectal distension<sup>[83,84]</sup>. In healthy volunteers and in small numbers of IBS-D patients, octreotide administered in a 50 mg bolus was shown to prolong orocecal transit times and inhibit small bowel transit times<sup>[85,86]</sup>. While these results suggest that octreotide may have benefits in patients with IBS-D, its intravenous preparation precludes daily use, and to date, no studies with oral somatostatin analogues exist for the treatment of IBS.

### Opioid agents

Opioid receptors are found throughout the enteric nervous system and on nociceptive pathways that conduct pain to the central nervous system<sup>[87]</sup>. Altered bowel transit and visceral hypersensitivity are important components in IBS pathophysiology and peripherally-acting opioid receptors may be effective drug targets. Alvimopan is a peripherally acting  $\mu$ -opioid antagonist that is effective in treatment of post-operative ileus<sup>[88]</sup>. In a study of 74 healthy volunteers, it normalized colonic transit delays induced by the administration of codeine, and alone was shown to accelerate colonic transit<sup>[89]</sup>.

The  $\kappa$ -opioid agonist, asimadoline, exerts nociceptive properties on the GI tract at least in part by blocking Na receptor channels. It has been shown to decrease colonic tone during fasting and decrease colonic pain at low levels of distension<sup>[90]</sup>. In a study of 20 IBS-C patients, asimadoline was shown to be effective in decreasing pain perception from colonic distension without affecting colonic compliance or tone<sup>[91]</sup>.

More recently, a randomized, placebo-controlled trial by Szarka and colleagues showed that asimadoline (up to 1 mg four times daily) did not statistically improve abdominal pain when taken on an "as needed basis" compared to placebo<sup>[92]</sup>. Further studies are needed to determine the efficacy of this medication.

### CRH receptor antagonists

Corticotropin-releasing hormone (CRH) is a key mediator that regulates changes in colonic motility, visceral hypersensitivity, and autonomic function in response to stress<sup>[93,94]</sup>. Stress is processed within the CNS and increases both ACTH and CRH secretion, the latter stimulates colonic motility and inhibits gastric emptying<sup>[93,95]</sup>. Distension of the colon also activates CRH pathways in the brain, providing a plausible rationale for why visceral stimulation is perceived as anxiety or stress in some patients with IBS<sup>[93,96]</sup>. Fukudo and colleagues demonstrated that administration of intravenous CRH was associated with exaggeration of colonic motility and increased ACTH secretion in IBS patients compared to healthy controls<sup>[94]</sup>. More recently,  $\alpha$ -Helical CRH (a non-selective CRH receptor antagonist) has been shown to significantly reduce abdominal pain and anxiety ratings induced by electrical stimulation of the rectum of IBS patients, but not of controls<sup>[97]</sup>. Two CRH receptors appear important in the pathogenesis of IBS. Stimulation of the CRH-1 receptor is anxiogenic and associated with pro-inflammatory states, while CRH-2 receptors are involved in the inhibition of gastric emptying<sup>[96]</sup>. CRH-1 antagonists are currently under development for clinical use in IBS.

### Chloride channel activators

Lubiprostone is a member of a new class of bicyclic fatty-acid derivatives known as prostones, and was approved by the FDA in 2006 for the treatment of chronic constipation. It acts on type-2 chloride channels located on the apical side of gastrointestinal epithelial cells, and increases secretion of electrolyte-rich fluid into the small intestine, promoting increased motility<sup>[98,99]</sup>. Several studies have shown that in

patients with chronic constipation, lubiprostone is effective in improving the spontaneous bowel movement and is associated with less bloating, straining and abdominal discomfort<sup>[99,100]</sup>. The role of lubiprostone in IBS-C has recently been evaluated in a randomized, double-blinded placebo-controlled trial of approximately 200 patients<sup>[101]</sup>. The results of this study showed an overall improvement in abdominal symptoms and bowel movement when compared to placebo and the most frequently reported side-effect was mild to moderate nausea. To date, no serious adverse events have been noted in clinical trials<sup>[100,102]</sup>. Sucampo Pharmaceuticals submitted a supplemental New Drug Application to the FDA in September 2007 requesting approval for the use of lubiprostone in IBS-C. A decision is expected sometime in early 2008.

### **CCK antagonists**

Cholecystokinin (CCK) is a neuropeptide released by duodenal and jejunal enterochromaffin cells that stimulates secretion of pancreatic enzymes and decreases gastric emptying in response to dietary fat. CCK1 receptors are distributed within enteric neurons and vagal afferents, and antagonism at this receptor has been postulated to stimulate gut motility in patients with IBS-C. A recent double-blinded study found that the CCK antagonist, dexloxiglumide (200 mg three times daily) had no significant effect on satisfactory relief of IBS, and that it did not alter transit times in IBS-C<sup>[103]</sup>. Furthermore, two large phase III clinical studies of 1400 women with IBS-C found no statistical improvement in the symptoms of abdominal pain, discomfort or altered bowel habits when compared to placebo<sup>[103]</sup>. As a result, Forest Laboratories have discontinued development of this drug, though Rotta Research is pursuing additional placebo-controlled studies in Europe.

### **Neurokinin antagonists**

Substance P and neurokinin A are excitatory co-transmitters of cholinergic enteric neurons that have been well-linked to the pathophysiology of several neurologic and psychiatric disorders. Their receptors, neurokinin 1 (NK1) and NK2, play important roles in nociception and smooth muscle contraction, and the regulation of visceral sensitivity and mucosal inflammatory processes<sup>[104-107]</sup>. Clinical data is currently deficient. However, in one study of healthy volunteers using the selective NK2 antagonist nepadutant (MEN11420), IBS-like symptoms triggered by the infusion of neurokinin A were reduced<sup>[108]</sup>. Saredutant (SR48968), another NK2 antagonist, is also being developed for the treatment of IBS, but clinical results are not yet available.

### **Antidepressants**

Antidepressants are frequently prescribed by gastroenterologists for the treatment of IBS<sup>[109]</sup>. Their mechanism in IBS is not completely understood, but has been postulated to relate to an ability to modulate central and peripheral pain perception<sup>[110,111]</sup>, improve underlying psychiatric conditions, and possibly improve gut motility through modification of neurotransmitter activity<sup>[112,113]</sup>. Unfortunately, randomized-controlled trials to date

have largely been hindered by poor study design and methodological flaws, making it difficult to judge the therapeutic value of these agents<sup>[114-116]</sup>. Most studies provide little evidence that antidepressants are superior to placebo in improving specific IBS-related symptoms, but some do suggest that overall global well-being may be improved. In a recent meta-analysis evaluating 12 randomized-controlled trials, Jackson and colleagues concluded that tricyclic antidepressants are effective in improving global IBS symptoms (OR, 4.2; 95% CI, 2.3-7.9), and calculated an NNT of only 3 in order to see an effect<sup>[117]</sup>. Fewer studies have been conducted with SSRIs. In one randomized-controlled trial comparing paroxetine (10-40 mg/d) to placebo, patients treated with the SSRI reported a significant improvement in overall well-being, but did not experience improvement in abdominal pain<sup>[118]</sup>. Other studies with paroxetine have shown an improvement in abdominal pain and discomfort, and suggest that further studies with this class of medication are needed<sup>[119,120]</sup>. While certain subgroups of IBS patients (particularly those with psychiatric comorbidities such as depression or anxiety) will likely benefit from the use of antidepressants, their use in other patients (particularly the elderly) should be met with caution as side-effects are common. Furthermore, the anticholinergic nature of some of these medications limits their use in patients with IBS-C. Antidepressants should not be used to relieve the target symptoms of IBS, and their use will not likely alter GI motility or physiology. Instead, antidepressants should be used as an adjunct in patients with moderate to severe IBS to help improve overall quality of life, well-being, and patient satisfaction with treatment.

### **Antispasmodics**

Antispasmodic agents are believed to work in IBS based on their ability to decrease intestinal smooth muscle activity. There are two broad categories of antispasmodic agents: anticholinergics/antimuscarinic agents (e.g. hyoscyamine, dicyclomine, cimetropium) and direct smooth muscle relaxing agents (e.g. mebeverine, pinaverine, octylonium bromide). Most studies of smooth muscle relaxants have been hindered by poor study design, high drop-out rates, and low patient enrollment which have made assessment of their therapeutic value in IBS difficult<sup>[121-123]</sup>. In a recent meta-analysis, only octylonium bromide was found to have some benefit after excluding poor quality studies<sup>[122]</sup>.

In a review of anticholinergic drugs, Schoenfeld and colleagues found similar problems with study design, and based on the poor quality of trials and marginal data, concluded that any benefit observed with these agents was likely due to placebo effect<sup>[123]</sup>. Additionally, their substantial side-effect profile makes these agents a suboptimal choice for IBS therapy.

### **Antidiarrheals**

Loperamide is one of the most frequently used drugs for IBS-D. It is a synthetic opioid that decreases intestinal transit, and increases intestinal water and ion absorption. Several RCTs have provided a good evidence that loperamide decreases stool frequency and improves stool



consistency in IBS-D<sup>[124-126]</sup>. Loperamide does not appear to improve abdominal pain and should only be considered in cases of painless diarrhea.

### Benzodiazepines

Dextroisopam is a 2, 3 benzodiazepine that has been used outside of the U.S. for treatment of anxiety and autonomic and stress-related disorders. Unlike typical benzodiazepines which bind GABA receptors, dextroisopam binds 2, 3 benzodiazepine receptors. Results from a 12-wk placebo-controlled phase IIb trial showed that dextroisopam (200 mg twice daily) was associated with longer periods of overall relief from IBS symptoms than placebo during the treatment period (57% *vs* 43%). Stool frequency and consistency were also improved<sup>[127]</sup>. Further trials are underway.

### Antibiotics

Small intestinal bacterial overgrowth (SIBO) has been proposed to be common in patients with IBS. Using lactulose breath tests (LBT), Pimental and colleagues showed that 78% of IBS patients had SIBO<sup>[128]</sup> and that eradication with a seven-day course of neomycin was associated with a significant reduction in symptoms<sup>[129]</sup>. However, these results are somewhat controversial, as both the accuracy of the LBT and its ability to gauge treatment response has been questioned<sup>[130-133]</sup>. More recently, Posserud and colleagues conducted a study of 162 IBS patients using cultures of jejunal aspirates to detect SIBO<sup>[134]</sup>. They found that higher bacterial counts were present in IBS patients compared to placebo (43% *vs* 12%), but this finding was not related to small intestinal motility. Furthermore, using a standard definition of SIBO ( $> 10^5$  bacteria/mL), there was no difference between IBS patients and healthy controls. Further research is needed in this area, including a better evaluation of the long-term effects of SIBO eradication.

## NON-PHARMACEUTICAL THERAPY

### Bulking agents

The use of fiber and bulking agents remains a mainstay of therapy for patients with IBS-C, though their efficacy is controversial. The proposed mechanism for fiber is a decrease in intra-colonic pressures and an acceleration of oroanal transit<sup>[135,136]</sup> though results from studies on transit times are conflicting<sup>[137,138]</sup>. There have been a number of trials evaluating the effectiveness of fiber, but many have methodological limitations including small sample sizes, short follow-up periods, selection bias, and lack of well-defined endpoints<sup>[123,139]</sup>. In a recent systematic review of 17 randomized controlled trials examining the role of fiber in the treatment of IBS, Bijkerk *et al* found only a minimal improvement in overall relief of IBS global symptoms (RR 1.33; 95% CI, 1.19-1.50), and found no evidence that fiber reduces abdominal pain<sup>[140]</sup>. Furthermore, the effectiveness on individual symptoms was variable and insoluble fiber (corn, wheat bran) was found to actually worsen symptoms in some patients<sup>[140]</sup>. In another meta-analysis of 13 placebo-controlled studies, fiber was found to improve global symptoms of IBS. But after exclusion of low-quality

studies, the result did not reach statistical significance<sup>[122]</sup>. Abnormal bacterial fermentation and disturbed gas handling may also cause bloating and abdominal pain and may worsen clinical outcome<sup>[141]</sup>. The American College of Gastroenterology Functional Gastrointestinal Disorders Task Force currently recommends the use of fiber in patients with constipation, but does not recommend its use in IBS<sup>[142]</sup>.

### Probiotics

The presence of low-grade inflammation and immune activation in some patients with IBS suggests that alterations in indigenous gut flora may play an important role in this disorder. Probiotics may work by helping restore both qualitative (i.e. depleted bifidobacteria species) and quantitative (i.e. small intestinal bacterial overgrowth) alterations in intestinal flora<sup>[143,144]</sup>. Lactobacilli and bifidobacteria are two of the most frequently studied probiotics, and several trials have shown that their use is associated with improvement in IBS symptoms<sup>[145-148]</sup>. Niedzielin and colleagues demonstrated a complete resolution of abdominal pain in patients who took *L. plantarum* compared to approximately 50% of placebo controls<sup>[146]</sup>. Other studies have shown that *L. plantarum* is associated with a decrease in bloating and may reduce global IBS symptom index scores<sup>[145,147]</sup>. Post-infectious IBS may lead to abnormal immune activation and a persistent inflammatory state<sup>[149]</sup>. The bifidobacterial species may be beneficial in this subset of IBS as it appears to possess immune-modulating activity through an ability to alter levels of IL-10 and IL-12<sup>[150]</sup>.

To date, most studies assessing the efficacy of probiotics have been small, and it has been difficult to compare results across studies largely because of non-standardized formulations of probiotics. Nonetheless, data suggests that probiotics may be beneficial, and a good safety profile makes them a reasonable choice for IBS.

### Diet

Two-thirds of IBS patients believe that their disorder is related to diet<sup>[23]</sup>. They often complain of postprandial worsening of symptoms, and are intolerant to certain foods. Visceral hypersensitivity, motility disorders, gas-handling disturbances, and abnormal carbohydrate absorption are abnormalities that may explain some of these findings. However, presence of underlying psychological issues also affects the diet-related symptoms<sup>[151]</sup>. In a recent pilot study of 20 IBS patients, Drisko and colleagues found that elimination and rotation diets based on the results of IgG and IgE food and mold panels led to a substantial improvement in stool frequency, abdominal pain, and quality-of-life scores<sup>[152]</sup>. These results were sustained at the one year follow-up. Previous studies on elimination diets have produced more conflicting results, showing an effectiveness rate ranging from 15% to 71%<sup>[153]</sup>. Nonetheless, dietary modification may be an option for patients who fail in the standard therapy.

## CONCLUSION

The complex nature of IBS continues to pose a significant

treatment challenge for patients and practitioners. Traditional medications such as antispasmodics, bulking agents, and antidepressants are frequently prescribed for IBS. But, they are seldom efficacious in patients with advanced symptoms. Fortunately, the recent efforts of basic scientists and clinician investigators have elucidated many of the neurotransmitters, effectors and neuroenteric interactions involved in the pathophysiology of IBS, and have led to the development of several new and promising therapeutic agents. Over the past years, the serotonergic medications, tegaserod and alosetron, have been proven to significantly improve patients' overall quality of life and effectively manage many of the motor and sensory abnormalities in IBS. With more progress made in our understanding of IBS and more data obtained from phase III trials, we can expect to see several other classes of medications, such as CCK antagonists, NK antagonists, CRH antagonists, opioid-receptor agents and chloride channel activators, in the near future.

## REFERENCES

- Drossman DA**, Camilleri M, Mayer EA, Whitehead WE. AGA technical review on irritable bowel syndrome. *Gastroenterology* 2002; **123**: 2108-2131
- Cremonini F**, Talley NJ. Irritable bowel syndrome: epidemiology, natural history, health care seeking and emerging risk factors. *Gastroenterol Clin North Am* 2005; **34**: 189-204
- Drossman DA**, Li Z, Andruzzi E, Temple RD, Talley NJ, Thompson WG, Whitehead WE, Janssens J, Funch-Jensen P, Corazziari E. U.S. householder survey of functional gastrointestinal disorders. Prevalence, sociodemography, and health impact. *Dig Dis Sci* 1993; **38**: 1569-1580
- Morris-Yates A**, Talley NJ, Boyce PM, Nandurkar S, Andrews G. Evidence of a genetic contribution to functional bowel disorder. *Am J Gastroenterol* 1998; **93**: 1311-1317
- Levy RL**, Jones KR, Whitehead WE, Feld SI, Talley NJ, Corey LA. Irritable bowel syndrome in twins: heredity and social learning both contribute to etiology. *Gastroenterology* 2001; **121**: 799-804
- Lydiard RB**, Falsetti SA. Experience with anxiety and depression treatment studies: implications for designing irritable bowel syndrome clinical trials. *Am J Med* 1999; **107**: 65S-73S
- Whitehead WE**, Crowell MD. Psychologic considerations in the irritable bowel syndrome. *Gastroenterol Clin North Am* 1991; **20**: 249-267
- Spiller RC**. Inflammation as a basis for functional GI disorders. *Best Pract Res Clin Gastroenterol* 2004; **18**: 641-661
- Spiller R**, Campbell E. Post-infectious irritable bowel syndrome. *Curr Opin Gastroenterol* 2006; **22**: 13-17
- O'Mahony L**, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, O'Sullivan GC, Kiely B, Collins JK, Shanahan F, Quigley EM. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. *Gastroenterology* 2005; **128**: 541-551
- Serra J**, Azpiroz F, Malagelada JR. Impaired transit and tolerance of intestinal gas in the irritable bowel syndrome. *Gut* 2001; **48**: 14-19
- Kellow JE**, Eckersley CM, Jones MP. Enhanced perception of physiological intestinal motility in the irritable bowel syndrome. *Gastroenterology* 1991; **101**: 1621-1627
- Drossman DA**, Sandler RS, McKee DC, Lovitz AJ. Bowel patterns among subjects not seeking health care. Use of a questionnaire to identify a population with bowel dysfunction. *Gastroenterology* 1982; **83**: 529-534
- Thompson WG**, Heaton KW, Smyth GT, Smyth C. Irritable bowel syndrome in general practice: prevalence, characteristics, and referral. *Gut* 2000; **46**: 78-82
- Hulisz D**. The burden of illness of irritable bowel syndrome: current challenges and hope for the future. *J Manag Care Pharm* 2004; **10**: 299-309
- Leong SA**, Barghout V, Birnbaum HG, Thibeault CE, Ben-Hamadi R, Frech F, Ofman JJ. The economic consequences of irritable bowel syndrome: a US employer perspective. *Arch Intern Med* 2003; **163**: 929-935
- Gralnek IM**, Hays RD, Kilbourne A, Naliboff B, Mayer EA. The impact of irritable bowel syndrome on health-related quality of life. *Gastroenterology* 2000; **119**: 654-660
- Drossman DA**, Thompson WG. The irritable bowel syndrome: review and a graduated multicomponent treatment approach. *Ann Intern Med* 1992; **116**: 1009-1016
- Drossman DA**. Diagnosing and treating patients with refractory functional gastrointestinal disorders. *Ann Intern Med* 1995; **123**: 688-697
- Halpert A**, Dalton CB, Palsson O, Morris C, Hu Y, Bangdiwala S, Hankins J, Norton N, Drossman D. What patients know about irritable bowel syndrome (IBS) and what they would like to know. National Survey on Patient Educational Needs in IBS and development and validation of the Patient Educational Needs Questionnaire (PEQ). *Am J Gastroenterol* 2007; **102**: 1972-82
- Owens DM**, Nelson DK, Talley NJ. The irritable bowel syndrome: long-term prognosis and the physician-patient interaction. *Ann Intern Med* 1995; **122**: 107-112
- North CS**, Hong BA, Alpers DH. Relationship of functional gastrointestinal disorders and psychiatric disorders: implications for treatment. *World J Gastroenterol* 2007; **13**: 2020-2027
- Lacy BE**, Weiser K, Noddin L, Robertson DJ, Crowell MD, Parratt-Engstrom C, Grau MV. Irritable bowel syndrome: patients' attitudes, concerns and level of knowledge. *Aliment Pharmacol Ther* 2007; **25**: 1329-1341
- Greenwood-van Meerveld B**. Importance of 5-hydroxytryptamine receptors on intestinal afferents in the regulation of visceral sensitivity. *Neurogastroenterol Motil* 2007; **19** Suppl 2: 13-18
- Gershon MD**. Review article: serotonin receptors and transporters -- roles in normal and abnormal gastrointestinal motility. *Aliment Pharmacol Ther* 2004; **20** Suppl 7: 3-14
- Gershon MD**. Review article: roles played by 5-hydroxytryptamine in the physiology of the bowel. *Aliment Pharmacol Ther* 1999; **13** Suppl 2: 15-30
- Crowell MD**. Role of serotonin in the pathophysiology of the irritable bowel syndrome. *Br J Pharmacol* 2004; **141**: 1285-1293
- Gershon MD**. Serotonin and its implication for the management of irritable bowel syndrome. *Rev Gastroenterol Disord* 2003; **3** Suppl 2: S25-S34
- Gershon MD**. 5-HT (serotonin) physiology and related drugs. *Curr Opin Gastroenterol* 2000; **16**: 113-120
- Bardhan KD**, Bodemar G, Geldof H, Schutz E, Heath A, Mills JG, Jacques LA. A double-blind, randomized, placebo-controlled dose-ranging study to evaluate the efficacy of alosetron in the treatment of irritable bowel syndrome. *Aliment Pharmacol Ther* 2000; **14**: 23-34
- Houghton LA**, Foster JM, Whorwell PJ. Alosetron, a 5-HT<sub>3</sub> receptor antagonist, delays colonic transit in patients with irritable bowel syndrome and healthy volunteers. *Aliment Pharmacol Ther* 2000; **14**: 775-782
- Grider JR**, Foxx-Orenstein AE, Jin JG. 5-Hydroxytryptamine<sub>4</sub> receptor agonists initiate the peristaltic reflex in human, rat, and guinea pig intestine. *Gastroenterology* 1998; **115**: 370-380
- Hegde SS**, Eglén RM. Peripheral 5-HT<sub>4</sub> receptors. *FASEB J* 1996; **10**: 1398-1407
- Coates MD**, Mahoney CR, Linden DR, Sampson JE, Chen J, Blaszyk H, Crowell MD, Sharkey KA, Gershon MD, Mawe GM, Moses PL. Molecular defects in mucosal serotonin content and decreased serotonin reuptake transporter in ulcerative colitis and irritable bowel syndrome. *Gastroenterology* 2004; **126**: 1657-1664
- Yeo A**, Boyd P, Lumsden S, Saunders T, Handley A, Stubbins

- M, Knaggs A, Asquith S, Taylor I, Bahari B, Crocker N, Rallan R, Varsani S, Montgomery D, Alpers DH, Dukes GE, Purvis I, Hicks GA. Association between a functional polymorphism in the serotonin transporter gene and diarrhoea predominant irritable bowel syndrome in women. *Gut* 2004; **53**: 1452-1458
- 36 **Camilleri M**, Atanasova E, Carlson PJ, Ahmad U, Kim HJ, Viramontes BE, McKinzie S, Urrutia R. Serotonin-transporter polymorphism pharmacogenetics in diarrhea-predominant irritable bowel syndrome. *Gastroenterology* 2002; **123**: 425-432
- 37 **Delvaux M**, Louvel D, Mamet JP, Campos-Oriola R, Frexinos J. Effect of alosetron on responses to colonic distension in patients with irritable bowel syndrome. *Aliment Pharmacol Ther* 1998; **12**: 849-855
- 38 **Berman SM**, Chang L, Suyenobu B, Derbyshire SW, Stains J, Fitzgerald L, Mandelkern M, Hamm L, Vogt B, Naliboff BD, Mayer EA. Condition-specific deactivation of brain regions by 5-HT<sub>3</sub> receptor antagonist Alosetron. *Gastroenterology* 2002; **123**: 969-977
- 39 **Mayer EA**, Berman S, Derbyshire SW, Suyenobu B, Chang L, Fitzgerald L, Mandelkern M, Hamm L, Vogt B, Naliboff BD. The effect of the 5-HT<sub>3</sub> receptor antagonist, alosetron, on brain responses to visceral stimulation in irritable bowel syndrome patients. *Aliment Pharmacol Ther* 2002; **16**: 1357-1366
- 40 **Camilleri M**, Mayer EA, Drossman DA, Heath A, Dukes GE, McSorley D, Kong S, Mangel AW, Northcutt AR. Improvement in pain and bowel function in female irritable bowel patients with alosetron, a 5-HT<sub>3</sub> receptor antagonist. *Aliment Pharmacol Ther* 1999; **13**: 1149-1159
- 41 **Camilleri M**, Northcutt AR, Kong S, Dukes GE, McSorley D, Mangel AW. Efficacy and safety of alosetron in women with irritable bowel syndrome: a randomised, placebo-controlled trial. *Lancet* 2000; **355**: 1035-1040
- 42 **Camilleri M**, Chey WY, Mayer EA, Northcutt AR, Heath A, Dukes GE, McSorley D, Mangel AM. A randomized controlled clinical trial of the serotonin type 3 receptor antagonist alosetron in women with diarrhea-predominant irritable bowel syndrome. *Arch Intern Med* 2001; **161**: 1733-1740
- 43 **Lembo T**, Wright RA, Bagby B, Decker C, Gordon S, Jhingan P, Carter E. Alosetron controls bowel urgency and provides global symptom improvement in women with diarrhea-predominant irritable bowel syndrome. *Am J Gastroenterol* 2001; **96**: 2662-2670
- 44 **Cremonini F**, Delgado-Aros S, Camilleri M. Efficacy of alosetron in irritable bowel syndrome: a meta-analysis of randomized controlled trials. *Neurogastroenterol Motil* 2003; **15**: 79-86
- 45 **Chang L**, Chey WD, Harris L, Olden K, Surawicz C, Schoenfeld P. Incidence of ischemic colitis and serious complications of constipation among patients using alosetron: systematic review of clinical trials and post-marketing surveillance data. *Am J Gastroenterol* 2006; **101**: 1069-1079
- 46 **Horton R**. Lotronex and the FDA: a fatal erosion of integrity. *Lancet* 2001; **357**: 1544-1545
- 47 **Krause R**, Ameen V, Gordon SH, West M, Heath AT, Perschy T, Carter EG. A randomized, double-blind, placebo-controlled study to assess efficacy and safety of 0.5 mg and 1 mg alosetron in women with severe diarrhea-predominant IBS. *Am J Gastroenterol* 2007; **102**: 1709-1719
- 48 **Cilansetron**: KC 9946. *Drugs R D* 2005; **6**: 169-173
- 49 **Chey WD**, Cash BD. Cilansetron: a new serotonergic agent for the irritable bowel syndrome with diarrhoea. *Expert Opin Investig Drugs* 2005; **14**: 185-193
- 50 **Olden KW**, Crowell MD. Cilansetron. *Drugs Today (Barc)* 2005; **41**: 661-666
- 51 **Stacher G**. Cilansetron. Solvay. *Curr Opin Investig Drugs* 2001; **2**: 1432-1436
- 52 **Rabasseda X**. Ramosetron, a 5-HT<sub>3</sub> receptor antagonist for the control of nausea and vomiting. *Drugs Today (Barc)* 2002; **38**: 75-89
- 53 **Shi Y**, He X, Yang S, Ai B, Zhang C, Huang D, Dong M, Liu P, Zhou S, Han X. Ramosetron versus ondansetron in the prevention of chemotherapy-induced gastrointestinal side effects: A prospective randomized controlled study. *Chemotherapy* 2007; **53**: 44-50
- 54 **Hirata T**, Keto Y, Funatsu T, Akuzawa S, Sasamata M. Evaluation of the pharmacological profile of ramosetron, a novel therapeutic agent for irritable bowel syndrome. *J Pharmacol Sci* 2007; **104**: 263-273
- 55 **Funatsu T**, Takeuchi A, Hirata T, Keto Y, Akuzawa S, Sasamata M. Effect of ramosetron on conditioned emotional stress-induced colonic dysfunction as a model of irritable bowel syndrome in rats. *Eur J Pharmacol* 2007; **573**: 190-195
- 56 **Bharucha AE**, Camilleri M, Haydock S, Ferber I, Burton D, Cooper S, Tompson D, Fitzpatrick K, Higgins R, Zinsmeister AR. Effects of a serotonin 5-HT<sub>4</sub> receptor antagonist SB-207266 on gastrointestinal motor and sensory function in humans. *Gut* 2000; **47**: 667-674
- 57 **Houghton LA**, Jackson NA, Whorwell PJ, Cooper SM. 5-HT<sub>4</sub> receptor antagonism in irritable bowel syndrome: effect of SB-207266-A on rectal sensitivity and small bowel transit. *Aliment Pharmacol Ther* 1999; **13**: 1437-1444
- 58 **Prather CM**, Camilleri M, Zinsmeister AR, McKinzie S, Thomforde G. Tegaserod accelerates orocecal transit in patients with constipation-predominant irritable bowel syndrome. *Gastroenterology* 2000; **118**: 463-468
- 59 **Muller-Lissner SA**, Fumagalli I, Bardhan KD, Pace F, Pecher E, Nault B, Ruegg P. Tegaserod, a 5-HT<sub>4</sub> receptor partial agonist, relieves symptoms in irritable bowel syndrome patients with abdominal pain, bloating and constipation. *Aliment Pharmacol Ther* 2001; **15**: 1655-1666
- 60 **Novick J**, Miner P, Krause R, Glebas K, Bliesath H, Ligozio G, Ruegg P, Lefkowitz M. A randomized, double-blind, placebo-controlled trial of tegaserod in female patients suffering from irritable bowel syndrome with constipation. *Aliment Pharmacol Ther* 2002; **16**: 1877-1888
- 61 **Kellow J**, Lee OY, Chang FY, Thongsawat S, Mazlam MZ, Yuen H, Gwee KA, Bak YT, Jones J, Wagner A. An Asia-Pacific, double blind, placebo controlled, randomised study to evaluate the efficacy, safety, and tolerability of tegaserod in patients with irritable bowel syndrome. *Gut* 2003; **52**: 671-676
- 62 **Nyhlin H**, Bang C, Elsborg L, Silvennoinen J, Holme I, Ruegg P, Jones J, Wagner A. A double-blind, placebo-controlled, randomized study to evaluate the efficacy, safety and tolerability of tegaserod in patients with irritable bowel syndrome. *Scand J Gastroenterol* 2004; **39**: 119-126
- 63 **Evans BW**, Clark WK, Moore DJ, Whorwell PJ. Tegaserod for the treatment of irritable bowel syndrome. *Cochrane Database Syst Rev* 2004; CD003960
- 64 **Quigley EM**, Wald A, Fidelholtz J, Boivin M, Pecher E, Earnest D. Safety and tolerability of tegaserod in patients with chronic constipation: pooled data from two phase III studies. *Clin Gastroenterol Hepatol* 2006; **4**: 605-613
- 65 **Muller-Lissner S**, Kamm MA, Musoglu A, Earnest DL, Dunger-Baldauf C, Shetzline MA. Safety, tolerability, and efficacy of tegaserod over 13 months in patients with chronic constipation. *Am J Gastroenterol* 2006; **101**: 2558-2569; quiz 2671
- 66 **Muller-Lissner S**, Holtmann G, Rueegg P, Weidinger G, Loffler H. Tegaserod is effective in the initial and retreatment of irritable bowel syndrome with constipation. *Aliment Pharmacol Ther* 2005; **21**: 11-20
- 67 **Harish K**, Hazeena K, Thomas V, Kumar S, Jose T, Narayanan P. Effect of tegaserod on colonic transit time in male patients with constipation-predominant irritable bowel syndrome. *J Gastroenterol Hepatol* 2007; **22**: 1183-1189
- 68 **Coremans G**, Kerstens R, De Pauw M, Stevens M. Prucalopride is effective in patients with severe chronic constipation in whom laxatives fail to provide adequate relief. Results of a double-blind, placebo-controlled clinical trial. *Digestion* 2003; **67**: 82-89
- 69 **Kamm MA**. Review article: the complexity of drug development for irritable bowel syndrome. *Aliment Pharmacol Ther* 2002; **16**: 343-351
- 70 **Camilleri M**, McKinzie S, Fox J, Foxx-Orenstein A, Burton D, Thomforde G, Baxter K, Zinsmeister AR. Effect of renzapride

- on transit in constipation-predominant irritable bowel syndrome. *Clin Gastroenterol Hepatol* 2004; **2**: 895-904
- 71 **Tack J**, Middleton SJ, Horne MC, Piessevaux H, Bloor JS, Meyers NL, Palmer RM. Pilot study of the efficacy of renzapride on gastrointestinal motility and symptoms in patients with constipation-predominant irritable bowel syndrome. *Aliment Pharmacol Ther* 2006; **23**: 1655-1665
  - 72 **Liu Z**, Sakakibara R, Odaka T, Uchiyama T, Uchiyama T, Yamamoto T, Ito T, Asahina M, Yamaguchi K, Yamaguchi T, Hattori T. Mosapride citrate, a novel 5-HT<sub>4</sub> agonist and partial 5-HT<sub>3</sub> antagonist, ameliorates constipation in parkinsonian patients. *Mov Disord* 2005; **20**: 680-686
  - 73 **Mine Y**, Yoshikawa T, Oku S, Nagai R, Yoshida N, Hosoki K. Comparison of effect of mosapride citrate and existing 5-HT<sub>4</sub> receptor agonists on gastrointestinal motility in vivo and in vitro. *J Pharmacol Exp Ther* 1997; **283**: 1000-1008
  - 74 **Inui A**, Yoshikawa T, Nagai R, Yoshida N, Ito T. Effects of mosapride citrate, a 5-HT<sub>4</sub> receptor agonist, on colonic motility in conscious guinea pigs. *Jpn J Pharmacol* 2002; **90**: 313-320
  - 75 **Wei W**, Ge ZZ, Lu H, Gao YJ, Hu YB, Xiao SD. Effect of mosapride on gastrointestinal transit time and diagnostic yield of capsule endoscopy. *J Gastroenterol Hepatol* 2007; **22**: 1605-1608
  - 76 **Carlsson L**, Amos GJ, Andersson B, Drews L, Duker G, Wadstedt G. Electrophysiological characterization of the prokinetic agents cisapride and mosapride in vivo and in vitro: implications for proarrhythmic potential? *J Pharmacol Exp Ther* 1997; **282**: 220-227
  - 77 **Foxx-Orenstein AE**, Camilleri M, Szarka LA, McKinzie S, Burton D, Thomforde G, Baxter K, Zinsmeister AR. Does co-administration of a non-selective opiate antagonist enhance acceleration of transit by a 5-HT<sub>4</sub> agonist in constipation-predominant irritable bowel syndrome? A randomized controlled trial. *Neurogastroenterol Motil* 2007; **19**: 821-830
  - 78 **Bharucha AE**, Camilleri M, Zinsmeister AR, Hanson RB. Adrenergic modulation of human colonic motor and sensory function. *Am J Physiol* 1997; **273**: G997-G1006
  - 79 **Malcolm A**, Camilleri M, Kost L, Burton DD, Fett SL, Zinsmeister AR. Towards identifying optimal doses for alpha-2 adrenergic modulation of colonic and rectal motor and sensory function. *Aliment Pharmacol Ther* 2000; **14**: 783-793
  - 80 **Viramontes BE**, Malcolm A, Camilleri M, Szarka LA, McKinzie S, Burton DD, Zinsmeister AR. Effects of an alpha(2)-adrenergic agonist on gastrointestinal transit, colonic motility, and sensation in humans. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G1468-G1476
  - 81 **Malcolm A**, Phillips SF, Camilleri M, Hanson RB. Pharmacological modulation of rectal tone alters perception of distention in humans. *Am J Gastroenterol* 1997; **92**: 2073-2079
  - 82 **Camilleri M**, Kim DY, McKinzie S, Kim HJ, Thomforde GM, Burton DD, Low PA, Zinsmeister AR. A randomized, controlled exploratory study of clonidine in diarrhea-predominant irritable bowel syndrome. *Clin Gastroenterol Hepatol* 2003; **1**: 111-121
  - 83 **Schwetz I**, Naliboff B, Munakata J, Lembo T, Chang L, Matin K, Ohning G, Mayer EA. Anti-hyperalgesic effect of octreotide in patients with irritable bowel syndrome. *Aliment Pharmacol Ther* 2004; **19**: 123-131
  - 84 **Hasler WL**, Soudah HC, Owyang C. Somatostatin analog inhibits afferent response to rectal distention in diarrhea-predominant irritable bowel patients. *J Pharmacol Exp Ther* 1994; **268**: 1206-1211
  - 85 **O'Donnell LJ**, Watson AJ, Cameron D, Farthing MJ. Effect of octreotide on mouth-to-caecum transit time in healthy subjects and in the irritable bowel syndrome. *Aliment Pharmacol Ther* 1990; **4**: 177-181
  - 86 **von der Ohe MR**, Camilleri M, Thomforde GM, Klee GG. Differential regional effects of octreotide on human gastrointestinal motor function. *Gut* 1995; **36**: 743-748
  - 87 **Sternini C**. Receptors and transmission in the brain-gut axis: potential for novel therapies. III. Mu-opioid receptors in the enteric nervous system. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G8-G15
  - 88 **Delaney CP**, Wolff BG, Viscusi ER, Senagore AJ, Fort JG, Du W, Techner L, Wallin B. Alvimopan, for postoperative ileus following bowel resection: a pooled analysis of phase III studies. *Ann Surg* 2007; **245**: 355-363
  - 89 **Gonenne J**, Camilleri M, Ferber I, Burton D, Baxter K, Keyashian K, Foss J, Wallin B, Du W, Zinsmeister AR. Effect of alvimopan and codeine on gastrointestinal transit: a randomized controlled study. *Clin Gastroenterol Hepatol* 2005; **3**: 784-791
  - 90 **Delgado-Aros S**, Chial HJ, Camilleri M, Szarka LA, Weber FT, Jacob J, Ferber I, McKinzie S, Burton DD, Zinsmeister AR. Effects of a kappa-opioid agonist, asimadoline, on satiation and GI motor and sensory functions in humans. *Am J Physiol Gastrointest Liver Physiol* 2003; **284**: G558-G566
  - 91 **Delvaux M**, Beck A, Jacob J, Bouzamondo H, Weber FT, Frexinos J. Effect of asimadoline, a kappa opioid agonist, on pain induced by colonic distension in patients with irritable bowel syndrome. *Aliment Pharmacol Ther* 2004; **20**: 237-246
  - 92 **Szarka LA**, Camilleri M, Burton D, Fox JC, McKinzie S, Stanislav T, Simonson J, Sullivan N, Zinsmeister AR. Efficacy of on-demand asimadoline, a peripheral kappa-opioid agonist, in females with irritable bowel syndrome. *Clin Gastroenterol Hepatol* 2007; **5**: 1268-1275
  - 93 **Tache Y**, Monnikes H, Bonaz B, Rivier J. Role of CRF in stress-related alterations of gastric and colonic motor function. *Ann N Y Acad Sci* 1993; **697**: 233-243
  - 94 **Fukudo S**, Nomura T, Hongo M. Impact of corticotropin-releasing hormone on gastrointestinal motility and adrenocorticotrophic hormone in normal controls and patients with irritable bowel syndrome. *Gut* 1998; **42**: 845-849
  - 95 **Vale W**, Spiess J, Rivier C, Rivier J. Characterization of a 41-residue ovine hypothalamic peptide that stimulates secretion of corticotropin and beta-endorphin. *Science* 1981; **213**: 1394-1397
  - 96 **Fukudo S**. Role of corticotropin-releasing hormone in irritable bowel syndrome and intestinal inflammation. *J Gastroenterol* 2007; **42** Suppl 17: 48-51
  - 97 **Sagami Y**, Shimada Y, Tayama J, Nomura T, Satake M, Endo Y, Shoji T, Karahashi K, Hongo M, Fukudo S. Effect of a corticotropin releasing hormone receptor antagonist on colonic sensory and motor function in patients with irritable bowel syndrome. *Gut* 2004; **53**: 958-964
  - 98 **Cuppoletti J**, Malinowska DH, Tewari KP, Li QJ, Sherry AM, Patchen ML, Ueno R. SPI-0211 activates T84 cell chloride transport and recombinant human CIC-2 chloride currents. *Am J Physiol Cell Physiol* 2004; **287**: C1173-C1183
  - 99 **Camilleri M**, Bharucha AE, Ueno R, Burton D, Thomforde GM, Baxter K, McKinzie S, Zinsmeister AR. Effect of a selective chloride channel activator, lubiprostone, on gastrointestinal transit, gastric sensory, and motor functions in healthy volunteers. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G942-G947
  - 100 **Johanson JF**, Ueno R. Lubiprostone, a locally acting chloride channel activator, in adult patients with chronic constipation: a double-blind, placebo-controlled, dose-ranging study to evaluate efficacy and safety. *Aliment Pharmacol Ther* 2007; **25**: 1351-1361
  - 101 **Johanson JF WA**, Ueno R. Efficacy and safety of lubiprostone in a subgroup of constipation patients diagnosed with irritable bowel syndrome with constipation (IBS-C). *J Gastroenterol* 2006; **101** (suppl 2): S491
  - 102 **Ueno R WA**, Rivera E. Pooled analysis of the most frequent adverse events associated with the use of lubiprostone (abstract). *J Gastroenterol* 2006; **101** (suppl 2): S489
  - 103 **Cremonini F**, Camilleri M, McKinzie S, Carlson P, Camilleri CE, Burton D, Thomforde G, Urrutia R, Zinsmeister AR. Effect of CCK-1 antagonist, dexloxiglumide, in female patients with irritable bowel syndrome: a pharmacodynamic and pharmacogenomic study. *Am J Gastroenterol* 2005; **100**: 652-663
  - 104 **Holzer P**. Tachykinins as targets of gastroenterological

- pharmacotherapy. *Drug News Perspect* 1998; **11**: 394-401
- 105 **Holzer P**, Holzer-Petsche U. Tachykinins in the gut. Part I. Expression, release and motor function. *Pharmacol Ther* 1997; **73**: 173-217
  - 106 **Holzer P**, Holzer-Petsche U. Tachykinins in the gut. Part II. Roles in neural excitation, secretion and inflammation. *Pharmacol Ther* 1997; **73**: 219-263
  - 107 **Holzer P**, Lippe IT, Heinemann A, Bartho L. Tachykinin NK1 and NK2 receptor-mediated control of peristaltic propulsion in the guinea-pig small intestine in vitro. *Neuropharmacology* 1998; **37**: 131-138
  - 108 **Lordal M**, Navalesi G, Theodorsson E, Maggi CA, Hellstrom PM. A novel tachykinin NK2 receptor antagonist prevents motility-stimulating effects of neurokinin A in small intestine. *Br J Pharmacol* 2001; **134**: 215-223
  - 109 **Clouse RE**, Lustman PJ. Use of psychopharmacological agents for functional gastrointestinal disorders. *Gut* 2005; **54**: 1332-41
  - 110 **Clouse RE**. Antidepressants for functional gastrointestinal syndromes. *Dig Dis Sci* 1994; **39**: 2352-2363
  - 111 **Mertz H**, Fass R, Kodner A, Yan-Go F, Fullerton S, Mayer EA. Effect of amitriptyline on symptoms, sleep, and visceral perception in patients with functional dyspepsia. *Am J Gastroenterol* 1998; **93**: 160-165
  - 112 **Gorard DA**, Libby GW, Farthing MJ. Influence of antidepressants on whole gut and orocaecal transit times in health and irritable bowel syndrome. *Aliment Pharmacol Ther* 1994; **8**: 159-166
  - 113 **Gorard DA**, Libby GW, Farthing MJ. 5-Hydroxytryptamine and human small intestinal motility: effect of inhibiting 5-hydroxytryptamine reuptake. *Gut* 1994; **35**: 496-500
  - 114 **Heefner JD**, Wilder RM, Wilson ID. Irritable colon and depression. *Psychosomatics* 1978; **19**: 540-547
  - 115 **Steinhart MJ**, Wong PY, Zarr ML. Therapeutic usefulness of amitriptyline in spastic colon syndrome. *Int J Psychiatry Med* 1981; **11**: 45-57
  - 116 **Greenbaum DS**, Mayle JE, Vanegeren LE, Jerome JA, Mayor JW, Greenbaum RB, Matson RW, Stein GE, Dean HA, Halvorsen NA. Effects of desipramine on irritable bowel syndrome compared with atropine and placebo. *Dig Dis Sci* 1987; **32**: 257-266
  - 117 **Jackson JL**, O'Malley PG, Tomkins G, Balden E, Santoro J, Kroenke K. Treatment of functional gastrointestinal disorders with antidepressant medications: a meta-analysis. *Am J Med* 2000; **108**: 65-72
  - 118 **Tabas G**, Beaves M, Wang J, Friday P, Mardini H, Arnold G. Paroxetine to treat irritable bowel syndrome not responding to high-fiber diet: a double-blind, placebo-controlled trial. *Am J Gastroenterol* 2004; **99**: 914-920
  - 119 **Masand PS**, Gupta S, Schwartz TL, Virk S, Lockwood K, Hameed A, King M, Kaplan DS. Paroxetine in Patients With Irritable Bowel Syndrome: A Pilot Open-Label Study. *Prim Care Companion J Clin Psychiatry* 2002; **4**: 12-16
  - 120 **Creed F**, Fernandes L, Guthrie E, Palmer S, Ratcliffe J, Read N, Rigby C, Thompson D, Tomenson B. The cost-effectiveness of psychotherapy and paroxetine for severe irritable bowel syndrome. *Gastroenterology* 2003; **124**: 303-317
  - 121 **Klein KB**. Controlled treatment trials in the irritable bowel syndrome: a critique. *Gastroenterology* 1988; **95**: 232-241
  - 122 **Lesbros-Pantoflickova D**, Michetti P, Fried M, Beglinger C, Blum AL. Meta-analysis: The treatment of irritable bowel syndrome. *Aliment Pharmacol Ther* 2004; **20**: 1253-1269
  - 123 **Schoenfeld P**. Efficacy of current drug therapies in irritable bowel syndrome: what works and does not work. *Gastroenterol Clin North Am* 2005; **34**: 319-335, viii
  - 124 **Cann PA**, Read NW, Holdsworth CD, Barends D. Role of loperamide and placebo in management of irritable bowel syndrome (IBS). *Dig Dis Sci* 1984; **29**: 239-247
  - 125 **Lavo B**, Stenstam M, Nielsen AL. Loperamide in treatment of irritable bowel syndrome--a double-blind placebo controlled study. *Scand J Gastroenterol Suppl* 1987; **130**: 77-80
  - 126 **Efskind PS**, Bernklev T, Vatn MH. A double-blind placebo-controlled trial with loperamide in irritable bowel syndrome. *Scand J Gastroenterol* 1996; **31**: 463-468
  - 127 **Leventer S**, Raudibaugh K, Frisora C. The safety and efficacy of dextro-isopropamide in patients with diarrhea-predominant or alternating irritable bowel syndrome. *Gastroenterology* 2005; **128** (4 Suppl 2): A94
  - 128 **Pimentel M**, Chow EJ, Lin HC. Eradication of small intestinal bacterial overgrowth reduces symptoms of irritable bowel syndrome. *Am J Gastroenterol* 2000; **95**: 3503-3506
  - 129 **Pimentel M**, Chow EJ, Lin HC. Normalization of lactulose breath testing correlates with symptom improvement in irritable bowel syndrome. a double-blind, randomized, placebo-controlled study. *Am J Gastroenterol* 2003; **98**: 412-419
  - 130 **Parisi G**, Leandro G, Bottona E, Carrara M, Cardin F, Faedo A, Goldin D, Pantalena M, Tafner G, Verdianelli G, Zilli M. Small intestinal bacterial overgrowth and irritable bowel syndrome. *Am J Gastroenterol* 2003; **98**: 2572; author reply 2573-2574
  - 131 **Riordan SM**, McIver CJ, Walker BM, Duncombe VM, Bolin TD, Thomas MC. The lactulose breath hydrogen test and small intestinal bacterial overgrowth. *Am J Gastroenterol* 1996; **91**: 1795-1803
  - 132 **O'Leary C**, Quigley EM. Small bowel bacterial overgrowth, celiac disease, and IBS: what are the real associations? *Am J Gastroenterol* 2003; **98**: 720-722
  - 133 **Hasler WL**. Lactulose breath testing, bacterial overgrowth, and IBS: just a lot of hot air? *Gastroenterology* 2003; **125**: 1898-1900; discussion 1900
  - 134 **Posserud I**, Stotzer PO, Bjornsson ES, Abrahamsson H, Simren M. Small intestinal bacterial overgrowth in patients with irritable bowel syndrome. *Gut* 2007; **56**: 802-808
  - 135 **Cann PA**, Read NW, Holdsworth CD. What is the benefit of coarse wheat bran in patients with irritable bowel syndrome? *Gut* 1984; **25**: 168-173
  - 136 **Camilleri M**, Heading RC, Thompson WG. Clinical perspectives, mechanisms, diagnosis and management of irritable bowel syndrome. *Aliment Pharmacol Ther* 2002; **16**: 1407-1430
  - 137 **Cook IJ**, Irvine EJ, Campbell D, Shannon S, Reddy SN, Collins SM. Effect of dietary fiber on symptoms and rectosigmoid motility in patients with irritable bowel syndrome. A controlled, crossover study. *Gastroenterology* 1990; **98**: 66-72
  - 138 **Ashraf W**, Lof J, Jin G, Quigley EM. Comparative effects of intraduodenal psyllium and senna on canine small bowel motility. *Aliment Pharmacol Ther* 1994; **8**: 329-336
  - 139 **Zuckerman MJ**. The role of fiber in the treatment of irritable bowel syndrome: therapeutic recommendations. *J Clin Gastroenterol* 2006; **40**: 104-108
  - 140 **Bijkerk CJ**, Muris JW, Knottnerus JA, Hoes AW, de Wit NJ. Systematic review: the role of different types of fibre in the treatment of irritable bowel syndrome. *Aliment Pharmacol Ther* 2004; **19**: 245-251
  - 141 **Haderstorfer B**, Psychoglin D, Whitehead WE, Schuster MM. Intestinal gas production from bacterial fermentation of undigested carbohydrate in irritable bowel syndrome. *Am J Gastroenterol* 1989; **84**: 375-378
  - 142 **Brandt LJ**, Bjorkman D, Fennerty MB, Locke GR, Olden K, Peterson W, Quigley E, Schoenfeld P, Schuster M, Talley N. Systematic review on the management of irritable bowel syndrome in North America. *Am J Gastroenterol* 2002; **97**: S7-S26
  - 143 **Quigley EM**. Bacterial flora in irritable bowel syndrome: role in pathophysiology, implications for management. *J Dig Dis* 2007; **8**: 2-7
  - 144 **Quigley EM**, Flourie B. Probiotics and irritable bowel syndrome: a rationale for their use and an assessment of the evidence to date. *Neurogastroenterol Motil* 2007; **19**: 166-172
  - 145 **Halpern GM**, Prindiville T, Blankenburg M, Hsia T, Gershwin ME. Treatment of irritable bowel syndrome with Lacteol Fort: a randomized, double-blind, cross-over trial. *Am J Gastroenterol* 1996; **91**: 1579-1585
  - 146 **Niedzielin K**, Kordecki H, Birkenfeld B. A controlled, double-blind, randomized study on the efficacy of Lactobacillus plantarum 299V in patients with irritable bowel syndrome. *Eur J Gastroenterol Hepatol* 2001; **13**: 1143-1147



- 147 **Nobaek S**, Johansson ML, Molin G, Ahrne S, Jeppsson B. Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome. *Am J Gastroenterol* 2000; **95**: 1231-1238
- 148 **O'Sullivan MA**, O'Morain CA. Bacterial supplementation in the irritable bowel syndrome. A randomised double-blind placebo-controlled crossover study. *Dig Liver Dis* 2000; **32**: 294-301
- 149 **Chadwick VS**, Chen W, Shu D, Paulus B, Bethwaite P, Tie A, Wilson I. Activation of the mucosal immune system in irritable bowel syndrome. *Gastroenterology* 2002; **122**: 1778-1783
- 150 **Hart AL**, Lammers K, Brigidi P, Vitali B, Rizzello F, Gionchetti P, Campieri M, Kamm MA, Knight SC, Stagg AJ. Modulation of human dendritic cell phenotype and function by probiotic bacteria. *Gut* 2004; **53**: 1602-1609
- 151 **Addolorato G**, Marsigli L, Capristo E, Caputo F, Dall'Aglio C, Baudanza P. Anxiety and depression: a common feature of health care seeking patients with irritable bowel syndrome and food allergy. *Hepatogastroenterology* 1998; **45**: 1559-1564
- 152 **Drisko J**, Bischoff B, Hall M, McCallum R. Treating irritable bowel syndrome with a food elimination diet followed by food challenge and probiotics. *J Am Coll Nutr* 2006; **25**: 514-522
- 153 **Niec AM**, Frankum B, Talley NJ. Are adverse food reactions linked to irritable bowel syndrome? *Am J Gastroenterol* 1998; **93**: 2184-2190

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## TOPIC HIGHLIGHT

Lynne V McFarland, PhD, Series Editor

# Meta-analysis of probiotics for the treatment of irritable bowel syndrome

Lynne V McFarland, Sascha Dublin

Lynne V McFarland, Department of Health Services Research and Development, VA Puget Sound Health Care System, Metropolitan Park West, 1100 Olive Way, Suite #1400, Seattle WA 98101, United States

Lynne V McFarland, Department of Medicinal Chemistry, School of Pharmacy, University of Washington, Seattle WA 98101, United States

Sascha Dublin, Group Health Center for Health Studies, 1730 Minor Avenue, Suite 1600, Seattle 98101, United States

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**Correspondence to:** Lynne V McFarland, PhD, Department of Health Services Research and Development, VA Puget Sound Health Care System, Metropolitan Park West, 1100 Olive Way, Suite #1400, Seattle WA 98101, United States. [lynne.mcfarland@va.gov](mailto:lynne.mcfarland@va.gov)

Telephone: +1-206-2771095 Fax: +1-206-7642935

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estimation of a pooled RR. While our analyses suggest that probiotic use may be associated with improvement in IBS symptoms compared to placebo, these results should be interpreted with caution, given the methodological limitations of contributing studies. Probiotics warrant further study as a potential therapy for IBS.

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**Key words:** Probiotics; Meta-analysis; Irritable bowel syndrome

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## Abstract

Irritable bowel syndrome (IBS) is a chronic condition affecting 3%-25% of the general population. As no curative treatment is available, therapy is aimed at reducing symptoms, often with little success. Because alteration of the normal intestinal microflora has been observed in IBS, probiotics (beneficial microbes taken to improve health) may be useful in reducing symptoms. This paper systematically reviews randomized, controlled, blinded trials of probiotics for the treatment of IBS and synthesizes data on efficacy across trials of adequate quality. PubMed, Medline, Google Scholar, NIH registry of clinical trials, metaRegister, and the Cochrane Central Register of Controlled Trials were searched from 1982-2007. We also conducted secondary searches of reference lists, reviews, commentaries, relevant articles on associated diseases, books and meeting abstracts. Twenty trials with 23 probiotic treatment arms and a total of 1404 subjects met inclusion criteria. Probiotic use was associated with improvement in global IBS symptoms compared to placebo [pooled relative risk (RR<sub>pooled</sub>) 0.77, 95% confidence interval (95% CI) 0.62-0.94]. Probiotics were also associated with less abdominal pain compared to placebo [RR<sub>pooled</sub> = 0.78 (0.69-0.88)]. Too few studies reported data on other IBS symptoms or on specific probiotic strains to allow

## INTRODUCTION

Irritable bowel syndrome (IBS) is a chronic condition that severely impacts the quality of life of affected individuals<sup>[1,2]</sup>. The prevalence of IBS in the general population ranges from 3%-25%<sup>[3]</sup>. IBS is characterized by intermittent abdominal pain, altered bowel habits (diarrhea and/or constipation) and other gastrointestinal symptoms such as bloating and flatulence in the absence of structural abnormalities in the intestine. The pathophysiology of IBS is multifactorial and may include motor and sensory dysfunction, immune responses, food sensitivities and genetic predisposition<sup>[3,4]</sup>. Risk factors include female gender (2-3 times more common), acute gastrointestinal infections (e.g. *Campylobacter* or *Salmonella*) and psychological factors<sup>[3,5,6]</sup>. As no curative treatments are available, therapy for IBS is palliative and supportive, targeting specific symptoms, but is notoriously unsatisfactory<sup>[7,8]</sup>. Although 30% of patients report resolution of symptoms within one year, nearly 70% report that symptoms recur within five years<sup>[3]</sup>.

Studies have observed altered intestinal microflora in IBS patients and an increase in symptoms after enteric infections<sup>[9-12]</sup>, suggesting that restoration of the intestinal

microflora may be a useful therapeutic goal. One strategy to restore normal flora is the use of probiotics<sup>[13,14]</sup>. Probiotics are “beneficial bacteria or yeasts that are ingested to improve health”<sup>[15]</sup>. Probiotics are also known to modulate the immune response and reduce cytokine production<sup>[9,16-18]</sup>. Strong evidence for the beneficial role of probiotics exists for the prevention of antibiotic-associated diarrhea, traveler’s diarrhea and pediatric diarrhea<sup>[19-22]</sup>. There is emerging evidence that probiotics may be useful in preventing or treating *Clostridium difficile* diarrhea and pouchitis<sup>[20,23,24]</sup>. Studies of probiotics for IBS have yielded contradictory results, which may be due to a variety of factors: small sample size; variability in trial design; heterogeneity of probiotic strain, dose and treatment duration; and patient characteristics. The wide availability of probiotics as non-prescription products and the lack of a synthesis of data regarding efficacy have prompted us to conduct this meta-analysis.

We conducted a systematic review of randomized, controlled trials published as full articles or meeting abstracts to: (1) assess the characteristics and quality of randomized clinical trials in this area and (2) synthesize data across studies regarding the efficacy of probiotics for IBS.

## SEARCH STRATEGY

PubMed, Medline and Google Scholar were searched from 1982-2007 for articles unrestricted by language. Three on-line clinical trial registers were searched: Cochrane Central Register of Controlled Trials ([www.cochrane.org](http://www.cochrane.org)), metaRegister of Controlled Trials ([www.controlled-trials.com/mrct](http://www.controlled-trials.com/mrct)) and National Institutes of Health ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)). Secondary and hand searches of reference lists, other studies cross-indexed by authors, reviews, commentaries, books and meeting abstracts also were performed. Search terms included: irritable bowel syndrome, diarrhea, probiotics, risk factors, Rome criteria, Manning criteria, randomized controlled trials, placebo-controlled, bloating and associated author names. Search strategies were broad-based initially, then narrowed to the disease of interest to increase the search network<sup>[25]</sup>. The procedure for this meta-analysis was designed as suggested by Egger *et al* with clearly delineated parameters, *a priori* inclusion and exclusion criteria and standardized data extraction<sup>[26,27]</sup>. Abstracts of all citations and retrieved studies were reviewed and rated for inclusion. Full articles were retrieved if specific treatments were given for IBS. In some cases, only published abstracts from meetings were available. Published abstracts from meetings were included to lessen the potential for publication bias due to failure to publish negative findings.

## INCLUSION AND EXCLUSION CRITERIA

The primary objective of this meta-analysis was to determine the overall efficacy of probiotics for IBS by comparing a common outcome in treated patients with a control group. Inclusion criteria included: randomized, controlled, blinded efficacy trials in humans published as full articles or meeting abstracts in peer-reviewed journals.

Exclusion criteria included: pre-clinical studies, safety studies, case reports or case series, phase 1 studies in volunteers, reviews, duplicate reports, trials of unspecified treatments, uncontrolled studies, prebiotic treatments only (no living organisms) or insufficient data in article.

## ASSESSMENT OF METHODOLOGICAL QUALITY

Studies that met the inclusion criteria were graded for quality using the Linde Internal Validity Scale (LIVS), which includes the following six items: method of allocation to groups, concealment of allocation, baseline comparability of intervention and placebo groups, blinding of patients, blinding of evaluators, and intention to treat/handling of withdrawals and drop-outs<sup>[28-29]</sup>. If no information was provided for an item or it was unclear, authors were contacted for more information. If available information was still inadequate, then zero points were given for that item. Total possible scores range from 0 to 6. All trials included in the meta-analysis had a total quality score of 3 or more and those with a score less than 3 were excluded. Two independent reviewers independently assessed inclusion criteria and quality of the trials. Inconsistencies were resolved by discussion.

## INTENT-TO-TREAT (ITT) ANALYSIS

Studies were considered to have adhered to intention-to-treat principles if all subjects who were randomized were analyzed with the group to which they were originally assigned and if exclusions were primarily due to patient withdrawal or loss to follow-up. If the investigators excluded patients after randomization due to use of non-study medications or antibiotics, noncompliance with assigned treatment, or non-response to therapy, the analysis was not considered to be ITT.

## DATA EXTRACTION

Information on study design, methods, interventions, outcomes, adverse effects and treatments was extracted from each article using a standardized extraction table. When necessary, authors were contacted for data not reported in the original article.

## OUTCOMES AND DEFINITIONS

We documented the types of outcomes for trials involving IBS and probiotic in the literature. Outcomes were reported by different studies as either the proportion of subjects reporting improvement or the change in symptom scores from baseline. We did not attempt to synthesize results from studies reporting changes in symptom scores because of numerous challenges including heterogeneity in scales and scoring systems across studies and inconsistent or incomplete reporting of numeric symptom scores. Thus, we selected the proportion of subjects with improvement in global IBS symptoms as the primary outcome for this meta-analysis. Secondary outcomes

included the proportion of subjects with improvement in one of three common IBS symptoms: abdominal pain, bloating or flatulence. Documentation of the outcome was based on subject self-report and/or clinician assessment.

## META-ANALYSIS METHODS

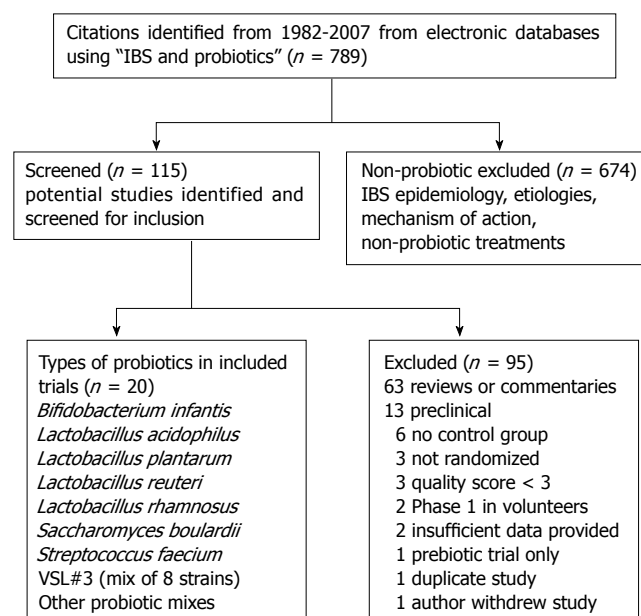
To estimate pooled relative risks across studies, we first evaluated heterogeneity between and within trials using the  $\chi^2$  test<sup>[30]</sup>. The relative risks of responding to probiotic therapy were pooled using a random-effects model if significant heterogeneity was found or a fixed-effects model if the studies were homogenous<sup>[31]</sup>. The number needed to treat (NNT) was calculated using the reciprocal of the pooled absolute risk reduction. *P* values less than 0.05 were considered significant. Analyses were performed using Stata software version 9.2 (Stata Corporation, College Station, Texas).

## PUBLICATION BIAS

We used a funnel scatterplot to assess the potential for publication bias<sup>[32]</sup>. Risk ratios were plotted against the standard error of the risk ratio (a surrogate for study size) of each study to detect asymmetry in the distribution of trials. Larger studies usually provide a more precise estimate of the true effect of the treatment and form the narrow spout of the funnel plot. Smaller trials provide less precise estimates, and the increased variability results in a wider cone of the funnel plot. A gap in the funnel plot (commonly, the absence of small studies with negative findings) suggests potential publication bias or methodological problems in smaller studies. Begg's test was also used to assess potential publication bias<sup>[33,34]</sup>.

## STUDY CHARACTERISTICS PREDICTIVE OF POSITIVE FINDINGS

Because there was heterogeneity across studies, we examined study design characteristics that we hypothesized could be associated with results favoring probiotics over placebo. These analyses examined results for the primary outcome variable, reduction in global IBS symptoms. We classified studies as favoring probiotics if the unpooled RR was 0.67 or less. The study by Whorwell *et al* included 3 different probiotic dose arms but was considered as a single study for the purposes of this analysis<sup>[35]</sup>. Since one of the 3 arms showed results favoring probiotic, we classified this study as favoring probiotics. Characteristics examined as possible predictors included sample size, LIVS quality score, proportion of female subjects, probiotic dose, treatment duration, attrition > 20%, ITT analysis and use of a proprietary (commercial) *vs* nonproprietary product. To explore possible predictive variables, we first examined descriptive statistics (median and interquartile range for continuous variables, proportions for categorical variables). To test for statistical significance, we used the Wilcoxon rank-sum test for continuous data and Fisher's exact test for categorical data.



**Figure 1** QUOROM flow diagram of included and excluded studies of probiotics for the treatment of Irritable Bowel Syndrome.

## LITERATURE SCREENING

The literature search yielded 3552 citations on probiotics, of which 789 addressed probiotics and IBS. Based on review of abstracts, 115 were selected for detailed screening.

## STUDY SELECTION

The study selection process is shown in a QUOROM (Quality of Reporting of Meta-analysis) flow diagram (Figure 1)<sup>[27]</sup>. Overall, 95 studies that were screened failed to meet 1 or more of the inclusion criteria: 63 (66%) were reviews, 13 (14%) were pre-clinical studies, 6 (6%) had no control group<sup>[36-41]</sup>, 3 (3%) were not randomized<sup>[42-44]</sup>, and 10 (10%) were excluded for a variety of reasons. A total of 20 articles met inclusion criteria and provided data on 23 probiotic treatment arms for 1404 patients with IBS (Table 1)<sup>[17,35,45-62]</sup>. An additional seven trials were excluded after article retrieval and screening for issues related to quality and/or study design (Table 2)<sup>[63-68]</sup>.

## STUDY QUALITY

The study quality of 23 treatments was assessed, and 20 trials with LIVS quality scores > 3.0 were included (Table 3). The median quality score was 4 (range 3 to 6). Nine studies did not describe the method of randomization, 8 did not provide baseline comparison of groups, 14 did not specifically state that evaluators were blinded and 20 did not perform intention-to-treat analysis and/or did not fully describe withdrawals. For six studies, the published article or abstract did not contain sufficient information to allow quality scoring, requiring communication with the authors. Only three studies (15%) clearly documented their adherence to intention-to-treat principles<sup>[45,50,60]</sup>.

There were a variety of ways in which studies failed to

Table 1 Description of 20 randomized, controlled trials of probiotics for IBS included in systematic review

Reference	Probiotic	Type of control	Number of subjects randomized	Number analyzed	Dose (cfu/d)	Duration of treatment (wk)	% attrition
Maupas <sup>[45]</sup>	<i>Saccharomyces cerevisiae boulardii lyo</i>	Placebo capsules	34	34	$9 \times 10^9$	4	0
Gade <sup>[46]</sup>	<i>Streptococcus faecium</i> 40371	Placebo tablets	58	54	$1 \times 10^{12}$	4	7
Halpern <sup>[47]</sup>	<i>L. acidophilus</i> (heat killed) "Lacteol Fort"	Placebo capsules	29	18	$2 \times 10^{10}$	6	38
Nobaek <sup>[48]</sup>	<i>Lactobacillus plantarum</i> DSM9843, in rose hip drink	Placebo plain rose hip drink	60	52	$5 \times 10^7$	4	13
O'Sullivan <sup>[49]</sup>	<i>Lactobacillus rhamnosus</i> GG	Placebo tablets	24	19	$1 \times 10^{10}$	8	21
Niedzielin <sup>[50]</sup>	<i>Lactobacillus plantarum</i> 299v, "ProViva" drink	Placebo drink	40	40	$2 \times 10^{10}$	4	0
Kim <sup>[51]</sup>	VSL#3 (mix of 8 strains) powder packet <sup>1</sup>	Placebo powder	25	25	$9 \times 10^{11}$	8	4
Bausserman <sup>[52]</sup>	<i>Lactobacillus rhamnosus</i> GG	Placebo capsules	58	50	$2 \times 10^{10}$	6	22
Bittner <sup>[53]</sup>	Prescript-assist <sup>®</sup> 29 soil strains and prebiotic "leonardite"	Placebo capsules	27	25	$2.6 \times 10^8$	2	7
Kajander <sup>[54]</sup>	<i>L. rhamnosus</i> GG + <i>L. rham.</i> LC705 + <i>Bifido. breve</i> Bb99 + <i>Prop. freudenreichii</i>	Placebo capsules	103	81	$8-9 \times 10^9$	24	21
Kim <sup>[55]</sup>	VSL#3 yogurt <sup>1</sup>	Placebo yogurt	48	48	$8 \times 10^9$	4	0
Niv <sup>[56]</sup>	<i>Lactobacillus reuteri</i> 55730	Placebo capsules	54	39	$2 \times 10^8$	24	28
O'Mahony <sup>[17]</sup>	<i>L. salivarius</i> UCC4331	Placebo drink	54	51	$1 \times 10^{10}$	8	16
O'Mahony <sup>[17]</sup>	<i>Bifido. infantis</i> 35624	Placebo drink	53	49	$1 \times 10^{10}$	8	16
Kim <sup>[57]</sup>	<i>B. subtilis</i> + <i>Strept. faecalis</i>	Placebo capsules	40	34	$3 \times 10^{10}$	4	15
Simren <sup>[58]</sup>	<i>L. plantarum</i> 299v in rose hip drink	plain rose hip drink	66	58	$2 \times 10^9$	6	12
Whorwell <sup>[35]</sup>	<i>Bifido. infantis</i> 35624 in 3 doses	Placebo capsules	362	292	$1 \times 10^6$ $1 \times 10^8$ $1 \times 10^{10}$	4	19
Enck <sup>[59]</sup>	<i>E. coli</i> + <i>Strept faecalis</i> drink	Placebo drink	297	264	$4.5 \times 10^2$	8	11
Gawronska <sup>[60]</sup>	<i>L. rhamnosus</i> GG	Placebo capsules	37	37	$6 \times 10^9$	4	0
Marteau <sup>[61]</sup>	<i>Bifido. longum</i> , <i>Lact acidophilus</i> , <i>Lactococcus lactis</i> , <i>Strept. thermophilus</i>	Placebo capsules	106	100	$1 \times 10^{10}$	4	6
Simren <sup>[62]</sup>	<i>Lact. paracasei</i> , <i>Lact acidophilus</i> , <i>Bifido. lactis</i> in yoghurt	Control milk	74	67	$2 \times 10^{10}$	8	9

IBS: Irritable bowel syndrome; cfu/d: Colony forming units per day; *Bifido.*: *Bifidobacterium*; *B.*: *Bacillus*; *E.*: *Escherichia*; *L.*: *Lactobacillus*; *Prop.*: *Propionibacterium*.  
<sup>1</sup>VSL#3 is a mixture of 8 probiotic strains (*Lactobacillus casei*, *L. plantarum*, *L. acidophilus*, *L. bulgaricus*, *Bifido. longum*, *Bifido. breve*, *Bifido. infantis* and *Streptococcus thermophilus*).

Table 2 Examples of excluded randomized, controlled trials of probiotics for IBS

Reference	Probiotic	Number of subjects randomized	Number of subjects analyzed	Dose (cfu/mL)	Duration	Exclusion reason
DiBaise <sup>[63]</sup>	<i>L. plantarum</i> 299v vs placebo	29	20	$6 \times 10^9$	4 wk	Withdrawn by author
Saggioro <sup>[64]</sup>	<i>L. plantarum</i> + <i>L. acidophilus</i>	46	39	$1 \times 10^{11}$	4 wk	Quality score = 2.0
Saggioro <sup>[64]</sup>	<i>L. plantarum</i> + <i>Bifido. breve</i>	44	37	$1 \times 10^{10}$	4 wk	Quality score = 2.0
Long <sup>[65]</sup>	<i>Bifido. (species not given)</i>	60	60	$6 \times 10^9$	2 wk	Quality score = 2.5
Kajander <sup>[66]</sup>	<i>L. rhamnosus</i> GG + <i>L. rham.</i> LC705 + <i>Bifid. breve</i> Bb99 + <i>Prop. freudenreichii</i>	103	83	$8-9 \times 10^9$	6 mo	Duplicate study of Kajander K 2005
Bittner <sup>[67]</sup>	Prescript-assist <sup>®</sup> , 29 soil strains and prebiotic	24	24	$2.6 \times 10^8$	Varied	Controls from Bittner 2005 study, phase 4 study
Moon <sup>[68]</sup>	<i>Bacillus subtilis</i> + <i>St. faecium</i>	34	34	750 mL/d, cfu/d not given	4 wk	Outcome data not provided for each group in abstract

cfu/mL: Colony forming units per milliliter; IBS: Irritable bowel syndrome; *L.*: *Lactobacillus*; *Bifido.*: *Bifidobacterium*.

adhere to ITT principles. Seven studies excluded participants who used prohibited/non-study medications, including antibiotics, during the treatment phase<sup>[17,46,48,49,51,55,59]</sup>, while five studies excluded subjects who demonstrated poor compliance with study medications<sup>[47,52,54,56,61]</sup>. Three studies reported that subjects either dropped out or were excluded due to inadequate response to treatment<sup>[49,58,62]</sup>, while in 4 studies, subjects were excluded for worsening abdominal pain<sup>[51,52,54,56]</sup>. Often, it was unclear whether subjects with inadequate response or worsening symptoms were excluded

at the investigators' discretion or withdrew from the study of their own accord.

## DESCRIPTION OF INCLUDED STUDIES

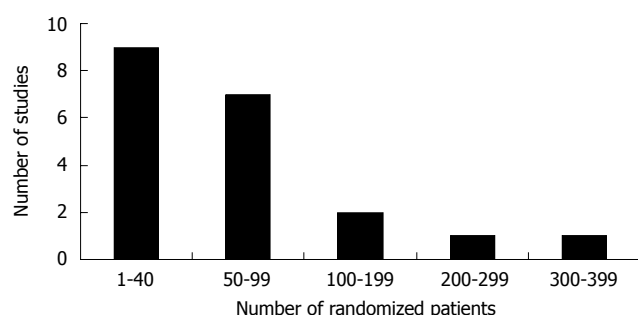
A standardized data extraction table (Table 1) was used to characterize each clinical trial. Twenty randomized controlled trials provided adequate data regarding efficacy in a total of 1404 patients with IBS. In 20 trials, 23 probiotic treatment arms were compared to placebo control arms.



Table 3 Quality scoring for 20 randomized, controlled trials of probiotics for IBS (Linde Internal Validity Scale)

Reference	Total quality score <sup>1</sup>	Treatment allocation	Randomization method	Baseline comparison	Patients blinded	Evaluators blinded	Handling and reporting of withdrawals/use of ITT	Data source <sup>2</sup>
Maupas <sup>[45]</sup>	6	1	1	1	1	1	1	Paper
Gade <sup>[46]</sup>	4.5	1	1	0	1	1	0.5	Paper
Halpern <sup>[47]</sup>	4	1	1	0.5	1	0.5	0	Paper
Nobaek <sup>[48]</sup>	3	1	0	0	1	0.5	0.5	Paper
O'Sullivan <sup>[49]</sup>	3	1	0	1	0.5	0.5	0	Author
Niedzielin <sup>[50]</sup>	4	1	0	1	1	0	1	Paper
Kim <sup>[51]</sup>	4.5	1	0	0.5	1	1	1	Paper
Bausserman <sup>[52]</sup>	5.5	1	1	1	1	1	0.5	Paper
Bittner <sup>[53]</sup>	3	1	0	0	0.5	0.5	1	Author
Kajander <sup>[54]</sup>	4.5	1	1	1	0.5	0.5	0	Paper
Kim <sup>[55]</sup>	4	1	0	1	1	0.5	0.5	Paper
Niv <sup>[56]</sup>	3.5	1	0	0.5	1	0.5	0.5	Paper
O'Mahony <sup>[17]</sup>	4.5	1	1	0	1	1	0.5	Paper
Kim <sup>[57]</sup>	4	1	0	1	0.5	0.5	1	Paper
Simren <sup>[58]</sup>	3	1	0.5	0	1	0.5	0	Author
Whorwell <sup>[35]</sup>	3.5	1	0	0.5	1	0.5	0.5	Paper
Enck <sup>[59]</sup>	4	1	0.5	0	1	0.5	1	Author
Gawronska <sup>[60]</sup>	4.5	1	1	0	1	0.5	1	Paper
Marteau <sup>[61]</sup>	4.5	1	0.5	1	1	0.5	0.5	Author
Simren <sup>[62]</sup>	3.5	1	0.5	0	1	0	1	Author

<sup>1</sup>Linde Internal Validity Scale score is based on columns 3-8; range, 0 (poor) to 6 (excellent). (Linde 1996)<sup>[29]</sup>; <sup>2</sup>Indicates whether additional contact with authors was required to obtain information needed for quality scoring; <sup>3</sup>Data from published meeting abstract only.



**Figure 2** Number of randomized patients in 20 randomized, controlled clinical trials of probiotics for the treatment of Irritable Bowel Syndrome.

Eighteen studies compared a single probiotic treatment arm to placebo, one study compared two probiotic treatments to placebo<sup>[17]</sup>, and one study compared three doses of one probiotic against placebo<sup>[35]</sup>. The number of patients in each of these studies was generally small, with a median of 54 randomized subjects (range, 25-363; Figure 2). The daily dose of probiotic treatment ranged from 450 to  $1 \times 10^{12}$  colony-forming units (cfu)/day (median =  $9 \times 10^9$ ). For the most part, the length of treatment in these studies was brief (median = 4 wk), with 90% of studies having a treatment phase of 8 wk or less.

## PROBIOTIC STRAIN

Only two probiotics were tested in multiple trials: *Lactobacillus rhamnosus* GG in three trials<sup>[49,52,60]</sup> and *Bifidobacterium infantis* in two trials<sup>[17,35]</sup>. None of the *L. rhamnosus* GG trials provided evaluable data on either the primary or secondary outcomes, which prevented analysis by strain type.

## ASSESSMENT AND REPORTING OF OUTCOMES

The outcomes assessed and reported varied widely across the 20 studies. The effect on global IBS symptoms (measured as either proportion with symptom improvement or a reduction in severity scores) was reported in 15/20 (75%) of studies (Table 4) and was the primary outcome for 7 (35%) of studies. Effects on abdominal pain were reported by all studies. But, only 4 (20%) used this as a primary outcome measure<sup>[35,50,52,60]</sup>.

Other symptoms were less consistently assessed (e.g. flatulence, 13/20 studies; mucus in stool, 3/20 studies; bloating, 15/20 studies). Only five studies collected some measure of quality of life<sup>[17,54,56,61,62]</sup>. Seven studies reported data for 3 or more symptoms or outcomes without specifying a primary outcome<sup>[17,46,48,49,53,54,56]</sup>.

Some studies reported the number and proportion of subjects with improvement, while others reported change in numeric symptom scores since baseline. The scales used to measure the severity of IBS symptoms varied widely between studies, making it challenging to compare results across studies. Visual analogue scales were most often used, but still only used by 6 studies<sup>[17,46,48,51,55,57]</sup>. Likert scales were used by 3 studies<sup>[17,49,52]</sup>, and specific validated scales were used by several studies: Gastrointestinal Symptom Rating Scale (GSRS)<sup>[52,58]</sup> and IBS Severity Scoring System (IBS-SSS)<sup>[56,58,62]</sup>. Several studies used their own study-specific scale or scoring system<sup>[17,35,45,47,50,53,54,59-61]</sup>. Often it was unclear whether this scale had been validated.

While many studies assessed a wide range of IBS symptoms, few reported detailed results across the spectrum of symptoms (Table 4), making it more difficult to combine data across studies. For instance, only 8 of 13 studies reporting that they had collected data on flatulence

Table 4 Outcome assessment and reporting for 20 included clinical trials of probiotics for IBS

Reference	Outcome							
	Global response	Abdominal pain	Bloating/distension	Flatulence	Stool frequency	Mucous	Stool consistency	Dyspepsia
Maupas <sup>[45]</sup>	<b>R</b>	R	R		R		R	R
Gade <sup>[46]</sup>	R	R	R	R			R	
Halpern <sup>[47]</sup>	<b>R</b>	A	A		A	A	A	
Nobaek <sup>[48]</sup>	R	R		R	A		R	
O'Sullivan <sup>[49]</sup>		R	R	A	R		A	
Niedzielin <sup>[50]</sup>	R	<b>R</b>		R	A		A	
Kim <sup>[51]</sup>	<b>R</b>	R	R	R	R		R	
Bausserman <sup>[52]</sup>	A	<b>R</b>	R	A			A	
Bittner <sup>[53]</sup>		A		A				A
Kajander <sup>[54]</sup>	R	R	R	R	R	A	A	
Kim <sup>[55]</sup>		R	<b>R</b>	R	R		R	
Niv <sup>[56]</sup>		R	R	R	A			
O'Mahony <sup>[17]</sup>	R	R	R		A		R	
Kim <sup>[57]</sup>		R	A	A	A		A	
Simren <sup>[58]</sup>	<b>R</b>	A	A	A	A	A	A	
Whorwell <sup>[35]</sup>	R	<b>R</b>	R	R	A			
Enck <sup>[59]</sup>	R	R	A		A		A	
Gawronska <sup>[60]</sup>		<b>R</b>						
Marteau <sup>[61]</sup>	<b>R</b>	R			A		A	
Simren <sup>[62]</sup>	<b>R</b>	A	A					
Percent reporting	65%	80%	50%	40%	25%	0	30%	5%

A: Assessed; R: Reported in sufficient detail to allow extraction of data. Bold font indicates that this was the primary outcome identified by the authors for analysis. If author reported no difference between active and placebo groups for a given symptom, but did provide further details, the outcome was classified as assessed only.

Table 5 Global Improvement in IBS Symptoms in 14 probiotic/placebo treatment arms

Reference	Probiotic	Global improvement in IBS symptoms		Definition of primary outcome <sup>1</sup>
		Probiotic <i>n/n</i> (%)	Placebo <i>n/n</i> (%)	
Maupas <sup>[45]</sup>	<i>Saccharomyces cerevisiae boulardii</i> lyo	13/16 (81)	13/18 (72)	Improvement of symptoms
Gade <sup>[46]</sup>	<i>Strept faecalis</i>	26/32 (81)	9/22 (41)	Improvement of symptoms based on physician assessment
Halpern <sup>[47]</sup>	<i>L. acidophilus</i>	17/18 (94)	13/18 (72)	Absence of symptoms
Nobaek <sup>[48]</sup>	<i>L. plantarum</i>	11/25 (44)	7/27 (26)	Decrease $\geq 1.5$ on VAS symptom scale
Niedzielin <sup>[50]</sup>	<i>L. plantarum</i>	9/20 (45)	3/20 (15)	Absence of symptoms
Kim <sup>[51]</sup>	VSL#3 <sup>2</sup>	4/12 (34)	5/13 (38)	Satisfactory relief of IBS symptoms
Kajander <sup>[54]</sup>	<i>L. rhamnosus</i> GG + <i>L. rham.</i> LC705 + <i>Bifid. breve</i> Bb99 + <i>Prop. freudenreichii</i>	31/41 (76)	17/40 (43)	Symptoms alleviated based on significant reduction of symptom scores
Simren <sup>[58]</sup>	<i>L. plantarum</i>	10/29 (35)	11/29 (38)	Reduction $\geq 50\%$ of total symptom score
Whorwell <sup>[35]</sup>	<i>Bifido. infantis</i> (dose, 10 <sup>6</sup> cfu/mL)	33/74 (44)	32/76 (42)	Adequate relief of symptoms
Whorwell <sup>[35]</sup>	<i>Bifido. infantis</i> (dose, 10 <sup>8</sup> cfu/mL)	45/72 (62)	32/76 (42)	Adequate relief of symptoms
Whorwell <sup>[35]</sup>	<i>Bifido. infantis</i> (dose, 10 <sup>10</sup> cfu/mL)	26/71 (37)	32/76 (42)	Adequate relief of symptoms
Enck <sup>[59]</sup>	<i>E. coli</i> + <i>Strept faecalis</i>	102/149 (68)	56/148 (38)	Reduction of $\geq 50\%$ in total symptom score
Marteau <sup>[61]</sup>	<i>Bifido. longum</i> , <i>L. acidophilus</i> , <i>Lactococcus lactis</i> , <i>Strept thermophilus</i>	20/47 (42.6)	22/52 (42.3)	Relief of discomfort
Simren <sup>[62]</sup>	<i>L. paracasei</i> , <i>L. acidophilus</i> , <i>Bifido. lactis</i> in <i>yoghurt</i>	14/33 (42)	17/34 (50)	Reduction of $\geq 50\%$ in total symptom score

<sup>1</sup>Unless otherwise stated, all primary outcomes are defined based on patient report. <sup>2</sup>VSL#3 is a mixture of 8 probiotic strains (*Lactobacillus casei*, *L. plantarum*, *L. acidophilus*, *L. bulgaricus*, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium infantis* and *Streptococcus thermophilus*).

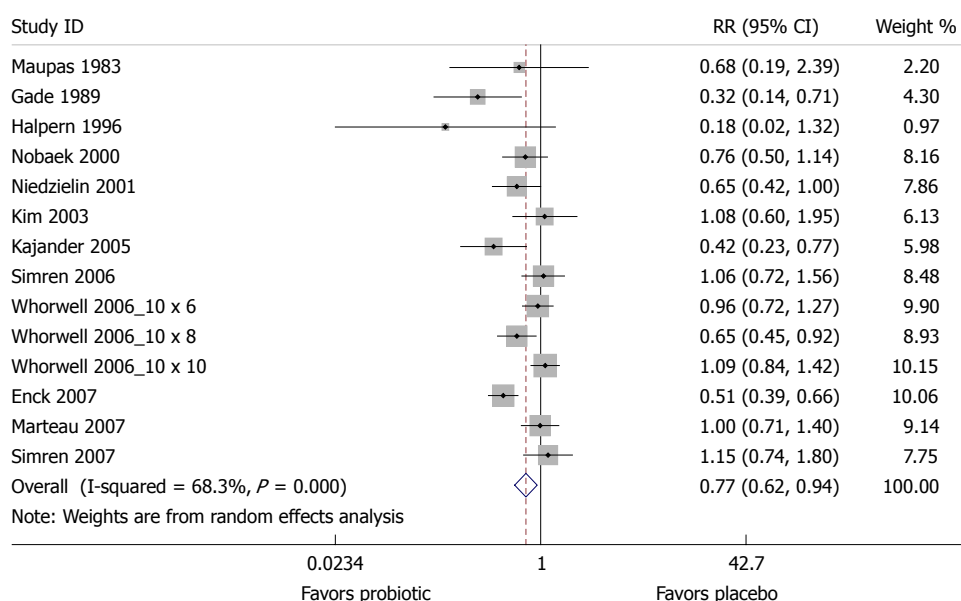
provided this data in their paper and only 5 of 15 reporting they had collected data on stool frequency reported any such data in their paper.

## GLOBAL RESPONDERS

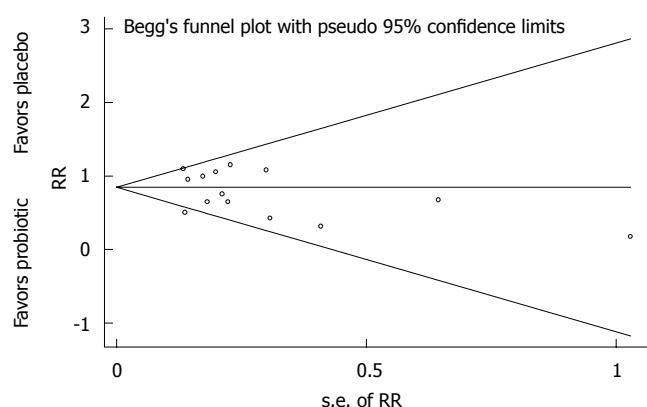
The primary outcome selected for this analysis was the proportion of patients in each group with global IBS symptoms by the end of treatment, with 'responders'

being a dichotomous variable defined by study investigators (Table 5). Of the 23 treatment arms, 14 (61%) had evaluable data for this outcome. Eight treatment arms either did not collect data on global symptom relief<sup>[49,52,55,60]</sup> or reported change in symptom scores rather than proportion with improvement<sup>[17,53,56,57]</sup>.

When the meta-analysis model was fitted, the  $\chi^2$  test for heterogeneity was 41.0 ( $P < 0.001$ ), indicating a high degree of heterogeneity; so a random-effects model was used to



**Figure 3** Forest Plot of randomized controlled trials of 14 treatment arms from 12 studies measuring relative risk of IBS symptoms after probiotic treatment compared to placebo. X-axis is relative risk, with black dot indicating the relative risk, line indicating 95% confidence interval and the size of the grey box proportional to sample size.



**Figure 4** Funnel plots of randomized controlled trials for examining presence of IBS symptoms with probiotic or placebo treatments. RR: Relative risk of global IBS symptoms; s.e. of RR: Standard error of relative risk, an indicator of sample size.

pool these results. The forest plot, weighted on sample size, is shown in Figure 3. Compared to placebo, probiotics were significantly protective (less global IBS symptoms compared to placebo at the end of the study) [pooled relative risk ( $RR_{pooled}$ ) = 0.77; 95% confidence interval (95% CI), 0.62-0.94]. The number needed to treat was 7.3. The funnel plot (Figure 4) is generally symmetrical, showing little evidence of publication bias. Begg's test did not show statistically significant publication bias ( $z = 0.93$ ,  $P = 0.35$ ).

## SENSITIVITY ANALYSES

We repeated the meta-analysis weighting by study quality score rather than sample size, with similar results ( $RR_{pooled} = 0.65$ ; 95% CI, 0.52-0.82). As it appeared that the pooled risk estimate was heavily influenced by one large study<sup>[59]</sup>, we re-ran the analysis excluding the study, but similar results were found ( $RR_{pooled} = 0.82$ ; 95% CI, 0.67-0.99).

## SECONDARY OUTCOMES

*A priori* secondary outcomes for this study included

the proportion of subjects who reported one of three IBS symptoms: abdominal pain, bloating/distension, or flatulence (gas). Of 23 treatment arms, 12 (52%) had evaluable data on at least one of these secondary outcomes. Fourteen treatment arms either did not collect data on these secondary outcomes<sup>[45,47,51,58,61,62]</sup> or reported symptom scores rather than proportion with symptoms<sup>[17,53,56,57]</sup>. As only five treatment arms reported proportion of subjects with reduced bloating<sup>[46,49,52,54,55]</sup> and four reported proportion with improved flatulence<sup>[46,48,50,54]</sup>, further statistical analyses were not performed for these outcomes.

Eight trials (11 probiotic treatment arms) had evaluable data for the proportion of patients reporting abdominal pain at the end of follow-up (Table 6)<sup>[35,46,48-50,52,54,59,60]</sup>. There was a high degree of heterogeneity ( $\chi^2 = 36.6$ ,  $P < 0.001$ ), and so a random-effects model was used. The forest plot, weighted on sample size, is shown in Figure 5. Compared to placebo, probiotics were associated with less risk of abdominal pain ( $RR_{pooled} = 0.78$ ; 95% CI, 0.69-0.88). The number needed to treat was 8.9. The funnel plot was generally symmetrical, showing little evidence of publication bias, and Begg's test did not show statistically significant publication bias ( $z = -0.70$ ,  $P = 0.48$ ). The pooled relative risk for abdominal pain was similar when weighted by study quality ( $RR_{pooled} = 0.61$ ; 95% CI, 0.45-0.81) and after exclusion of the two trials conducted in children ( $RR_{pooled} = 0.77$ ; 95% CI, 0.68-0.88)<sup>[52,60]</sup>.

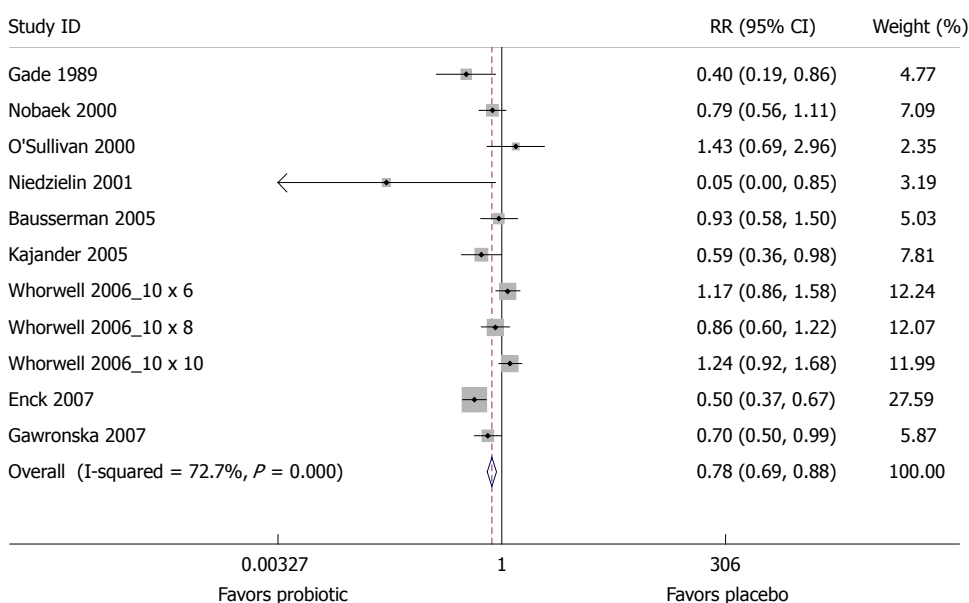
## STUDY CHARACTERISTICS PREDICTING POSITIVE RESULTS

We compared the characteristics of six studies that favored probiotics over placebo (study  $RR < 0.67$  for improvement in global IBS symptoms)<sup>[35,46,48,50,54,59]</sup> with six studies showing a weak effect or no benefit<sup>[45,47,51,58,61,62]</sup>. Studies with a stronger probiotic effect were larger than those showing weak or no effect (median 80.5 subjects *vs* 50 subjects,  $P = 0.20$ ) and had shorter duration of treatment (median 4 wk *vs* 6 wk,  $P = 0.60$ ). but, these

Table 6 Relief of abdominal pain in 11 probiotic/placebo treatment arms

Reference	Probiotic	Improvement in abdominal pain		Definition of secondary outcome <sup>1</sup>
		Probiotic n/n (%)	Placebo n/n (%)	
Gade <sup>[46]</sup>	<i>Strept faecalis</i>	25/32 (78)	10/22 (45)	Absence or presence of symptom
Nobaek <sup>[48]</sup>	<i>L. plantarum</i>	9/25 (36)	5/27 (18)	Decrease $\geq 1.5$ on VAS symptom scale
O'Sullivan <sup>[49]</sup>	<i>L. rhamnosus</i> GG	9/19 (47)	12/19 (63)	Symptom improved
Niedzielin <sup>[50]</sup>	<i>Lacto plantarum</i>	20/20 (100)	11/20 (55)	Absence of symptoms
Bausserman <sup>[52]</sup>	<i>Lacto rhamnosus</i> GG	11/25 (44)	10/25 (40)	Decrease of $\geq 1$ point symptom score
Kajander <sup>[54]</sup>	<i>L. rhamnosus</i> GG + <i>L. rham.</i> LC705 + <i>Bifid. breve</i> Bb99 + <i>Prop. freudenreichii</i>	27/41 (66)	17/40 (43)	Symptoms alleviated
Whorwell <sup>[35]</sup>	<i>Bifido. infantis</i> (10 <sup>6</sup> dose)	32/74 (43)	39/76 (52)	Adequate relief of symptoms
Whorwell <sup>[35]</sup>	<i>Bifido. infantis</i> (10 <sup>8</sup> dose)	42/72 (59)	39/76 (52)	Adequate relief of symptoms
Whorwell <sup>[35]</sup>	<i>Bifido. infantis</i> (10 <sup>10</sup> dose)	28/71 (39)	39/76 (52)	Adequate relief of symptoms
Enck <sup>[59]</sup>	<i>E. coli</i> + <i>Strept faecalis</i>	108/149 (72)	66/148 (45)	$\geq 50\%$ decrease in symptom score
Gawronska <sup>[60]</sup>	<i>Lacto rhamnosus</i> GG	6/18 (33)	1/19 (5)	Absence of pain

<sup>1</sup>All secondary outcomes are defined based on patient report.



**Figure 5** Forest plot of randomized controlled trials of 12 treatment arms from 10 studies measuring relative risk of abdominal pain after treatment with a probiotic compared to placebo. The X-axis depicts relative risk, with black dot indicating the relative risk, line indicating 95% CI and the size of the grey box proportional to sample size.

differences were not statistically significant. Two-thirds of studies showing strong protective effects used proprietary (commercial) products, compared to 100% of those showing weak or no effect ( $P = 0.46$ ). In bivariate analyses, no characteristics differed significantly between the two types of studies.

## ADVERSE EVENTS

Most studies (17/20, 85%) provided only minimal information about adverse events. Fourteen studies (70%) stated that no serious adverse reactions were noted, but failed to provide any information on how adverse events were ascertained or what types of reactions were considered. Three (15%) of the trials did provide limited data on adverse reactions, including reactions such as “increased intestinal symptoms”, “epistaxis”, “aftertaste”, “anxiety” and “angina”, but did not report rates of adverse reactions by treatment group<sup>[17,49,54]</sup>. Three trials (15%) did not report any safety data<sup>[47,58,59]</sup>.

We identified 20 clinical trials that met inclusion criteria

and provided relevant information about the efficacy of probiotics for IBS symptoms. These trials included 23 probiotic treatment arms and 1404 subjects. Trials were generally small and of short duration and had moderate quality. But, the majority did not follow intention-to-treat principles. Overall, probiotic use was associated with less likelihood of global IBS symptoms compared to placebo (RR = 0.77; 95% CI, 0.62-0.94) and with abdominal pain by the end of follow-up (RR = 0.78; 95% CI, 0.69-0.88). There was not sufficient data to examine other individual IBS symptoms or the efficacy of individual probiotic strains.

## STRENGTHS AND LIMITATIONS

We performed a comprehensive review of the literature and made an effort to minimize publication bias by including recent studies as well as those published only as meeting abstracts. Validated quality scoring and data extraction were performed by two reviewers independently, using standardized templates, and differences were resolved by

discussion. We excluded studies of poor quality, limiting the impact of serious study design flaws. We selected a primary outcome (global improvement in IBS symptoms) that is clinically relevant and of great concern to IBS patients, as is also true for our secondary outcome (relief of abdominal pain). Communication with study authors was a productive tool for obtaining data not reported in detail in some studies.

Our findings should be interpreted with caution due to important limitations of the existing literature. Two important limitations in the existing trials included the lack of ITT analysis and the presence of heterogeneity in both outcome assessment and study design. A crucial issue is the quality of included studies, with only 3 of 20 studies performing true intention-to-treat analyses. In many studies, participants were excluded from final analyses for reasons such as noncompliance, failure to respond to treatment, or use of prohibited medications. It is difficult to predict how these exclusions may have affected results. But, it is certainly possible that substantial bias could have been introduced, which could account for the apparent beneficial effects observed when data were pooled across studies. Missing values may cause both systematic and unpredictable bias in controlled trial results<sup>[69-71]</sup>. A recent meta-analysis of chondroitin for osteoarthritis found that small trials and those not analyzed according to ITT principles were more likely to report benefits from chondroitin, while larger studies with greater methodological rigor did not find an effect<sup>[72]</sup>. Larger studies utilizing ITT have not been performed to examine probiotics as potential therapy for IBS.

Heterogeneity was another important limitation of the published literature, including heterogeneity in the strain and dose of probiotic (which prevented analysis of effects of specific strains); sample size (smaller studies resulted in low power to detect effects in individual studies); duration of treatment and follow-up (short trials do not allow adequate follow-up given the chronic relapsing nature of IBS); and in the assessment and reporting of outcomes. All these sources of heterogeneity made it difficult to combine data from all twenty studies. Another important problem is the lack of systematic data collection and reporting about adverse effects. As a result, it is difficult to be sure that the probiotics studied have been adequately evaluated for safety.

## COMPARISON WITH OTHER SYSTEMATIC REVIEWS

To date, no other meta-analysis of probiotics for IBS has been published. Recent published reviews of probiotics for IBS included fewer studies (range, 4 to 12) and focused primarily on evaluating the rationale and potential mechanisms for probiotics as treatment for IBS<sup>[9,13,73]</sup>. No prior reviews have attempted to calculate a pooled estimate of efficacy, and few reviews provided a detailed summary of individual studies' outcome data or unpooled risk estimates.

## IMPLICATIONS FOR FUTURE RESEARCH

This review highlights important considerations for the design of future studies of probiotics as a potential

**Table 7 Recommendations for future research studies examining probiotics as a treatment for irritable bowel syndrome**

### Recommendations

- More trials testing the same probiotic strain
- Larger sample size
- Longer duration of treatment and follow-up
- Intention to treat analysis:
  - All participants analyzed with the group to which they were originally assigned, regardless of compliance with treatment, response to treatment, or use of prohibited (non-study) medications.
- Greater efforts to minimize loss to follow-up
- Standardized assessment of IBS outcomes
  - Provide some assessment of global relief of IBS symptoms
  - Detailed collection and reporting of data on potential adverse reactions

treatment for IBS (Table 7). There is a need for standardized outcome assessments and larger studies, preferably with longer duration of treatment and follow-up. Future studies should make every effort to minimize loss-to-follow-up and to adhere to ITT principles, analyzing all subjects with the group to which they were originally assigned, notwithstanding potential noncompliance with treatment or the use of other (non-study) medications. Following these methodological principles will provide greater assurance that results are not due to bias. Future studies would benefit from better standardization of outcomes to be studied, including the use of uniform symptom scales. We recommend that future studies examine overall relief of IBS symptoms as an outcome. Although many prior studies primarily reported symptom scores, a statistically significant reduction in symptom score may not be meaningful to an individual patient suffering from IBS. Bijkerk *et al* examined the validity of 10 methods to assess IBS response and found a single question asking about 'adequate relief of IBS-related symptoms' was as valid as more detailed questionnaires on outcome<sup>[74]</sup>. In order to determine if one probiotic strain is more effective for IBS than others, confirmatory trials with the same probiotic strains are required.

Finally, it is important that future studies systematically assess potential adverse effects and provide detailed results, including rates of adverse effects in the treatment and placebo groups.

## IMPLICATIONS FOR CLINICAL PRACTICE

While our findings provide preliminary evidence that probiotics may be useful in treating IBS, it is too soon to recommend their use in clinical practice. The pooled relative risks reported here are based on studies with significant methodological limitations, and bias cannot be ruled out as the explanation for these positive findings. Since we did not find any evidence of significant adverse effects from these treatments, and given the lack of available conventional treatments, clinicians should strongly consider discussing the evidence of benefits and risks of probiotics with their patients with IBS. Although the costs of probiotics vary widely, the cost may be similar to other over-the-counter remedies for IBS (such as loperamide).



An important consideration is the lack of regulation of the commercial probiotic products that are currently available. No universal quality assurance programs exist to ensure that commercial products contain the probiotic strain and concentration that are claimed, or to ensure the absence of contamination that could pose risks to consumers. Some resources are available to provide further information about product testing; for example, ConsumerLab is an independent company in the U.S. that tests commercially available health and nutrition products and publishes data about the contents of various commercial products, including the presence of contaminants (<http://www.consumerlab.com>). They also offer a voluntary certification program. In the summer of 2007, the Food and Drug Administration issued new rules regarding good manufacturing practices for supplement manufacturers, aimed at ensuring more uniform quality of supplements. It remains to be seen whether these new rules will substantially improve the quality and safety of nutritional supplements.

## CONCLUSION

In summary, the present meta-analysis suggests that probiotics offer promise for the treatment of IBS. Results should be interpreted cautiously given the methodological limitations of published studies. Future studies are needed, in particular larger studies of longer duration with greater methodological rigor. In addition, more data are needed regarding which specific strains and doses are most likely to be effective. The use of probiotics for IBS warrants further study, particularly given the chronic nature of this condition, its major impact on patients' quality of life, and the dearth of other effective treatments.

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## REFERENCES

- Cain KC, Headstrom P, Jarrett ME, Motzer SA, Park H, Burr RL, Surawicz CM, Heitkemper MM. Abdominal pain impacts quality of life in women with irritable bowel syndrome. *Am J Gastroenterol* 2006; **101**: 124-132
- Ford AC, Forman D, Bailey AG, Axon AT, Moayyedi P. Initial poor quality of life and new onset of dyspepsia: results from a longitudinal 10-year follow-up study. *Gut* 2007; **56**: 321-327
- Cremonini F, Talley NJ. Irritable bowel syndrome: epidemiology, natural history, health care seeking and emerging risk factors. *Gastroenterol Clin North Am* 2005; **34**: 189-204
- Saito YA, Cremonini F, Talley NJ. Association of the 1438G/A and 102T/C polymorphism of the 5-HT<sub>2A</sub> receptor gene with irritable bowel syndrome 5-HT<sub>2A</sub> gene polymorphism in irritable bowel syndrome. *J Clin Gastroenterol* 2005; **39**: 835; author reply 835-836
- Spiller R, Aziz Q, Creed F, Emmanuel A, Houghton L, Hungin P, Jones R, Kumar D, Rubin G, Trudgill N, Whorwell P. Guidelines on the irritable bowel syndrome: mechanisms and practical management. *Gut* 2007; **56**: 1770-1798
- Ruigomez A, Garcia Rodriguez LA, Panes J. Risk of irritable bowel syndrome after an episode of bacterial gastroenteritis in general practice: influence of comorbidities. *Clin Gastroenterol Hepatol* 2007; **5**: 465-469
- Agrawal A, Whorwell PJ. Irritable bowel syndrome: diagnosis and management. *BMJ* 2006; **332**: 280-283
- Cremonini F, Talley NJ. Treatments targeting putative mechanisms in irritable bowel syndrome. *Nat Clin Pract Gastroenterol Hepatol* 2005; **2**: 82-88
- Quigley EM, Flourie B. Probiotics and irritable bowel syndrome: a rationale for their use and an assessment of the evidence to date. *Neurogastroenterol Motil* 2007; **19**: 166-172
- Spiller RC. Role of infection in irritable bowel syndrome. *J Gastroenterol* 2007; **42** Suppl 17: 41-47
- Lin HC. Small intestinal bacterial overgrowth: a framework for understanding irritable bowel syndrome. *JAMA* 2004; **292**: 852-858
- Malinen E, Rinttila T, Kajander K, Matto J, Kassinen A, Krogius L, Saarela M, Korpela R, Palva A. Analysis of the fecal microbiota of irritable bowel syndrome patients and healthy controls with real-time PCR. *Am J Gastroenterol* 2005; **100**: 373-382
- Andresen V, Baumgart DC. Role of probiotics in the treatment of irritable bowel syndrome: potential mechanisms and current clinical evidence. *Inter J Probiotics Prebiotics* 2006; **1**: 11-18
- McFarland LV. Normal flora: diversity and functions. *Microb Ecol Health Dis* 2000; **12**: 193-207
- Elmer GW, McFarland LV, McFarland M. Introduction. Chapter 1. In: *The Power of Probiotics: Improving Your Health with Beneficial Microbes.*, Binghamton, New York: Haworth Press, 2007: 3-5
- McCarthy J, O'Mahony L, O'Callaghan L, Sheil B, Vaughan EE, Fitzsimons N, Fitzgibbon J, O'Sullivan GC, Kiely B, Collins JK, Shanahan F. Double blind, placebo controlled trial of two probiotic strains in interleukin 10 knockout mice and mechanistic link with cytokine balance. *Gut* 2003; **52**: 975-980
- O'Mahony L, McCarthy J, Kelly P, Hurley G, Luo F, Chen K, O'Sullivan GC, Kiely B, Collins JK, Shanahan F, Quigley EM. Lactobacillus and bifidobacterium in irritable bowel syndrome: symptom responses and relationship to cytokine profiles. *Gastroenterology* 2005; **128**: 541-551
- Verdu EF, Bercik P, Verma-Gandhu M, Huang XX, Blennerhassett P, Jackson W, Mao Y, Wang L, Rochat F, Collins SM. Specific probiotic therapy attenuates antibiotic induced visceral hypersensitivity in mice. *Gut* 2006; **55**: 182-190
- Szajewska H, Ruszczynski M, Radzikowski A. Probiotics in the prevention of antibiotic-associated diarrhea in children: a meta-analysis of randomized controlled trials. *J Pediatr* 2006; **149**: 367-372
- McFarland LV. Meta-analysis of probiotics for the prevention of antibiotic associated diarrhea and the treatment of Clostridium difficile disease. *Am J Gastroenterol* 2006; **101**: 812-822
- McFarland LV, Elmer GW and McFarland M. Meta-analysis of Probiotics for the Prevention and Treatment of Acute Pediatric Diarrhea. *Internl J Probiotics Prebiotics* 2006; **1**: 63-76
- McFarland LV. Meta-analysis of probiotics for the prevention of traveler's diarrhea. *Travel Med Infect Dis* 2007; **5**: 97-105
- Kuhbacher T, Ott SJ, Helwig U, Mimura T, Rizzello F, Kleessen B, Gionchetti P, Blaut M, Campieri M, Folsch UR, Kamm MA, Schreiber S. Bacterial and fungal microbiota in relation to probiotic therapy (VSL#3) in pouchitis. *Gut* 2006; **55**: 833-841
- Mimura T, Rizzello F, Helwig U, Poggioli G, Schreiber S, Talbot IC, Nicholls RJ, Gionchetti P, Campieri M, Kamm MA. Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. *Gut* 2004; **53**: 108-114
- Shaw RL, Booth A, Sutton AJ, Miller T, Smith JA, Young B, Jones DR, Dixon-Woods M. Finding qualitative research: an evaluation of search strategies. *BMC Med Res Methodol* 2004; **4**: 5

- 26 **Egger M**, Smith GD, Phillips AN. Meta-analysis: principles and procedures. *BMJ* 1997; **315**: 1533-1537
- 27 **Moher D**, Cook DJ, Eastwood S, Olkin I, Rennie D, Stroup DF. Improving the quality of reports of meta-analyses of randomised controlled trials: the QUOROM statement. QUOROM Group. *Br J Surg* 2000; **87**: 1448-1454
- 28 **Lim B**, Manheimer E, Lao L, Ziea E, Wisniewski J, Liu J, Berman B. Acupuncture for treatment of irritable bowel syndrome. *Cochrane Database Syst Rev* 2006; CD005111
- 29 **Linde K**, Ramirez G, Mulrow CD, Pauls A, Weidenhammer W, Melchart D. St John's wort for depression—an overview and meta-analysis of randomised clinical trials. *BMJ* 1996; **313**: 253-258
- 30 **Clarke M**, Oxman AD, eds. Analyzing and Presenting Results: Cochrane Reviewers' Handbook 4.2 (updated November 2002-Section 8.) In: the Cochrane Library. Oxford: Update Software; 2003, issue 1, cited January 23, 2008. Available from: URL: <http://www.cochrane.org/resources/handbook/index.htm>
- 31 **DerSimonian R**, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177-188
- 32 **Egger M**, Davey Smith G, Schneider M, Minder C. Bias in meta-analysis detected by a simple, graphical test. *BMJ* 1997; **315**: 629-634
- 33 **Begg CB**, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. *Biometrics* 1994; **50**: 1088-1101
- 34 **Sutton AJ**, Duval SJ, Tweedie RL, Abrams KR, Jones DR. Empirical assessment of effect of publication bias on meta-analyses. *BMJ* 2000; **320**: 1574-1577
- 35 **Whorwell PJ**, Altringer L, Morel J, Bond Y, Charbonneau D, O'Mahony L, Kiely B, Shanahan F, Quigley EM. Efficacy of an encapsulated probiotic Bifidobacterium infantis 35624 in women with irritable bowel syndrome. *Am J Gastroenterol* 2006; **101**: 1581-1590
- 36 **Adler SN**. The probiotic agent Escherichia coli M-17 has a healing effect in patients with IBS with proximal inflammation of the small bowel. *Dig Liver Dis* 2006; **38**: 713
- 37 **Bazzocchi G**, Gionchetti P, Almerigi PF, Amadini C, Campieri M. Intestinal microflora and oral bacteriotherapy in irritable bowel syndrome. *Dig Liver Dis* 2002; **34** Suppl 2: S48-S53
- 38 **Brigidi P**, Vitali B, Swennen E, Bazzocchi G, Matteuzzi D. Effects of probiotic administration upon the composition and enzymatic activity of human fecal microbiota in patients with irritable bowel syndrome or functional diarrhea. *Res Microbiol* 2001; **152**: 735-741
- 39 **Colecchia A**, Vestito A, La Rocca A, Pasqui F, Nikiforaki A, Festi D. Effect of a symbiotic preparation on the clinical manifestations of irritable bowel syndrome, constipation-variant. Results of an open, uncontrolled multicenter study. *Minerva Gastroenterol Dietol* 2006; **52**: 349-358
- 40 **Drisko J**, Bischoff B, Hall M, McCallum R. Treating irritable bowel syndrome with a food elimination diet followed by food challenge and probiotics. *J Am Coll Nutr* 2006; **25**: 514-522
- 41 **Fan YJ**, Chen SJ, Yu YC, Si JM, Liu B. A probiotic treatment containing Lactobacillus, Bifidobacterium and Enterococcus improves IBS symptoms in an open label trial. *J Zhejiang Univ Sci B* 2006; **7**: 987-991
- 42 **Astegiano M**, Pellicano R, Terzi E, Simondi D, Rizzetto M. Treatment of irritable bowel syndrome. A case control experience. *Minerva Gastroenterol Dietol* 2006; **52**: 359-363
- 43 **Tsuchiya J**, Barreto R, Okura R, Kawakita S, Fesce E, Marotta F. Single-blind follow-up study on the effectiveness of a symbiotic preparation in irritable bowel syndrome. *Chin J Dig Dis* 2004; **5**: 169-174
- 44 **Sen S**, Mullan MM, Parker TJ, Woolner JT, Tarry SA, Hunter JO. Effect of Lactobacillus plantarum 299v on colonic fermentation and symptoms of irritable bowel syndrome. *Dig Dis Sci* 2002; **47**: 2615-2620
- 45 **Maupas JL**, Champemont P, Delforge M. Treatment of irritable bowel syndrome. Double blind trial of Saccharomyces boulardii. *Medecine Chirurgie Digestives* 1983; **12**: 77-79
- 46 **Gade J**, Thorn P. Paragurth for patients with irritable bowel syndrome. A controlled clinical investigation from general practice. *Scand J Prim Health Care* 1989; **7**: 23-26
- 47 **Halpern GM**, Prindiville T, Blankenburg M, Hsia T, Gershwin ME. Treatment of irritable bowel syndrome with Lacteol Fort: a randomized, double-blind, cross-over trial. *Am J Gastroenterol* 1996; **91**: 1579-1585
- 48 **Nobaek S**, Johansson ML, Molin G, Ahrne S, Jeppsson B. Alteration of intestinal microflora is associated with reduction in abdominal bloating and pain in patients with irritable bowel syndrome. *Am J Gastroenterol* 2000; **95**: 1231-1238
- 49 **O'Sullivan MA**, O'Morain CA. Bacterial supplementation in the irritable bowel syndrome. A randomised double-blind placebo-controlled crossover study. *Dig Liver Dis* 2000; **32**: 294-301
- 50 **Niedzielin K**, Kordecki H, Birkenfeld B. A controlled, double-blind, randomized study on the efficacy of Lactobacillus plantarum 299V in patients with irritable bowel syndrome. *Eur J Gastroenterol Hepatol* 2001; **13**: 1143-1147
- 51 **Kim HJ**, Camilleri M, McKinzie S, Lempke MB, Burton DD, Thomforde GM, Zinsmeister AR. A randomized controlled trial of a probiotic, VSL#3, on gut transit and symptoms in diarrhoea-predominant irritable bowel syndrome. *Aliment Pharmacol Ther* 2003; **17**: 895-904
- 52 **Bausserman M**, Michail S. The use of Lactobacillus GG in irritable bowel syndrome in children: a double-blind randomized control trial. *J Pediatr* 2005; **147**: 197-201
- 53 **Bittner AC**, Croffut RM, Stranahan MC. Prescript-Assist probiotic-prebiotic treatment for irritable bowel syndrome: a methodologically oriented, 2-week, randomized, placebo-controlled, double-blind clinical study. *Clin Ther* 2005; **27**: 755-761
- 54 **Kajander K**, Hatakka K, Poussa T, Farkkila M, Korpela R. A probiotic mixture alleviates symptoms in irritable bowel syndrome patients: a controlled 6-month intervention. *Aliment Pharmacol Ther* 2005; **22**: 387-394
- 55 **Kim HJ**, Vazquez Roque MI, Camilleri M, Stephens D, Burton DD, Baxter K, Thomforde G, Zinsmeister AR. A randomized controlled trial of a probiotic combination VSL# 3 and placebo in irritable bowel syndrome with bloating. *Neurogastroenterol Motil* 2005; **17**: 687-696
- 56 **Niv E**, Naftali T, Hallak R, Vaisman N. The efficacy of Lactobacillus reuteri ATCC 55730 in the treatment of patients with irritable bowel syndrome--a double blind, placebo-controlled, randomized study. *Clin Nutr* 2005; **24**: 925-931
- 57 **Kim YG**, Moon JT, Lee KM, Chon NR, Park H. The effects of probiotics on symptoms of irritable bowel syndrome. *Korean J Gastroenterol* 2006; **47**: 413-419
- 58 **Simren M**, Syrous A, Lindh A, Abrahamsson H. Effects of lactobacillus plantarum 299v on symptoms and rectal sensitivity in patients with irritable bowel syndrome (IBS) - A randomized, double-blind controlled trial. *Gastroenterology* 2006; **130** Suppl 2: A600
- 59 **Enck P**, Menke G, Zimmermann K, Martens U, Klosterhalfen S. Effective probiotic therapy of the irritable bowel syndrome (IBS): A multi-center clinical trial with primary care physicians. *Gastroenterology* 2007; **132** Suppl 2: A79
- 60 **Gawronska A**, Dziechciarz P, Horvath A, Szajewska H. A randomized double-blind placebo-controlled trial of Lactobacillus GG for abdominal pain disorders in children. *Aliment Pharmacol Ther* 2007; **25**: 177-184
- 61 **D'haens GR**, Kovacs G, Vergauwe P, Lonovics J, Bouhnik Y, Weiss W, Brunner H, Lavergne-Slove A, Di Stefano AF, Marteau P. A randomized controlled trial of the probiotic combination Lactibiane (R) in irritable bowel syndrome, the Lactibiane (R) study group. *Gastroenterology* 2007; **132** Suppl 2: A371
- 62 **Simren M**, Lindh A, Samelsson L, Olsson J, Posserud I, Strid H, Abrahamsson H. Effect of yoghurt containing three probiotic bacteria in patients with irritable bowel syndrome (IBS) - A randomized, double-blind, controlled trial. *Gastroenterology* 2007; **132** Suppl 2: A210
- 63 **DiBaise JK**, Lof J, Taylor K, Quigley EM. Lactobacillus plantarum 299V in the irritable bowel syndrome: A randomized, double-blind, placebo-controlled crossover study. *Gastroenterology* 2000; **118** Suppl 2: A3163
- 64 **Saggioro A**. Probiotics in the treatment of irritable bowel

- syndrome. *J Clin Gastroenterol* 2004; **38**: S104-S106
- 65 **Long ZR**, Yu CH, Yang Y, Wang HN, Chi XX. Clinical observation on acupuncture combined with microorganism pharmaceutical preparations for treatment of irritable bowel syndrome of constipation type. *Zhongguo Zhenjiu* 2006; **26**: 403-405
- 66 **Kajander K**, Korpela R. Clinical studies on alleviating the symptoms of irritable bowel syndrome. *Asia Pac J Clin Nutr* 2006; **15**: 576-580
- 67 **Bittner AC**, Croffut RM, Stranahan MC, Yokelson TN. Prescript-assist probiotic-prebiotic treatment for irritable bowel syndrome: an open-label, partially controlled, 1-year extension of a previously published controlled clinical trial. *Clin Ther* 2007; **29**: 1153-1160
- 68 **Moon JT**, Kim HS, Park HJ. Effects of probiotics on the intestinal gas volume score and symptoms in patients with irritable bowel syndrome. A randomized double-blind placebo-controlled study. *Gastroenterology* 2007; **132** Suppl 2: A688
- 69 **Porta N**, Bonet C, Cobo E. Discordance between reported intention-to-treat and per protocol analyses. *J Clin Epidemiol* 2007; **60**: 663-669
- 70 **Salim A**, Mackinnon A, Griffiths K. Sensitivity analysis of intention-to-treat estimates when withdrawals are related to unobserved compliance status. *Stat Med* 2008; **27**: 1164-1179
- 71 **Brittain E**, Lin D. A comparison of intent-to-treat and per-protocol results in antibiotic non-inferiority trials. *Stat Med* 2005; **24**: 1-10
- 72 **Reichenbach S**, Sterchi R, Scherer M, Trelle S, Burgi E, Burgi U, Dieppe PA, Juni P. Meta-analysis: chondroitin for osteoarthritis of the knee or hip. *Ann Intern Med* 2007; **146**: 580-590
- 73 **Camilleri M**. Probiotics and irritable bowel syndrome: rationale, putative mechanisms, and evidence of clinical efficacy. *J Clin Gastroenterol* 2006; **40**: 264-269
- 74 **Bijkerk CJ**, de Wit NJ, Muris JW, Jones RH, Knottnerus JA, Hoes AW. Outcome measures in irritable bowel syndrome: comparison of psychometric and methodological characteristics. *Am J Gastroenterol* 2003; **98**: 122-127

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## TOPIC HIGHLIGHT

Lynne V McFarland, PhD, Series Editor

# Colorectal cancer and dysplasia in inflammatory bowel disease

Timothy L Zisman, David T Rubin

Timothy L Zisman, Clinical Research Fellow in Gastro-enterology, the University of Chicago Medical Center, 5841 S. Maryland Avenue MC 4076, Chicago 60637, United States

David T Rubin, The University of Chicago Medical Center, 5841 S. Maryland Ave. MC 4080, Chicago, IL 60637, United States

Author contributions: Zisman TL performed the literature review and manuscript preparation; Rubin DT provided editing, revision and oversight.

Correspondence to: David T Rubin, MD, Associate Professor of Medicine, The University of Chicago Medical Center, 5841 S Maryland Avenue MC 4080, Chicago, IL 60637,

United States. [drubin@medicine.bsd.uchicago.edu](mailto:drubin@medicine.bsd.uchicago.edu)

Telephone: +1-773-7022950 Fax: +1-773-7025790

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## Abstract

Both ulcerative colitis and Crohn's disease carry an increased risk of developing colorectal cancer. Established risk factors for cancer among patients with inflammatory bowel disease (IBD) include the younger age at diagnosis, greater extent and duration of disease, increased severity of inflammation, family history of colorectal cancer and coexisting primary sclerosing cholangitis. Recent evidence suggests that current medical therapies and surgical techniques for inflammatory bowel disease may be reducing the incidence of this complication. Nonetheless heightened vigilance and a careful, comprehensive approach to prevent or minimize the complications of invasive cancer are warranted in this unique cohort of patients. Current guidelines for the prevention and early detection of cancer in this high risk population are grounded in the concept of an inflammation-dysplasia-carcinoma sequence. A thorough understanding of the definition and natural history of dysplasia in IBD, as well as the challenges associated with detection and interpretation of dysplasia are fundamental to developing an effective strategy for surveillance and prevention, and understanding the limitations of the current approach to prevention. This article reviews the current consensus guidelines for screening and surveillance of cancer in IBD, as well as presenting the evidence and rationale for chemoprevention of cancer and a discussion of emerging technologies for the detection of dysplasia.

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**Key words:** Cancer; Dysplasia; Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Chemoprevention

## INTRODUCTION

Patients with inflammatory bowel disease (IBD) have an increased risk of developing intestinal cancer. The magnitude of that increased risk as well as how best to mitigate it remain a topic of ongoing investigation in the field. Although only 1% of all cases of colorectal cancer (CRC) occur in patients with ulcerative colitis (UC) or Crohn's disease<sup>[1]</sup>, patients with IBD represent one of the highest risk groups for developing this dreaded complication. Strategies to reduce or prevent the complications associated with invasive cancer are essential in this high-risk population. Current guidelines advocate routine surveillance colonoscopy as the cornerstone of prevention. For patients in whom precancerous dysplastic lesions or early cancer are detected, surgical removal of the colon can be a potentially curative procedure for both the cancer and the colitis. This secondary prevention strategy has several drawbacks, however. Colonoscopy is less sensitive for detecting precancerous dysplasia in IBD patients than in the general population. Unlike in sporadic CRC in which dysplastic adenomas begin as raised polypoid lesions, dysplasia in IBD can arise in mucosa that is indistinct from surrounding mucosa, making it "invisible" to the endoscopist. Consequently many lesions may be missed. Additionally, the molecular biology of cancer in IBD is unique in that the accumulation of molecular and genetic alterations may occur more rapidly or in an unconventional sequence when compared to sporadic CRC. Given the limitations of the current surveillance approach, primary cancer prevention via chemoprevention has been proposed as an alternative or additive strategy. Although such a prospect would be ideal, the effectiveness of medications to mitigate cancer risk in IBD has not been firmly established. Further research is directed toward improving detection of dysplasia during colonoscopy through the use of novel endoscopic imaging techniques.

These exciting and promising developments are hoped to impact the approach to cancer prevention in patients with IBD. This article reviews the epidemiology of cancer and dysplasia in IBD, as well as the evidence and rationale behind consensus guidelines for screening and surveillance.

## EPIDEMIOLOGY OF CANCER IN ULCERATIVE COLITIS

The increased risk of colorectal cancer in UC has been recognized for decades, although estimates of the magnitude of that risk vary considerably in the literature. Some authors have described a cumulative probability as high as 60% for developing cancer after 40 years of disease<sup>[2]</sup>, while others have demonstrated a risk level on par with that of the general population<sup>[3]</sup>. Several reasons have been proposed to explain these discrepant results, including a lack of uniformity in study design and case definitions, geographic differences in incidence based on environmental factors, and referral center bias. In an effort to bring clarity to this issue, Eaden and colleagues performed a meta-analysis in 2001 in which they reviewed 116 studies (41 of which were included in the analysis), encompassing 54478 patients with UC and 1698 cases of CRC, to yield an overall prevalence of CRC in UC of 3.7%<sup>[4]</sup>. By pooling the results of the studies that reported data on duration of disease by decade the authors were able to calculate a cumulative probability of CRC in UC of 2% after 10 years, 8% after 20 years, and 18% after 30 years of disease. Since this landmark publication, however, several more recent population-based and referral-based studies suggest that this risk may be declining over time or may simply be lower than previously accepted. Bernstein *et al* conducted a large population-based study in Manitoba, Canada, including 19655 person years of follow-up in which they described an increased incidence rate ratio for developing CRC of 2.75 [95% confidence interval (95% CI), 1.91-3.97] compared to the general population<sup>[5]</sup>. This estimate of risk is approximately half that reported in the Eaden meta-analysis. These results were replicated in a population-based study in Hungary where the cumulative incidence of CRC was found to be 0.6% after 10 years, 5.4% after 20 years, and 7.5% after 30 years of chronic UC<sup>[6]</sup>. A small population study from Olmsted County in the United States followed 378 patients diagnosed with UC between 1940 and 2001<sup>[7]</sup>. Only 6 cases of CRC were discovered in 5567 person-years of follow-up, yielding a 30-year cumulative probability of CRC in this cohort of only 2%, not significantly different from the non-IBD patients in this population cohort. Interestingly, no cases of CRC were discovered in patients whose UC was diagnosed after 1980. Although the number of patients in this study was relatively small, these results were reinforced by a much larger study in Denmark involving 22290 person-years of follow-up that demonstrated no increase in CRC risk among UC patients<sup>[3]</sup>. The 30-year cumulative probability of CRC was only 2.1% among UC patients in this population. This declining trend in CRC incidence among UC patients holds true at referral centers as well. In 2006, Rutter and colleagues from St. Mark's Hospital in the

United Kingdom reported on their 30 year experience with the longest prospectively collected database on surveillance for dysplasia and cancer in UC<sup>[8]</sup>. The cumulative incidence of CRC in this referral population was 2.5% at 20 years, 7.6% at 30 years and 10.8% after 40 years of disease. The reasons for this observed change in incidence may be the more widespread use of surveillance colonoscopy, a chemoprotective effect attributable to the more widespread use of maintenance therapies, more aggressive surgical intervention or dietary or environmental factors. Despite the encouraging finding that CRC in UC appears to be less common than previously believed, one should take caution in interpreting the results of these more recent studies as evidence to relax the practice of routine screening and surveillance. Rather these results may indicate that a comprehensive approach to screening, surveillance and control of disease inflammation is highly effective and that physicians should maintain an appropriate level of vigilance in this high-risk patient population<sup>[9]</sup>.

## RISK FACTORS FOR CRC IN ULCERATIVE COLITIS

Several factors have been identified that increase the risk of CRC in patients with UC. The observation that cumulative cancer risk increases over time<sup>[4,10]</sup> establishes that increasing duration of disease is an important risk factor. Consistent with an intuitive understanding of CRC risk as being associated with the cumulative effect of chronic inflammation, the extent of colon involvement in UC is an independent predictor of cancer risk<sup>[10]</sup>. A Swedish study reported that the relative risk of CRC was 1.7 for ulcerative proctitis, whereas the risk in left-sided colitis was 2.8 and this risk rose to 14.8 in patients with extensive colitis<sup>[10]</sup>.

Until relatively recently there was no hard evidence to support the intuitive notion that the degree of inflammation correlates with cancer risk. This may in part be due to the difficulty of demonstrating an independent effect of inflammatory activity while controlling for duration and extent of disease, two factors that are surrogate measures of cumulative inflammation. Three recent studies have confirmed this association, however. In a retrospective analysis from the St. Mark's Hospital in the UK, Rutter and colleagues demonstrated that severity of inflammation on biopsy independently predicted risk of CRC<sup>[11]</sup>. This finding was reinforced by studies from the University of Chicago and Mt. Sinai Medical Center in New York in which inflammatory activity was shown to be independently associated with CRC risk<sup>[12,13]</sup>. In several studies, a younger age at diagnosis is also associated with an elevated risk of CRC, independent of disease duration<sup>[10]</sup>. Although the reason for this association is not known, it may in part be related to the finding that patients with an early age of diagnosis tend to have more severe inflammation<sup>[11]</sup>. Several studies have demonstrated that a family history of CRC, independent of a family history of IBD is associated with higher risk of developing cancer<sup>[14-17]</sup>. Additionally, coexistent primary sclerosing cholangitis (PSC) confers an elevated risk of CRC in UC



patients, with a meta-analysis by Soetikno *et al* describing an odds ratio of 4.09 (95% CI, 2.89-5.76) when compared to UC patients without PSC<sup>[18]</sup>. This finding has led to the recommendation of closer surveillance in this unique high-risk subset of UC patients. The reason for elevated CRC risk in PSC may be that PSC is a marker for longstanding subclinical disease<sup>[19]</sup>. However, the finding that treatment with ursodeoxycholic acid (UDCA) can lessen CRC risk suggests that the altered bile acid milieu of PSC may play a role in carcinogenesis<sup>[20,21]</sup>. Additionally, one study has demonstrated that backwash ileitis may be an independent predictor of increased CRC risk<sup>[22]</sup>. The finding of a stricture or dysplasia during colonoscopy also carries a heightened risk of malignancy<sup>[23,24]</sup>.

Thus, cancer risk in UC appears to result from the combined effects of chronic inflammation (as estimated by the extent and duration of disease and the degree of histologic inflammation) and an individual's underlying genetic predisposition (as suggested by family history, coexistent PSC and early age of diagnosis). Unfortunately, severity of inflammation appears to be the only modifiable risk factor, underscoring the importance of medical management in mitigating cancer risk, and highlighting the need for a preventive approach to cancer and pre-cancer detection.

## CANCER IN CROHN'S DISEASE

While the relationship between UC and CRC has been appreciated for many years, the association between Crohn's disease and CRC has gained increasing recognition recently. Measuring the risk of CRC in Crohn's disease poses several methodological challenges, relating to the heterogeneous nature of the disease, with many patients having no colonic involvement. Even among patients with Crohn's colitis it is difficult to control for the extent of colonic inflammation, given that the disease can involve any area of the colon in a patchy distribution, and many Crohn's patients have undergone partial surgical resection of the colon, removing some of the at-risk tissue. Consequently, there is substantial variation among the articles that attempt to quantify the risk of CRC in Crohn's, in terms of both study design and results.

Several publications offer estimates of CRC risk in colonic Crohn's disease. Gyde and colleagues reported the relative risk of CRC in Crohn's colitis to be 23.8, whereas the risk was 4.3 in the general Crohn's population<sup>[25]</sup>. Greenstein and colleagues calculated a relative risk of 6.9 for developing CRC in isolated colonic Crohn's<sup>[26]</sup>. A landmark study from Sweden demonstrated a relative risk of CRC of 5.6 for those with exclusively colonic involvement, as compared to a relative risk of 3.2 for patients with ileocolitis and 1.0 for patients with ileal involvement only<sup>[27]</sup>. This not only established that Crohn's carries a higher risk of CRC, but also that this risk correlates with the extent of colonic involvement. Additionally, a subset analysis revealed that patients whose IBD was diagnosed prior to age 30 had a higher relative risk than patients diagnosed at an older age, similar to patients with ulcerative colitis.

A meta-analysis of twelve hospital-based and population-

based studies of CRC risk in Crohn's disease revealed an overall relative risk of CRC in all Crohn's patients of 2.5 (95% CI, 1.3-4.7)<sup>[28]</sup>. In the subset of patients with colonic disease this risk rose to 4.5 (95% CI, 1.3-14.9), while for patients with ileal disease only the risk was not significantly different from the general population. The cumulative risk of CRC for all patients with Crohn's disease, regardless of disease distribution, was 2.9% after 10 years, 5.6% after 20 years and 8.3% after 30 years of disease. In contrast to the Canavan meta-analysis that included population and referral-based studies, Jess *et al* performed a meta-analysis restricted to population studies of intestinal cancer risk in Crohn's<sup>[29]</sup>. Six papers met the inclusion criteria and reported varying estimates of relative risk of CRC ranging from 0.9 to 2.2, with a pooled estimate of 1.9 (95% CI, 1.4-2.5).

The risk of CRC risk in Crohn's is equivalent to that in UC when comparison is controlled for similar extent of disease. In a study by Gillen and colleagues from the UK, patients with extensive Crohn's colitis were compared to patients with extensive ulcerative colitis with regard to CRC risk<sup>[30]</sup>. The results were astonishingly similar with a relative risk of developing CRC of 18 for Crohn's colitis and 19 for UC. The cumulative risk of CRC was 8% at 22 years for patients with Crohn's versus 7% at 22 years for patients with UC. A large population-based study in Canada demonstrated increased incidence rate ratio of CRC in Crohn's patients of 2.64 (95% CI, 1.69-4.12) compared to the general population<sup>[5]</sup>. This was remarkably similar to the risk of CRC in UC patients in the same study, reinforcing the finding that Crohn's and UC share a similar risk of CRC. In addition to a similar magnitude of risk, Crohn's patients share many of the same risk factors for CRC as UC patients, including younger age at diagnosis, greater extent of colonic involvement and longer duration of disease. In addition it appears that bypassed segments of bowel<sup>[31]</sup> and perianal fistulae<sup>[32]</sup> in Crohn's disease may also be sites at increased risk for neoplastic transformation and warrant heightened vigilance. Furthermore, bowel strictures in Crohn's disease may harbor dysplasia or cancer<sup>[33]</sup> and should be carefully biopsied and resected if a pediatric or upper endoscope cannot traverse them. Different from UC, however, is that benign strictures are considered a possible manifestation of the disease so may not need resection otherwise.

While it is technically difficult to determine the exact risk of CRC in Crohn's disease, it is generally accepted that patients with Crohn's disease of the colon are at increased risk for dysplasia and CRC, and that this risk is related to cumulative effect of colonic inflammation, akin to UC. With the exception of strictures as described above, screening and surveillance of CRC in patients with Crohn's should be handled identically to patients with UC, matched for extent of colonic involvement.

## DYSPLASIA IN INFLAMMATORY BOWEL DISEASE

The current approach to surveillance is grounded in the concept of an inflammation-dysplasia-carcinoma sequence, with dysplasia representing a premalignant

phase during which intervention can prevent or minimize the complications associated with invasive cancer. An understanding of the definition, diagnostic challenges and natural history of dysplasia in IBD is, therefore, essential when contemplating complex clinical management decisions.

Dysplasia is defined as unequivocal neoplasia of the epithelium confined to the basement membrane, without invasion into the lamina propria<sup>[34]</sup>. Dysplasia can be classified as raised or flat based on its endoscopic appearance. Flat dysplasia is classically thought to be endoscopically invisible and is detected only on random biopsy specimens. At least 2 authors, however, have demonstrated that many of these lesions are in fact visible through standard white light endoscopy using newer generation colonoscopes with higher optical resolution<sup>[35,36]</sup>. Elevated lesions that are endoscopically visible, but not amenable to endoscopic resection are often referred to as DALMs (dysplasia associated lesion or mass) a term with ominous connotation attributable to the high rate of synchronous malignancy associated with these lesions<sup>[24,37]</sup>. A newer term ALM (adenoma-like lesion or mass) has been introduced to describe the finding of a polypoid lesion resembling a sporadic adenoma that is found in an area of the colon not involved by chronic colitis. Irrespective of the endoscopic appearance of a lesion as raised or flat, pathologists use the same set of criteria to describe the histologic appearance of dysplasia in IBD. A standardized classification system introduced by Riddell and colleagues in 1983 divides dysplasia into categories, including indefinite dysplasia, low grade dysplasia (LGD), high grade dysplasia (HGD) and cancer<sup>[34]</sup>. Although this system remains widely employed, it has several acknowledged limitations, including poor inter-observer agreement and intra-observer reliability, even among expert gastrointestinal pathologists<sup>[34,38]</sup>. This lack of concordance of biopsy interpretations has led to the routine practice of requiring confirmation of a dysplasia diagnosis by a second expert pathologist prior to making critical treatment decisions.

Management of dysplasia, once diagnosed, relies on an understanding of the natural history. In 1994, two groups published data revealing that approximately one in 8 patients with UC will have dysplasia or cancer found on their initial screening colonoscopy, but that those with a negative initial exam have a low incidence (about 3%) of developing high grade dysplasia or cancer on subsequent surveillance colonoscopies<sup>[24,39]</sup>. Among patients with LGD who undergo immediate colectomy 19% will already harbor concurrent CRC or HGD<sup>[24]</sup> and an additional 29%-54% will subsequently develop advanced neoplasia over the next 5 years<sup>[24,39,40]</sup>. HGD carries a 43% risk of synchronous malignancy and is therefore considered to be an indication for immediate colectomy<sup>[24]</sup>. DALMs associated with a similarly high rate of CRC are likewise an indication for total proctocolectomy<sup>[24,37]</sup>. In contrast to DALMs, however, adenoma-like lesions can be safely managed by polypectomy with biopsies of the surrounding flat mucosa<sup>[41]</sup>. If the lesion is successfully removed in its entirety and the surrounding mucosa is free of dysplasia, a regimen of more frequent surveillance colonoscopy is

recommended. The finding of adjacent dysplasia in the flat mucosa prompts immediate colectomy by most experts, given the likelihood of concurrent cancer or progression to cancer.

## MOLECULAR BIOLOGY OF CANCER IN IBD

Several of the molecular alterations that contribute to sporadic CRC are also found in colitis-associated CRC, including loss of APC and p53 tumor suppressor gene function. However, the timing and sequence in which these genetic mutations occur differs from sporadic CRC. Whereas APC loss is considered an early development in the adenoma-carcinoma sequence of sporadic CRC, and p53 represents the final mutation that transforms adenoma into carcinoma, the opposite is often true in IBD-associated CRC. While the description of an inflammation-dysplasia-carcinoma sequence facilitates our understanding of the molecular alterations involved in IBD-associated CRC, it is important to recognize that this process does not necessarily occur in a systematic and sequential progression from inflammation to indefinite dysplasia to LGD to HGD and ultimately to carcinoma. Cancer can develop without any apparent preceding dysplasia, and the natural history of low grade dysplasia has been described to regress or to progress to cancer without evolving first into HGD<sup>[42]</sup>. This unpredictable course of dysplasia in IBD complicates efforts to develop molecular or histologic markers of neoplastic progression or future cancer risk. Currently our limited understanding of the molecular biology of IBD-associated cancer is not sufficient to use these markers for clinical management decisions. However, as our knowledge advances, it is possible that such markers will one day complement or supplant histologic evidence of dysplasia in assigning cancer risk in patients with IBD.

## CHEMOPREVENTION OF DYSPLASIA AND CANCER

Chemoprevention refers to the use of chemical compounds to prevent, halt, or reverse the development of cancer. One advantage of chemoprevention over the current secondary prevention strategy of routine colonoscopy is the potential to intervene early enough in the carcinogenic sequence to avoid not only cancer, but also the need for colectomy. The goal of chemoprevention should be to reduce CRC risk, allowing for less frequent surveillance exams and a reduction in the number of invasive cancers. The bulk of evidence for chemoprevention in IBD relates to the use of 5-aminosalicylates (5-ASA). Unfortunately, no prospective data exist, and retrospective studies have yielded mixed results with regard to the protective effect of 5-ASA medications. A meta-analysis by Velayos *et al* including 9 case control and cohort studies revealed a pooled odds ratio of 0.51 (95% CI, 0.38-0.69) for the development of dysplasia or cancer in patients with regular use of 5-ASA medications<sup>[43]</sup>. Given the substantial heterogeneity of individual study results, this pooled estimate signifies the most accurate estimate of the protective effect of 5-ASA.

The most compelling evidence for chemoprevention

in IBD comes from a prospective randomized placebo-controlled trial of UDCA in the high-risk subset of UC patients with coexisting PSC<sup>[20]</sup>. Compared to the placebo group, patients who received UDCA had a relative risk of 0.26 (95% CI, 0.06-0.92) for developing CRC or dysplasia. A retrospective study at the University of Washington of patients with PSC and UC corroborated these results by demonstrating a strong negative association between UDCA use and dysplasia, with an odds ratio of 0.18 ( $P = 0.005$ )<sup>[21]</sup>.

While other medications have been explored as potential chemopreventive agents, none have yielded satisfactory results. The adverse effects of corticosteroids and non-steroidal anti-inflammatory drugs preclude their long-term use for chemoprevention in IBD patients, despite some evidence to suggest a protective effect in both IBD and non-IBD patients. The use of folate for chemoprevention has sound rationale and an excellent safety profile, but inadequate evidence of a protective benefit. Likewise despite the rationale of medically controlling inflammation as a potential mechanism of cancer prevention, there are insufficient data to recommend azathioprine or 6-mercaptopurine for chemoprevention.

## SURVEILLANCE FOR DYSPLASIA AND CANCER

Periodic surveillance colonoscopy is the foundation of our current approach to cancer prevention in IBD. This strategy relies on the ability to detect CRC at a preclinical phase of dysplasia during which intervention can avert the adverse consequences of invasive cancer. Detection of dysplasia depends on the frequency and technique of surveillance colonoscopy, as well as the quality of pathologic review. Itzkowitz and Harpaz report that a typical biopsy samples less than 0.05% of the colon<sup>[44]</sup>, highlighting the potential for sampling error associated with nontargeted biopsies to look for flat dysplasia. Rubin *et al* retrospectively determined that 33 biopsies are required to detect dysplasia with 90% sensitivity, and 64 biopsies are needed to achieve 95% sensitivity<sup>[45]</sup>. Although consensus guidelines incorporate this finding and recommend 30-40 biopsies, this can be quite cumbersome to perform. Additionally, many gastroenterologists are either not fully aware of these recommendations or intentionally do not adhere to them<sup>[46,47]</sup>. Newer imaging technologies such as chromoendoscopy, magnification endoscopy and confocal laser microscopy offer the potential to enhance detection of dysplasia during surveillance colonoscopy, allowing endoscopists to take fewer high-yield biopsies of targeted abnormal mucosa.

The recommendation to perform surveillance in IBD patients comes from consensus expert opinion, supported by solid rationale and an ethical imperative to attempt prevention in an at risk population<sup>[48]</sup>. However, hard evidence of efficacy is lacking. A Cochrane review concluded that although there is no clear evidence that surveillance colonoscopy prolongs survival in IBD patients; there are data to suggest that cancers tend to be detected at an earlier stage with a correspondingly more

favorable prognosis<sup>[49]</sup>. The authors include the caveat that lead time bias may contribute substantially to these results. Additionally they conclude that indirect evidence supports surveillance as a cost-effective endeavor<sup>[49]</sup>.

A number of guidelines published over the past decade offer direction to gastroenterologists in their approach to surveillance of dysplasia and cancer in IBD<sup>[23,50-53]</sup>. An international panel of experts convened by the Crohn's and Colitis Foundation of America published consensus guidelines in 2005 suggesting that an initial screening colonoscopy be performed in all UC patients 8-10 years after onset of symptoms attributable to UC<sup>[23]</sup>. The dual purpose of this initial screening exam is to identify dysplasia or cancer, if present, as well as to evaluate for possible reclassification of disease extent. The extent of disease in a given UC patient should be considered the greatest extent of involvement documented on either gross or histologic exam at the time of diagnosis of UC or at initial screening colonoscopy. Patients with Crohn's disease should be managed in an identical manner to UC patients of comparable extent of colonic involvement. Crohn's patients with at least one third of their colon involved are considered to have extensive colitis. Those patients with left-sided or extensive colitis (UC or Crohn's) who have a negative screening examination should continue periodic surveillance at an interval of every 1 year to 2 years. In light of the increased risk imposed by coexistent PSC, annual surveillance is warranted beginning at the time of PSC diagnosis. The technique of colonoscopy should involve 4 quadrant random biopsies at 10 cm increments throughout the colon in addition to targeted biopsies of suspiciously abnormal mucosa. All abnormal biopsies results should be confirmed through independent review by a second pathologist. A finding of indefinite dysplasia should prompt accelerated surveillance with a repeat exam in 3 to 6 mo. Management of low grade dysplasia is a subject of debate among experts with no clear consensus on optimal management. In the setting of LGD, physicians should initiate an informed discussion with their patients regarding the risks and benefits of immediate surgery versus heightened colonoscopic surveillance. Prophylactic colectomy should be offered due to the about 20% prevalence of concurrent malignancy<sup>[8]</sup>, with counseling about possible surgical complications including incontinence, adhesions, pouchitis and decreased fertility in female patients. Patients who elect nonoperative management should be informed regarding the drawbacks of surveillance, including limitations with endoscopic detection and sampling and challenges with histologic interpretation. An accelerated program of surveillance colonoscopy every 6 mo should be pursued with adherence to an extensive biopsy protocol.

The finding of high grade dysplasia should prompt referral for immediate total proctocolectomy attributable to the high rate of concurrent or subsequent malignancy<sup>[24]</sup>. Raised lesions found within an area of colitis should be removed, and the surrounding mucosa biopsied. If the lesion is amenable to complete endoscopic resection and the adjacent mucosa is free of dysplasia, a regimen of more frequent surveillance colonoscopy is recommended. The



finding of adjacent dysplasia in the flat mucosa warrants referral for colectomy. Raised lesions resembling adenomas that are encountered in areas free of inflammation can be handled in accordance with standard guidelines for management of sporadic adenomas<sup>[23]</sup>.

Strictures represent a unique circumstance that merits a higher degree of vigilance. Colonic strictures in UC often harbor malignancy and are considered a strong indication for surgery, even if biopsies and brushing of that area are unrevealing<sup>[23]</sup>. In Crohn's disease, colonic strictures may be followed with annual surveillance and biopsy if the lesion can be traversed with a standard pediatric colonoscope. In the setting of longstanding Crohn's disease, consideration should be given to surgical resection of a stricture due to the heightened risk of CRC<sup>[23]</sup>.

## NOVEL IMAGING TECHNIQUES

Despite improvements in optical resolution of modern endoscopes, surveillance colonoscopy has suboptimal sensitivity for detecting flat dysplasia. Consequently, a protocol of nontargeted biopsies is still advocated to detect these "invisible" lesions. This approach is time-consuming and cumbersome, however, and adherence to this regimen by physicians is poor<sup>[47]</sup>. Endoscopic techniques to improve macroscopic and microscopic visibility of dysplastic lesions are crucial to enhancing the diagnostic yield of surveillance colonoscopy and reducing the number of missed lesions. Chromoendoscopy, magnification endoscopy, narrow band imaging and confocal laser endomicroscopy are evolving technologies that hold promise in this regard.

Chromoendoscopy involves the application of dye during colonoscopy to highlight subtle mucosal changes that cannot be appreciated by standard white light imaging techniques. Indigo carmine is a contrast dye that augments subtle mucosal alterations, whereas methylene blue is an absorptive dye that is avidly taken up by normal mucosa, but does not stain areas of inflammation or dysplasia, thereby creating a contrast gradient that enhances visualization. At least 3 prospective studies have demonstrated that chromoendoscopy improves the sensitivity of detecting neoplasia in UC patients<sup>[54-56]</sup>. In addition to this improved sensitivity, chromoendoscopy offers the potential to improve specificity as well, by facilitating enhanced endoscopic characterization of lesions, thereby allowing the endoscopist to perform fewer biopsies that are more targeted. The combination of chromoendoscopy with magnification permits a detailed analysis of the mucosal architecture, and can assist gastroenterologists in differentiating benign from neoplastic lesions during colonoscopy, improving the yield of targeted biopsies<sup>[57]</sup>.

Narrow band imaging uses specialized light filters to enhance visualization of the tissue microvasculature, facilitating distinction between normal mucosa and neoplasia. Although this novel and innovative technology remains to be thoroughly evaluated in the setting of surveillance in IBD, it holds the potential to offer the same benefits as chromoendoscopy with greater ease of application.

Confocal laser endomicroscopy (CLE) enables real-time histologic evaluation of the colonic mucosa during endoscopy and can be combined with chromoendoscopy. Suspicious lesions identified through application of dye can be subsequently examined with extreme detail at the subcellular level of resolution with CLE prior to targeted biopsy. In a randomized trial in UC patients of chromoendoscopy in conjunction with CLE compared to conventional colonoscopy, the presence of neoplasia could be predicted with 94.7% sensitivity, 98.3% specificity and 97.8% accuracy<sup>[58]</sup>. In this study of 153 patients, the mean examination time was 42 min using chromoendoscopy with CLE compared with 31 min in the standard colonoscopy group. This innovative imaging technique has major implications for the future of colonoscopic surveillance in IBD.

Despite the promise and emerging information about these new techniques, factors of cost and training remain far from answered, and chromoendoscopy is not yet considered a standard of care approach to surveillance in the United States.

## CONCLUSION

Patients with UC and Crohn's disease have an increased risk of developing CRC. This risk appears to be related to the cumulative effect of chronic inflammation and correlates directly with the extent and duration of disease as well as the severity of inflammatory activity. Additional factors that further increase CRC risk in IBD patients include a younger age at diagnosis, coexistent PSC and a family history of CRC. Despite varying estimates of the magnitude of cancer risk in IBD, it remains widely accepted that patients with IBD represent a high-risk group for developing CRC in whom current therapies and surgical techniques may be affecting the incidence of this complication, so a careful approach to prevention and surveillance is still warranted. The overall approach to cancer prevention in IBD should be a comprehensive strategy, including regular follow-up visits and intensive control of disease activity through medical therapy, in concert with routine surveillance colonoscopy involving extensive biopsies. Despite several acknowledged limitations, periodic surveillance colonoscopy continues to serve as the foundation of a prevention strategy, with colectomy reserved for patients in whom dysplasia or cancer are discovered. Cancer risk reduction through regular use of chemopreventive medications remains an attractive concept, and the most compelling data is in the setting of PSC and IBD, in which UDCA offers substantial benefit. The accumulated data appears to favor 5-ASA as a chemopreventive agent, but this remains inconclusive due to the retrospective nature of these studies. Novel endoscopic imaging technologies to enhance detection of neoplasia are under investigation and hold promise for improving the yield of surveillance colonoscopy.

## REFERENCES

- 1 Choi PM, Zelig MP. Similarity of colorectal cancer in Crohn's disease and ulcerative colitis: implications for carcinogenesis

- and prevention. *Gut* 1994; **35**: 950-954
- 2 **Devroede GJ**, Taylor WF, Sauer WG, Jackman RJ, Stickler GB. Cancer risk and life expectancy of children with ulcerative colitis. *N Engl J Med* 1971; **285**: 17-21
  - 3 **Winther KV**, Jess T, Langholz E, Munkholm P, Binder V. Long-term risk of cancer in ulcerative colitis: a population-based cohort study from Copenhagen County. *Clin Gastroenterol Hepatol* 2004; **2**: 1088-1095
  - 4 **Eaden JA**, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001; **48**: 526-535
  - 5 **Bernstein CN**, Blanchard JF, Kliwer E, Wajda A. Cancer risk in patients with inflammatory bowel disease: a population-based study. *Cancer* 2001; **91**: 854-862
  - 6 **Lakatos L**, Mester G, Erdelyi Z, David G, Pandur T, Balogh M, Fischer S, Vargha P, Lakatos PL. Risk factors for ulcerative colitis-associated colorectal cancer in a Hungarian cohort of patients with ulcerative colitis: results of a population-based study. *Inflamm Bowel Dis* 2006; **12**: 205-211
  - 7 **Jess T**, Loftus EV Jr, Velayos FS, Harmsen WS, Zinsmeister AR, Smyrk TC, Schleck CD, Tremaine WJ, Melton LJ 3rd, Munkholm P, Sandborn WJ. Risk of intestinal cancer in inflammatory bowel disease: a population-based study from olmsted county, Minnesota. *Gastroenterology* 2006; **130**: 1039-1046
  - 8 **Rutter MD**, Saunders BP, Wilkinson KH, Rumbles S, Schofield G, Kamm MA, Williams CB, Price AB, Talbot IC, Forbes A. Thirty-year analysis of a colonoscopic surveillance program for neoplasia in ulcerative colitis. *Gastroenterology* 2006; **130**: 1030-1038
  - 9 **Rubin DT**. The changing face of colorectal cancer in inflammatory bowel disease: progress at last! *Gastroenterology* 2006; **130**: 1350-1352
  - 10 **Ekbom A**, Helmick C, Zack M, Adami HO. Ulcerative colitis and colorectal cancer. A population-based study. *N Engl J Med* 1990; **323**: 1228-1233
  - 11 **Rutter M**, Saunders B, Wilkinson K, Rumbles S, Schofield G, Kamm M, Williams C, Price A, Talbot I, Forbes A. Severity of inflammation is a risk factor for colorectal neoplasia in ulcerative colitis. *Gastroenterology* 2004; **126**: 451-459
  - 12 **Rubin DT**, Huo D, Rothe JA, Hetzel JT, Sedrak M, Yadron N, Bunnag A, Hart J, Turner JR. Increased Inflammatory Activity Is An Independent Risk Factor for Dysplasia and Colorectal Cancer in Ulcerative Colitis: A Case-Control Analysis with Blinded Prospective Pathology Review. *Gastroenterology* 2006; **130**: A2
  - 13 **Gupta RB**, Harpaz N, Itzkowitz S, Hossain S, Matula S, Kornbluth A, Bodian C, Ullman T. Histologic inflammation is a risk factor for progression to colorectal neoplasia in ulcerative colitis: a cohort study. *Gastroenterology* 2007; **133**: 1099-1105; quiz 1340-1341
  - 14 **Nuako KW**, Ahlquist DA, Mahoney DW, Schaid DJ, Siems DM, Lindor NM. Familial predisposition for colorectal cancer in chronic ulcerative colitis: a case-control study. *Gastroenterology* 1998; **115**: 1079-1083
  - 15 **Eaden J**, Abrams K, Ekbom A, Jackson E, Mayberry J. Colorectal cancer prevention in ulcerative colitis: a case-control study. *Aliment Pharmacol Ther* 2000; **14**: 145-153
  - 16 **Askling J**, Dickman PW, Karlen P, Brostrom O, Lapidus A, Lofberg R, Ekbom A. Family history as a risk factor for colorectal cancer in inflammatory bowel disease. *Gastroenterology* 2001; **120**: 1356-1362
  - 17 **Velayos FS**, Loftus EV Jr, Jess T, Harmsen WS, Bida J, Zinsmeister AR, Tremaine WJ, Sandborn WJ. Predictive and protective factors associated with colorectal cancer in ulcerative colitis: A case-control study. *Gastroenterology* 2006; **130**: 1941-1949
  - 18 **Soetikno RM**, Lin OS, Heidenreich PA, Young HS, Blackstone MO. Increased risk of colorectal neoplasia in patients with primary sclerosing cholangitis and ulcerative colitis: a meta-analysis. *Gastrointest Endosc* 2002; **56**: 48-54
  - 19 **Broome U**, Lofberg R, Lundqvist K, Veress B. Subclinical time span of inflammatory bowel disease in patients with primary sclerosing cholangitis. *Dis Colon Rectum* 1995; **38**: 1301-1305
  - 20 **Pardi DS**, Loftus EV Jr, Kremers WK, Keach J, Lindor KD. Ursodeoxycholic acid as a chemopreventive agent in patients with ulcerative colitis and primary sclerosing cholangitis. *Gastroenterology* 2003; **124**: 889-893
  - 21 **Tung BY**, Emond MJ, Haggitt RC, Bronner MP, Kimmey MB, Kowdley KV, Brentnall TA. Ursodiol use is associated with lower prevalence of colonic neoplasia in patients with ulcerative colitis and primary sclerosing cholangitis. *Ann Intern Med* 2001; **134**: 89-95
  - 22 **Heuschen UA**, Hinz U, Allemeyer EH, Stern J, Lucas M, Autschbach F, Herfarth C, Heuschen G. Backwash ileitis is strongly associated with colorectal carcinoma in ulcerative colitis. *Gastroenterology* 2001; **120**: 841-847
  - 23 **Itzkowitz SH**, Present DH. Consensus conference: Colorectal cancer screening and surveillance in inflammatory bowel disease. *Inflamm Bowel Dis* 2005; **11**: 314-321
  - 24 **Bernstein CN**, Shanahan F, Weinstein WM. Are we telling patients the truth about surveillance colonoscopy in ulcerative colitis? *Lancet* 1994; **343**: 71-74
  - 25 **Gyde SN**, Prior P, Macartney JC, Thompson H, Waterhouse JA, Allan RN. Malignancy in Crohn's disease. *Gut* 1980; **21**: 1024-1029
  - 26 **Greenstein AJ**, Sachar DB, Smith H, Janowitz HD, Aufses AH Jr. A comparison of cancer risk in Crohn's disease and ulcerative colitis. *Cancer* 1981; **48**: 2742-2745
  - 27 **Ekbom A**, Helmick C, Zack M, Adami HO. Increased risk of large-bowel cancer in Crohn's disease with colonic involvement. *Lancet* 1990; **336**: 357-359
  - 28 **Canavan C**, Abrams KR, Mayberry J. Meta-analysis: colorectal and small bowel cancer risk in patients with Crohn's disease. *Aliment Pharmacol Ther* 2006; **23**: 1097-1104
  - 29 **Jess T**, Gamborg M, Matzen P, Munkholm P, Sorensen TI. Increased risk of intestinal cancer in Crohn's disease: a meta-analysis of population-based cohort studies. *Am J Gastroenterol* 2005; **100**: 2724-2729
  - 30 **Gillen CD**, Walmsley RS, Prior P, Andrews HA, Allan RN. Ulcerative colitis and Crohn's disease: a comparison of the colorectal cancer risk in extensive colitis. *Gut* 1994; **35**: 1590-1592
  - 31 **Greenstein AJ**, Sachar D, Pucillo A, Kreel I, Geller S, Janowitz HD, Aufses A Jr. Cancer in Crohn's disease after diversionary surgery. A report of seven carcinomas occurring in excluded bowel. *Am J Surg* 1978; **135**: 86-90
  - 32 **Connell WR**, Sheffield JP, Kamm MA, Ritchie JK, Hawley PR, Lennard-Jones JE. Lower gastrointestinal malignancy in Crohn's disease. *Gut* 1994; **35**: 347-352
  - 33 **Yamazaki Y**, Ribeiro MB, Sachar DB, Aufses AH Jr, Greenstein AJ. Malignant colorectal strictures in Crohn's disease. *Am J Gastroenterol* 1991; **86**: 882-885
  - 34 **Riddell RH**, Goldman H, Ransohoff DF, Appelman HD, Fenoglio CM, Haggitt RC, Ahren C, Correa P, Hamilton SR, Morson BC. Dysplasia in inflammatory bowel disease: standardized classification with provisional clinical applications. *Hum Pathol* 1983; **14**: 931-968
  - 35 **Rubin DT**, Rothe JA, Hetzel JT, Cohen RD, Hanauer SB. Are dysplasia and colorectal cancer endoscopically visible in patients with ulcerative colitis? *Gastrointest Endosc* 2007; **65**: 998-1004
  - 36 **Rutter MD**, Saunders BP, Wilkinson KH, Kamm MA, Williams CB, Forbes A. Most dysplasia in ulcerative colitis is visible at colonoscopy. *Gastrointest Endosc* 2004; **60**: 334-339
  - 37 **Blackstone MO**, Riddell RH, Rogers BH, Levin B. Dysplasia-associated lesion or mass (DALM) detected by colonoscopy in long-standing ulcerative colitis: an indication for colectomy. *Gastroenterology* 1981; **80**: 366-374
  - 38 **Odze RD**, Goldblum J, Noffsinger A, Alsaigh N, Rybicki LA, Fogt F. Interobserver variability in the diagnosis of ulcerative colitis-associated dysplasia by telepathology. *Mod Pathol* 2002; **15**: 379-386
  - 39 **Connell WR**, Lennard-Jones JE, Williams CB, Talbot IC,



- Price AB, Wilkinson KH. Factors affecting the outcome of endoscopic surveillance for cancer in ulcerative colitis. *Gastroenterology* 1994; **107**: 934-944
- 40 **Ullman T**, Croog V, Harpaz N, Sachar D, Itzkowitz S. Progression of flat low-grade dysplasia to advanced neoplasia in patients with ulcerative colitis. *Gastroenterology* 2003; **125**: 1311-1319
- 41 **Odze RD**, Farraye FA, Hecht JL, Hornick JL. Long-term follow-up after polypectomy treatment for adenoma-like dysplastic lesions in ulcerative colitis. *Clin Gastroenterol Hepatol* 2004; **2**: 534-541
- 42 **Itzkowitz SH**. Molecular biology of dysplasia and cancer in inflammatory bowel disease. *Gastroenterol Clin North Am* 2006; **35**: 553-571
- 43 **Velayos FS**, Terdiman JP, Walsh JM. Effect of 5-aminosalicylate use on colorectal cancer and dysplasia risk: a systematic review and metaanalysis of observational studies. *Am J Gastroenterol* 2005; **100**: 1345-1353
- 44 **Itzkowitz SH**, Harpaz N. Diagnosis and management of dysplasia in patients with inflammatory bowel diseases. *Gastroenterology* 2004; **126**: 1634-1648
- 45 **Rubin CE**, Haggitt RC, Burmer GC, Brentnall TA, Stevens AC, Levine DS, Dean PJ, Kimmey M, Perera DR, Rabinovitch PS. DNA aneuploidy in colonic biopsies predicts future development of dysplasia in ulcerative colitis. *Gastroenterology* 1992; **103**: 1611-1620
- 46 **Bernstein CN**, Weinstein WM, Levine DS, Shanahan F. Physicians' perceptions of dysplasia and approaches to surveillance colonoscopy in ulcerative colitis. *Am J Gastroenterol* 1995; **90**: 2106-2114
- 47 **Eaden JA**, Ward BA, Mayberry JF. How gastroenterologists screen for colonic cancer in ulcerative colitis: an analysis of performance. *Gastrointest Endosc* 2000; **51**: 123-128
- 48 **Rubin DT**, Kavitt RT. Surveillance for cancer and dysplasia in inflammatory bowel disease. *Gastroenterol Clin North Am* 2006; **35**: 581-604
- 49 **Mpofu C**, Watson AJ, Rhodes JM. Strategies for detecting colon cancer and/or dysplasia in patients with inflammatory bowel disease. *Cochrane Database Syst Rev* 2004; CD000279
- 50 **Carter MJ**, Lobo AJ, Travis SP. Guidelines for the management of inflammatory bowel disease in adults. *Gut* 2004; **53** Suppl 5: V1-V16
- 51 **Kornbluth A**, Sachar DB. Ulcerative colitis practice guidelines in adults (update): American College of Gastroenterology, Practice Parameters Committee. *Am J Gastroenterol* 2004; **99**: 1371-1385
- 52 **Eaden JA**, Mayberry JF. Guidelines for screening and surveillance of asymptomatic colorectal cancer in patients with inflammatory bowel disease. *Gut* 2002; **51** Suppl 5: V10-V12
- 53 **Winawer S**, Fletcher R, Rex D, Bond J, Burt R, Ferrucci J, Ganiats T, Levin T, Woolf S, Johnson D, Kirk L, Litin S, Simmang C. Colorectal cancer screening and surveillance: clinical guidelines and rationale-Update based on new evidence. *Gastroenterology* 2003; **124**: 544-560
- 54 **Kiesslich R**, Fritsch J, Holtmann M, Koehler HH, Stolte M, Kanzler S, Nafe B, Jung M, Galle PR, Neurath MF. Methylene blue-aided chromoendoscopy for the detection of intraepithelial neoplasia and colon cancer in ulcerative colitis. *Gastroenterology* 2003; **124**: 880-888
- 55 **Hurlstone DP**, Sanders DS, Lobo AJ, McAlindon ME, Cross SS. Indigo carmine-assisted high-magnification chromoscopic colonoscopy for the detection and characterisation of intraepithelial neoplasia in ulcerative colitis: a prospective evaluation. *Endoscopy* 2005; **37**: 1186-1192
- 56 **Rutter MD**, Saunders BP, Schofield G, Forbes A, Price AB, Talbot IC. Pancolonic indigo carmine dye spraying for the detection of dysplasia in ulcerative colitis. *Gut* 2004; **53**: 256-260
- 57 **Kudo S**, Tamura S, Nakajima T, Yamano H, Kusaka H, Watanabe H. Diagnosis of colorectal tumorous lesions by magnifying endoscopy. *Gastrointest Endosc* 1996; **44**: 8-14
- 58 **Kiesslich R**, Goetz M, Lammersdorf K, Schneider C, Burg J, Stolte M, Vieth M, Nafe B, Galle PR, Neurath MF. Chromoscopy-guided endomicroscopy increases the diagnostic yield of intraepithelial neoplasia in ulcerative colitis. *Gastroenterology* 2007; **132**: 874-882

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## TOPIC HIGHLIGHT

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# Controversies in the treatment of Crohn's disease: The case for an accelerated step-up treatment approach

Amandeep K Shergill, Jonathan P Terdiman

Amandeep K Shergill, Veterans Affairs Medical Center, Division of Gastroenterology, University of California, San Francisco 94143, United States

Jonathan P Terdiman, Division of Gastroenterology, University of California, San Francisco 94143, United States

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Correspondence to: Jonathan P Terdiman, MD, Division of Gastroenterology, University of California, San Francisco, Box 1623, San Francisco 94143,

United States. [jonathan.terdiman@ucsf.edu](mailto:jonathan.terdiman@ucsf.edu)

Telephone: +1-415-3537906 Fax: +1-415-5022249

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## Abstract

The ideal treatment strategy for Crohn's disease (CD) remains uncertain, as does the optimal endpoint of therapy. Top-down versus step-up describes two different approaches: early use of immunomodulators and biological agents in the former versus initial treatment with steroids in the latter, with escalation to immunomodulators or biological drugs in patients proven to be steroid refractory or steroid dependent. Top-down therapy has been associated with higher rates of mucosal healing. If mucosal healing proves to be associated with better long-term outcomes, such as a decreased need for hospitalization and surgery, top-down therapy may be the better approach for many patients. The main concern with the top-down approach is the toxicity of the immunomodulators and biological agents, which have been linked with infectious complications as well as an increased risk of lymphoma. It is unlikely that one strategy will be best for all patients given the underlying heterogeneity of CD presentation and severity. Ultimately, we must weigh the safety and efficacy of the therapies with the risks of the disease itself. Unfortunately our ability to risk stratify patients at diagnosis remains rudimentary. The purpose of this paper is to review the data that supports or refutes the differing treatment paradigms in CD, and to provide a rationale for an approach, termed the "accelerated step-up" approach, which attempts to balance the risks and benefits of our currently available therapies with the risk of disease related complications as we understand them in 2008.

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## INTRODUCTION

Crohn's disease (CD) is an idiopathic, chronic inflammatory disorder of the intestines with no known cure. Traditionally, the principal goal of treatment has been symptom control. Upon diagnosis, patients are usually started on medications based on the severity of the presenting symptoms. Patients with mild or moderate symptoms are often started on medications such as 5-aminosalicylates (5-ASA) or antibiotics. Though the efficacy of mesalamine is limited in CD<sup>[1]</sup>, therapy with mesalamine is initiated because of its minimal toxicity and excellent safety profile. For sicker patients, physicians often initiate therapy with corticosteroids, because of their superior ability to control symptoms<sup>[2]</sup>. In this setting, the short-term toxicities of steroids are tolerated with the hope that the patient will enter a durable symptomatic remission and stop steroid therapy before any long-term steroid-related complications develop. Therapy with the immunomodulators, azathioprine (AZA) or methotrexate, or biological agents, such as infliximab or adalimumab, is reserved for patients who either fail to respond to steroids or fail to enter a steroid free remission. Despite the recognized efficacy of the immunomodulators and biological agents to both induce and maintain remission in CD<sup>[3,4]</sup>, early use of these drugs is tempered by the fear

of their complications, such as life threatening infections or malignancy. Symptom abatement with the least toxic drug regimen is the guiding principle of this type of “step-up” approach to the management of CD. However, recent studies and emerging expert opinion have begun to question these traditional treatment goals and approaches<sup>[5,6]</sup>.

Not only can medical therapies lead to symptom control, but they may actually alter the natural history of CD by reducing the rates of surgery, hospitalizations and disability. In fact, using “aggressive” therapy earlier in the disease course, i.e. immunomodulators and/or biological agents, may be the best approach to prevent irreversible damage to the bowel. Currently, this relationship between medical therapy, mucosal healing and improved clinical outcomes is being investigated. Standard “step-up” therapies fail to induce mucosal healing in many patients, and the overall morbidity, and even mortality, of Crohn’s patients may be reduced by a more timely use of immunomodulators and biological agents<sup>[6-8]</sup>. This treatment paradigm in which immunomodulators and/or biological agents are used immediately in newly diagnosed Crohn’s patients to induce a rapid remission has been labeled the “top-down” approach. Once remission has been achieved, an attempt is made to reduce the maintenance therapy to the medications with the least presumed toxicity. The more potent medications are used again if the patient flares, on an on-demand basis. However, for many patients, tapering down these therapies may not be possible, or even prudent to try. Therefore, it is unclear if a top-down approach is practical; perhaps the optimal treatment approach is actually “top and STAY PUT”.

Ultimately, we must weigh the safety and efficacy of the therapies with the risks of the disease itself. The purpose of this paper is to review the data regarding the differing treatment approaches to CD and to synthesize these data into an optimal treatment approach circa 2008.

## NATURAL HISTORY OF CD

The natural history of CD remains poor for many, if not most, patients. Symptomatic flares, leading to reduced quality of life, are inevitable for almost all patients over a ten year period<sup>[9]</sup>. Steroid use is common, often incompletely effective, and is associated with many side-effects and complications<sup>[10]</sup>. For most patients there is an inexorable progression of bowel injury culminating in the need for intestinal resection in upwards of 80% of patients during their lifetime<sup>[11,12]</sup>. Hospitalization and surgery account for the majority of the direct cost of caring for patients with CD<sup>[13,14]</sup>. Unfortunately, surgery is not curative, with endoscopic recurrence in 75% of patients at 1 year after surgery, and symptomatic recurrence in 50% of patients at 5 years<sup>[15]</sup>.

## WHAT SHOULD BE THE GOALS OF THERAPY: SYMPTOM CONTROL OR MUCOSAL HEALING?

The traditional goal of therapy in CD in the clinical setting

has been relief of symptoms, such as abdominal pain and diarrhea, with restoration of a general sense of well-being. In research studies, the CD Activity Index (CDAI) has been used to define treatment responses and remission. However, many of the factors in the CDAI are considered subjective measures of patient symptoms, and more objective therapeutic endpoints, such as mucosal healing, are being evaluated.

The major morbidity from CD is a result of uncontrolled inflammation of the intestines and perianal area, leading to ulceration. Intestinal ulceration can lead to bleeding and anemia, perforation with abscess or fistula formation, or subsequent fibrosis with obstruction. Presumably, if the mucosa is healed, then these complications cannot occur. Interestingly, there appears to be a disconnect in CD between symptoms and mucosal healing. For example, in the endoscopic substudy of A Crohn's Disease Clinical Trial Evaluating Infliximab in a New Long-Term Treatment Regimen I (ACCENT I), mucosal healing did not correlate with CDAI. At 10 wk, only 36% of patients with mucosal healing were in remission as defined by the CDAI, and 40% of patients in clinical remission by CDAI did not have endoscopic remission<sup>[16]</sup>. So what is the optimal endpoint of therapy in CD? Ideally, patients should feel well, but should they also be treated to reduce intestinal injury in the hope of reducing the need for surgery?

Symptom control may not be enough, but unfortunately there is still no definite evidence that pushing therapy to achieve mucosal healing modifies the natural history of CD, though there are emerging data that this may be the case. In the postoperative setting, seminal work by Rutgeerts and colleagues has demonstrated that endoscopic recurrence precedes and predicts clinical recurrence<sup>[15]</sup>. Patients with no or very mild lesions (few aphthous ulcers) at follow-up endoscopy one year after resection had a low risk of endoscopic progression or clinical recurrence, with clinical recurrence rates remaining less than 10% over 5-10 years of follow-up. However, patients with advanced lesions (diffusely inflamed mucosa or worse) seen at follow-up endoscopy one year after resection had a poor prognosis with further endoscopic progression and clinical recurrence occurring in upwards of 90% over time<sup>[15]</sup>. In the ACCENT I study, patients on maintenance infliximab with mucosal healing required fewer hospitalizations and surgical interventions<sup>[17]</sup>. Nine patients had evidence of mucosal healing at both 10 wk and 54 wk endoscopic evaluations, and no CD related hospitalizations were required in this group. Patients with evidence of mucosal healing at 1 visit (either 10 wk or 54 wk) required fewer CD related hospitalizations as compared to patients with no healing at either visit (18.8% *vs* 28%)<sup>[16]</sup>. In a Norwegian population-based cohort, mucosal healing after one year of treatment was associated with decreased disease activity during the follow-up period and decreased need for active treatment over five years<sup>[18]</sup>.

Though the data to support mucosal healing as the optimal endpoint for CD therapies remains scant, one could argue that the reason we have not yet seen a change in the natural history of CD in the general population is because according to our standard treatment approaches,

we are only using drugs with a good potential to heal mucosa, such as the immunomodulators or biological agents, on the most refractory patients. Often, this is when the chronic inflammation of the bowel has already led to irreversible injury, manifest as high-grade strictures or bowel perforation with abscess or fistula formation. These complications may be beyond the capacity of any medicine to rectify.

## TO WHAT EXTENT DO THE CURRENT THERAPIES ACHIEVE THESE GOALS OF TREATMENTS?

First line therapies often include treatment with 5ASA or antibiotics as these are presumed to have low or limited toxicity. However, multiple studies have failed to demonstrate robust clinical efficacy for 5-ASA agents, particularly in patients with small bowel or ileocolic disease<sup>[1,2,19]</sup>. Limited data exist on the efficacy of antibiotics, as well. But, the use of antibiotics in CD has been poorly studied<sup>[20]</sup>. pH release formulations of budesonide (Entocort), a corticosteroid with limited systemic bioavailability, and therefore, less short term side-effects than prednisone, can be used to induce remission in right sided colonic and ileal disease, but at 1 year is unlikely to maintain remission<sup>[21,22]</sup>. For moderate to severe disease activity, systemic corticosteroids, such as prednisone, are often used, but are known to have significant short and long-term side effects. In addition, studies indicate that although steroids are effective at suppressing acute inflammation quickly, they have shown no benefit in maintaining a remission, preventing new flares or inducing mucosal healing<sup>[23]</sup>. Population based studies from Olmstead County have shown that 43% of CD patients required treatment with steroids; of these, the majority (85%) were able to achieve complete or partial response at one mo. However, at one year only one-third of patients had a sustained response: 28% patients were steroid dependent and 38% patients required surgical intervention<sup>[10]</sup>. In addition, less than one third of patients in a clinical remission on steroids had evidence of mucosal healing at 7 wk<sup>[24]</sup>. Steroids may also worsen disease, especially in patients with fistula, leading to higher rates of abscess formation and sepsis<sup>[25]</sup>.

Immunomodulators, such as azathioprine (AZA) or 6-mercaptopurine (6-MP), have a slow onset of action in CD, so their utility for the rapid induction of remission is limited<sup>[26]</sup>; however, they have been shown to maintain a remission in CD, and are able to induce mucosal healing<sup>[8]</sup>. Treatment with AZA/6MP results in an approximately 40% steroid-free remission rate at 1 year<sup>[27]</sup>. A study of 20 patients with Crohn's colitis or ileocolitis treated with azathioprine demonstrated that in the colon, 70% of patients had complete mucosal healing, while in the ileum, 54% had complete healing<sup>[28]</sup>. Even with their demonstrated ability to heal mucosa and be steroid sparing in CD, Cosnes *et al* did not find a decrease in rates of operations for Crohn's over the past two decades, despite an increased use of AZA/6-MP over this time period<sup>[29]</sup>. One major caveat to this finding is that the majority of

patients on immunosuppression were on therapy for less than 3 mo prior to their operation, which is often too short a treatment period to expect any real benefit from immunomodulators. Studies from the pediatric literature suggest that immunomodulators can have longstanding effects on CD that may alter the natural history. In one randomized controlled trial of children aged 13 (+/-2) years with moderate to severe CD on steroids and within 8 wk of CD diagnosis, immunomodulator use was able to induce a durable steroid-free remission, with a long term remission rate of 89% at 18 mo<sup>[30]</sup>.

The data is even more compelling for infliximab, an anti-tumor necrosis factor (anti-TNF) agent. In ACCENT I, regularly scheduled infusions of infliximab led to superior remission and response rates, superior mucosal healing, and decreased need for hospitalizations and surgery compared with placebo or episodic infusions of infliximab<sup>[17]</sup>. In the scheduled treatment group, 31% of patients had evidence of complete mucosal healing at 10 wk and 50% of patients had complete mucosal healing at wk 54<sup>[16]</sup>. Currently, these medications are often reserved for patients who are steroid refractory or steroid dependent, who are either not responding or too sick to wait for the effects of immunomodulators. The main reason for reserving anti-TNF therapy for sicker patients is the presumed risk-benefit profile.

## DOES TOP-DOWN THERAPY EXPOSE PATIENTS TO AN INCREASED RISK OF MEDICATION-RELATED COMPLICATIONS? THE RATIONALE FOR STEP-UP THERAPY

A small number of CD patient's will have a very mild course, requiring only treatment with 5-ASAs, antibiotics or a short course of budesonide, without further need for systemic steroids or immunomodulators. A top-down approach would unnecessarily expose these patients to the side effects of the immunomodulators and biologics. Immunomodulator use has been associated with an increased risk of infection, hepatitis, bone marrow suppression, pancreatitis and lymphoma<sup>[31]</sup>. Two to five percent of patients will experience bone marrow suppression secondary to azathioprine/6MP, and even in the absence of leukopenia, there is a risk of serious infection<sup>[4]</sup>. A recent meta-analysis suggests a four-fold increased risk of lymphoma in IBD patients treated with azathioprine/6-MP<sup>[32]</sup>. Side effects of the biologics can include infectious complications, malignancy, demyelinating disorders, autoimmunity and worsening of CHF<sup>[3]</sup>. Anti-TNF agents are associated with a 2.8%-4% increased risk of serious infections<sup>[3]</sup>. Analysis of the Crohn's Therapy, Resource, Evaluation and Assessment Tool (TREAT) registry demonstrated a relative risk of lymphoproliferative disorders of 1.3<sup>[14]</sup>. Recently, the risk of a rare form of lymphoma, hepatosplenic T-cell lymphoma, has been seen in association with infliximab and concomitant immunomodulator use. This type of lymphoma predominantly affects patients age 10-30 (the oldest reported patient is 31), and has not been seen in patients on infliximab alone<sup>[33]</sup>. This list of serious



and potentially life-threatening side effects has resulted in the limited use of biologic therapy to only the most severe cases of CD, which are resistant to conventional therapies. The recent American Gastroenterological Society (AGA) technical review on corticosteroids, immunomodulators and infliximab in IBD specifies that current indications for infliximab include severely active CD resistant to medical therapy or intolerant of medical therapy<sup>[4]</sup>.

Given these potential toxicities, a recent decision analysis evaluated the risks and benefits of infliximab as compared to standard therapy. This model suggested that despite an increased risk of lymphoma and death, infliximab results in increased quality of life years secondary to clinical response and remission rates and decreased surgical rates, and in properly selected patients, the benefits outweigh the risks<sup>[7]</sup>. The modeling likely underestimated the response and remission rates, and overestimated the mortality rates as data from the TREAT registry were excluded<sup>[34]</sup>. Interestingly, analysis of the TREAT registry revealed that infliximab, in multivariate, logistic regression analysis, was not an independent predictor of serious infections. Factors independently associated with serious infection included prednisone use, narcotic analgesic use, and moderate to severe disease activity. No increased risk of malignancy was associated with infliximab use, and the only factor associated with increased mortality was use of prednisone<sup>[14]</sup>. It is important to note that exposure to prednisone is likely greater over time with the step-up treatment approach.

### CAN THE TIMING AND ORDER OF MEDICINES CHANGE THE NATURAL HISTORY OF CD? THE RATIONALE FOR TOP-DOWN THERAPY

Studies in the pediatric population suggest that early treatment may alter the course of CD, and response to therapy may be related to disease duration. In a prospective, placebo controlled trial of newly diagnosed pediatric CD patients with moderate to severe disease on steroids, early (within 8 wk of initial diagnosis) treatment with 6-MP resulted in a 85% sustained steroid-free remission as compared to 54% of patients receiving placebo<sup>[30]</sup>. In a small study of 15 patients, Kugathesan *et al* treated 15 children with medically refractory CD in a prospective, open-label trial of a single, 5 mg/kg infliximab infusion. Fourteen of fifteen children responded. Of the 14 patients who responded, six had early CD (< 2 years from time of diagnosis) and eight had late CD (> 2 years from time of diagnosis). Three of six children with early disease maintained clinical response through the 12-mo trial period, compared to none of the eight children with late disease<sup>[35]</sup>. What is most remarkable about this study is that a single infusion of infliximab was able to induce such a profound and prolonged clinical response. This was a small study, but the positive findings suggest that there is something different about the newly presenting CD patient versus the relapsing/remitting CD patient. Based on this and other studies, there has been some speculation that earlier in the disease, medications such as infliximab may be able to

change the natural history of CD.

In the REACH (Response and Remission Related to Infliximab in Pediatric Patients with Moderate to Severe CD) trial, pediatric patients on concurrent immunomodulators, with mean disease duration of only 1.6 years, were given an infliximab induction regimen. Responders were randomized to infliximab 5 mg/kg every 8 wk or every 12 wk. At 10 wk there was an 88% clinical response and 59% clinical remission rate. At 54 wk, subjects receiving every 8 wk infliximab had a 64% response rate and 56% remission rate<sup>[36]</sup>. These response and remission rates are superior to those seen in ACCENT I study of infliximab in adults with a median disease duration of > 7 years, where the 10 wk remission rate was 40% and 54 wk remission rate was 30%<sup>[17]</sup>.

Retrospective analysis of data from Pegylated Antibody Fragment Evaluation in Crohn's Disease Safety and Efficacy 2 (PRECiSE 2) and Crohn's trial of the fully Human antibody Adalimumab for Remission Maintenance (CHARM) have demonstrated similar results. In PRECiSE 2, a higher percentage of patients were able to achieve a clinical response or remission with monthly certolizumab if they had < 1 year of disease activity versus those who had a longer duration of disease (> 2 years)<sup>[37]</sup>. A subanalysis of the CHARM study presented at DDW 2007 evaluated patients who responded to treatment with adalimumab at a 4 wk evaluation, and randomized these wk-4 responders to a maintenance regimen of placebo, adalimumab 40 mg subcutaneous weekly or adalimumab 40 mg subcutaneous every other week. In logistic regression analysis controlling for age, gender, CRP, concomitant therapies, presence of fistulas and active treatment with adalimumab, patients with disease duration of less than 2 years achieved the highest remission rates, confirming that disease duration had a significant effect on ability to achieve and maintain a remission<sup>[38]</sup>.

The potential for biologics to induce mucosal healing and potentially change the natural history of CD has been further illustrated by the Top Down/Step Up trial, which is currently awaiting publication, but has been presented in abstract form at DDW 2006<sup>[39,40]</sup>. This is an open-label, multicenter trial in 26 centers in the Netherlands and Belgium. Patients with active CD of less than 4 years duration were eligible for enrollment. Subjects were randomized to a top-down arm (infliximab induction 0, 2, 6 wk with AZA maintenance, with on-demand infliximab for flares; systemic steroids were added only if patients did not respond to the combination of infliximab and AZA) or a step-up arm (Prednisone 40 mg daily; permitted 2 steroid tapers before starting AZA; and then infliximab if failed treatment with immunomodulators). Co-primary endpoints were steroid-free remission and need for surgery at 6 and 12 mo. All patients underwent endoscopy at baseline with blinded scoring of ulcers. At 6 and 12 mo, significantly more top-down patients were in a steroid-free remission without resection as compared to the step-up arm (60% *vs* 36% at 6 mo, 62% *vs* 42% at 12 mo). At 24 mo, no statistically significant difference was found between the two groups. The most remarkable finding in this cohort was the results of the endoscopic substudy,



in which 44 patients were evaluated at 2 years after study initiation. 71% (17/24) of patients in the top-down arm achieved mucosal healing versus 30% (6/20) in the step-up arm. Some of the patients in the top-down arm had only received an induction dose of infliximab<sup>[39,40]</sup>.

Why might earlier use of immunomodulators or biological drugs lead to better long-term outcomes in CD? Later exposure to these potent therapies may be less effective because irreversible bowel injury may have occurred, with transformation of an inflammatory phenotype to a stricturing or penetrating phenotype, which is beyond the ability of medical therapy to repair. Alternatively, the immune dysregulation may be more difficult to reverse in long-standing disease, maybe more so in patients previously exposed to corticosteroids<sup>[41]</sup>.

### **ONCE YOU START BIOLOGICAL THERAPY, CAN YOU EVER STOP IT? IS THE NOTION OF TOP-DOWN THERAPY A MYTH?**

The idea of top-down therapy would be more palatable if many patients exposed to these more potent, but potentially toxic therapies can have these treatments tapered or discontinued over time. However, it is not clear if this is possible for many patients. ACCENT I data clearly support the use of scheduled infliximab therapy as compared to episodic therapy<sup>[17]</sup>, and this has become the standard of care when treating with infliximab. In the Top Down/Step Up trial, patients randomized to the Top-Down arm were given infliximab as induction therapy, and maintained with immunomodulators. Infliximab was given on-demand for symptom relapse<sup>[40]</sup>. Practically, it is rare for practitioners to use biologic agents as a bridge to other therapies or to use biologics episodically. Is a top-down approach realistic, or is it really a top and stay therapy?

Infliximab has been evaluated as a bridge to azathioprine therapy in steroid-dependent CD<sup>[42]</sup>. Patients received an induction regimen of infliximab or placebo, and all patients were given either Azathioprine or 6 MP; relapse was treated with steroids. At 52 wk, 40% of patients receiving infliximab induction followed by AZA/6MP were in a steroid-free remission as compared to 22% of patients who received placebo and then AZA/6MP. Although there is a statistically significant difference favoring induction with infliximab, a gradual loss of efficacy was seen when compared to the wk 12 steroid-free remission rates: 75% in the infliximab group and 38% in the placebo group<sup>[42]</sup>.

Data from the Step-Up/Top-Down trial also indicate that a substantial proportion of patients started on biologics have to remain on them. At one year, 41% of patients in the Top-Down group required at least one additional dose of infliximab, with a median interval of infusion of 16 wk<sup>[40]</sup>.

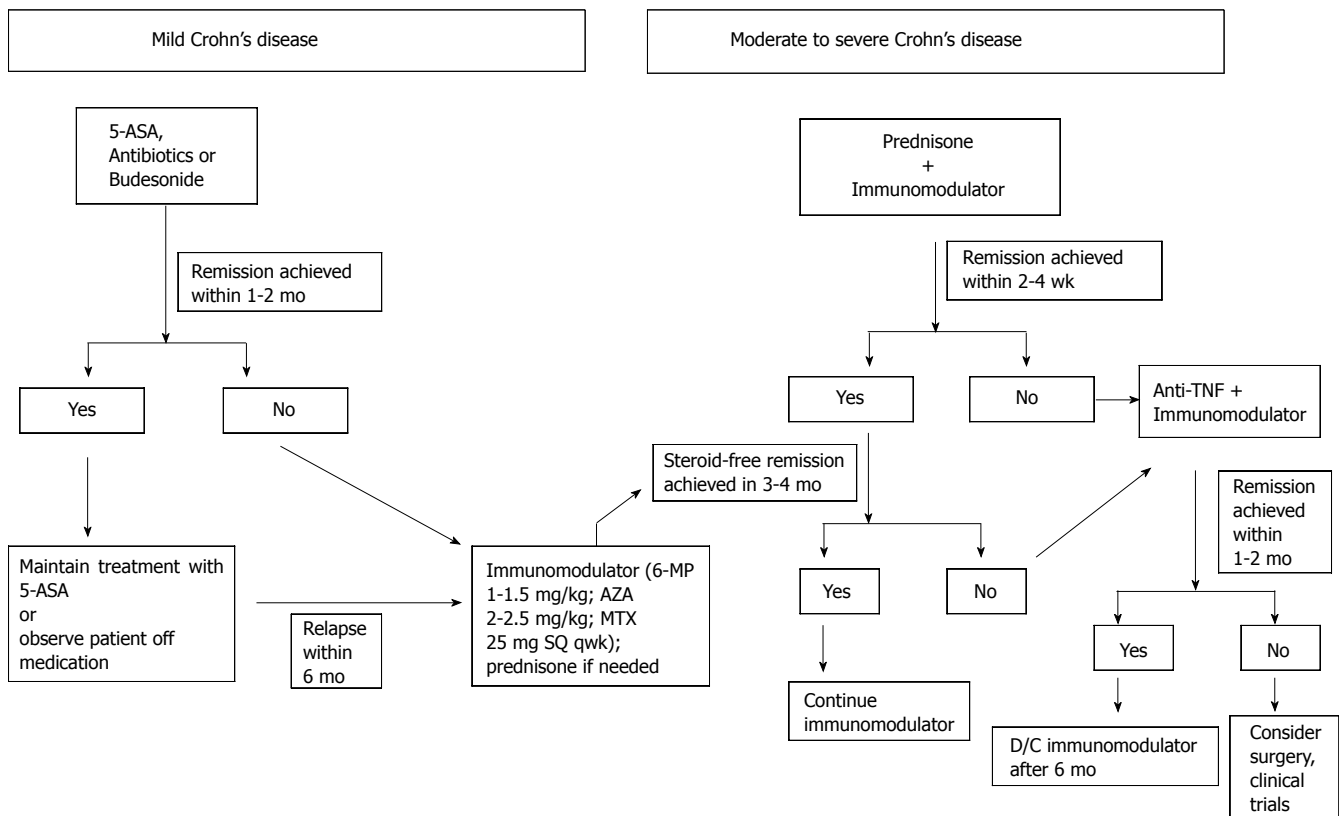
The association of combination infliximab and immunomodulator use (specifically AZA/6MP) with hepatosplenic T-cell lymphoma<sup>[33]</sup> has led many practitioners to move towards monotherapy, but most clinicians are choosing biologic monotherapy

over immunomodulator monotherapy. Initial results from the Infliximab Maintenance Immunosuppression Discontinuation (IMID) trial demonstrated no clinically significant benefit of combined therapy with infliximab and immunomodulator as compared to infliximab alone<sup>[43]</sup>. Patients in clinical remission on combination infliximab and immunomodulator (AZA/6MP) therapy were randomized to continue or discontinue immunomodulators after at least 6 mo of concomitant therapy. There was no difference in percent of patients requiring a change in infliximab dosing or discontinuation of infliximab therapy due to loss of response or intolerance between the two groups, and no difference in mucosal healing rates (61% in combination therapy group and 67% in infliximab monotherapy group). The only significant findings were that patients on combination therapy had higher median infliximab levels, higher trough serum infliximab levels, and lower incidence of antibodies to infliximab, although the clinical significance of these findings was unclear<sup>[43]</sup>. Additionally, data from ACCENT I and CHARM did not show any difference in remission rates with or without concomitant immunomodulator therapy at one year<sup>[44,45]</sup>.

### **IS ONE TREATMENT PARADIGM APPROPRIATE FOR ALL CROHN'S PATIENTS?**

A proportion of patients will have only very mild disease activity, and some may never have a flare requiring treatment with steroids. How do we differentiate patients who will never have a progressive, complicating disease course, in order to avoid the unnecessary exposure and the increased expense that would be associated with a top-down strategy? Ideally, we would apply a top-down approach to the subset of patients with the most aggressive disease, before the disease may become more resistant to treatment with biologics.

A retrospective study of 1123 CD patients at a tertiary referral center found a disabling disease course in 85% patients over 5 years of follow-up<sup>[46]</sup>. Disabling disease course was defined as greater than 2 steroid courses or steroid-dependence, hospitalizations, disabling chronic symptoms, need for immunosuppressive treatment, and intestinal resection or surgery for perianal disease. Clinical factors associated with a disabling disease course included: young age at onset of disease, presence of perianal lesions, early need for systemic steroids, and isolated small bowel involvement<sup>[46]</sup>. A second retrospective, cohort study of 83 patients undergoing surgery within the first three years of CD diagnosis found that smoking, isolated ileal involvement and oral corticosteroid use within the first 6 mo of diagnosis were associated with an increased risk of surgery<sup>[47]</sup>. Additional prognostic information may be attained from evaluation of serum markers such as ASCA, anti-OmpC, Anti-CBir1 and anti-I2. In a prospective study of 196 pediatric patients, the frequency of internal penetrating and/or stricturing disease increased with the number of positive markers, and the odds of developing internal penetrating and/or stricturing disease was highest



**Figure 1** "Accelerated" step-up treatment algorithm.

in patients with all four immune markers<sup>[48]</sup>. Genetic markers show promise; CARD15 mutations are thought to be responsible for approximately 20% of the genetic predisposition to CD<sup>[49]</sup>.

Ultimately, our ability to risk stratify patients remains crude. There is an urgent need for the ability to assess prognosis at the time of diagnosis, in order to personalize treatment options by targeting the patients who are at greatest risk of progressing to complicated disease with earlier, more potent anti-inflammatory therapy.

## MAKING PRACTICAL SENSE OF THE TREATMENT PARADIGMS: HOW MIGHT THEY BE IMPLEMENTED?

For now it seems that both the step-up and top-down treatment approaches, as they are conventionally defined, are lacking for many patients. It does not make sense to attempt to treat patients with severe symptoms at presentation with mesalamine, nor does it make sense to allow two attempts at a steroid taper before escalating therapy to an immunomodulator, as was done in Hommes' Step I Up/Top-Down study. At the same time, it makes little sense to expose all newly diagnosed patients to treatment with a biological drug, and it is increasingly unclear if patients treated with a biological drug should be treated concurrently with an immunomodulator. Based on the current data, we believe that a hybrid treatment approach, one that still "steps-up" therapy, but does so aggressively, is best. This approach can be described as an

"accelerated" step-up strategy (Figure 1).

The accelerated step-up treatment approach preserves the tactic of matching disease severity with treatment potency, but keeps in mind that earlier use of these potent therapies may have better outcomes. For patients with mild presenting symptoms, we still seek to achieve remission with mesalamine, antibiotics or budesonide. If remission is achieved within 1-2 mo, then options are to attempt to maintain remission with mesalamine, or observe the patient off medications. If the initial remission is not achieved within this short period of time, or for patients who relapse within 6 mo, therapy is started with an immunomodulator (6-MP with target dose of 1-1.5 mg/kg per day, AZA with target dose of 2-2.5 mg/kg per day or MTX with dose of 25 mg sq per week). Therapy with prednisone is initiated for patients with moderate or severe symptoms that do not permit one to wait the 2-4 mo required for the immunomodulators to take effect, though steroid use is avoided in patients with known perforating/fistulizing disease. Patients started on an immunomodulator are expected to be in a steroid free remission within 3-4 mo. For those that do not achieve this goal within that time frame, therapy with one of the anti-TNF drugs is initiated. At the present time, therapy with the immunomodulator is continued at the outset of therapy with a biological agent, but once the patient enters remission, concomitant therapy with the immunomodulator is stopped in most cases. For patients receiving infliximab infusions, intravenous hydrocortisone 200 mg is given as a premedication.

Biologics, i.e. anti-TNF drugs, are started even earlier if steroids are not able to induce a remission within 2-4 wk,

if a patient is intolerant of steroids or if a patient is intolerant of an immunomodulator. In addition, steroids are not used at all in patients with fistulizing or perforating disease, even at the outset. These patients are treated immediately with antibiotics, and in all but the mildest cases, one of the immunomodulators. If the patient is too ill to wait for the immunomodulators to take effect, then therapy is initiated immediately with a biological drug. In all cases therapy with immunomodulators and/or biological agents is not initiated until it is clear there is no infection that requires therapy and there is no indication for immediate operative intervention. With all of these therapies, the treatment endpoint remains symptom resolution. Though the authors concede the potential importance of mucosal healing in addition to symptom relief as a treatment goal, the authors do not yet document mucosal healing in all patients, nor do they push therapy in asymptomatic or minimally symptomatic patients if persistent inflammation is seen on endoscopy or imaging studies.

## CONCLUSIONS AND FUTURE DIRECTIONS

The ideal treatment approach in CD remains uncertain, as does the optimal endpoint of therapy. Since CD is heterogeneous with respect to phenotype and severity, it is unlikely that one approach will be best for all patients. Nevertheless, it is the authors' opinion that many physicians are not aggressive enough with therapy, delaying the use of potentially disease-modifying agents, such as the immunomodulators and/or biological agents, in patients with moderated to severe CD. In addition, there is a growing body of evidence that suggests that mucosal healing, which is most rapidly achieved with biologic therapy, is an appropriate treatment endpoint in CD patients. It does appear that earlier therapy with biologic drugs results in an increased response to therapy. Although no clear prospective study has been able to show that either mucosal healing or early biologic therapy are associated with a long-term change in the natural history of CD, there is a documented association with decreased hospitalizations and decreased need for surgery over one year of therapy. The greatest burden of CD comes from hospitalizations and surgeries, and thus early, aggressive therapy may have improved outcomes at an individual as well as a societal level. For now the authors recommend the "accelerated step-up" approach to therapy as outline above as the best way to induce and maintain CD remission while minimizing the risk of unnecessary medication toxicities. What is urgently needed are studies to improve the ability of clinicians to risk stratify patients upon diagnosis, and to predict response to therapies, so that treatment approaches can be individualized and optimized. Additional studies also are needed to demonstrate conclusively that mucosal healing, in addition to symptom control, should be a primary goal of therapy in CD.

## REFERENCES

- 1 Hanauer SB, Stromberg U. Oral Pentasa in the treatment of active Crohn's disease: A meta-analysis of double-blind, placebo-controlled trials. *Clin Gastroenterol Hepatol* 2004; **2**: 379-388
- 2 Summers RW, Switz DM, Sessions JT Jr, Bectel JM, Best WR, Kern F Jr, Singleton JW. National Cooperative Crohn's Disease Study: results of drug treatment. *Gastroenterology* 1979; **77**: 847-869
- 3 Clark M, Colombel JF, Feagan BC, Fedorak RN, Hanauer SB, Kamm MA, Mayer L, Regueiro C, Rutgeerts P, Sandborn WJ, Sands BE, Schreiber S, Targan S, Travis S, Vermeire S. American gastroenterological association consensus development conference on the use of biologics in the treatment of inflammatory bowel disease, June 21-23, 2006. *Gastroenterology* 2007; **133**: 312-339
- 4 Lichtenstein GR, Abreu MT, Cohen R, Tremaine W. American Gastroenterological Association Institute technical review on corticosteroids, immunomodulators, and infliximab in inflammatory bowel disease. *Gastroenterology* 2006; **130**: 940-987
- 5 Hanauer SB. Clinical perspectives in Crohn's disease. Turning traditional treatment strategies on their heads: current evidence for "step-up" versus "top-down". *Rev Gastroenterol Disord* 2007; **7** Suppl 2: S17-S22
- 6 Rutgeerts P, Vermeire S, Van Assche G. Mucosal healing in inflammatory bowel disease: impossible ideal or therapeutic target? *Gut* 2007; **56**: 453-455
- 7 Siegel CA, Hur C, Korzenik JR, Gazelle GS, Sands BE. Risks and benefits of infliximab for the treatment of Crohn's disease. *Clin Gastroenterol Hepatol* 2006; **4**: 1017-1024; quiz 976
- 8 Vermeire S, van Assche G, Rutgeerts P. Review article: Altering the natural history of Crohn's disease—evidence for and against current therapies. *Aliment Pharmacol Ther* 2007; **25**: 3-12
- 9 Munkholm P, Langholz E, Davidsen M, Binder V. Disease activity courses in a regional cohort of Crohn's disease patients. *Scand J Gastroenterol* 1995; **30**: 699-706
- 10 Faubion WA Jr, Loftus EV Jr, Harmsen WS, Zinsmeister AR, Sandborn WJ. The natural history of corticosteroid therapy for inflammatory bowel disease: a population-based study. *Gastroenterology* 2001; **121**: 255-260
- 11 Cosnes J, Cattan S, Blain A, Beaugerie L, Carbonnel F, Parc R, Gendre JP. Long-term evolution of disease behavior of Crohn's disease. *Inflamm Bowel Dis* 2002; **8**: 244-250
- 12 Munkholm P, Langholz E, Davidsen M, Binder V. Intestinal cancer risk and mortality in patients with Crohn's disease. *Gastroenterology* 1993; **105**: 1716-1723
- 13 Binder V, Hendriksen C, Kreiner S. Prognosis in Crohn's disease—based on results from a regional patient group from the county of Copenhagen. *Gut* 1985; **26**: 146-150
- 14 Lichtenstein GR, Feagan BG, Cohen RD, Salzberg BA, Diamond RH, Chen DM, Pritchard ML, Sandborn WJ. Serious infections and mortality in association with therapies for Crohn's disease: TREAT registry. *Clin Gastroenterol Hepatol* 2006; **4**: 621-630
- 15 Rutgeerts P, Geboes K, Vantrappen G, Beyls J, Kerremans R, Hiele M. Predictability of the postoperative course of Crohn's disease. *Gastroenterology* 1990; **99**: 956-963
- 16 Rutgeerts P, Diamond RH, Bala M, Olson A, Lichtenstein GR, Bao W, Patel K, Wolf DC, Safdi M, Colombel JF, Lashner B, Hanauer SB. Scheduled maintenance treatment with infliximab is superior to episodic treatment for the healing of mucosal ulceration associated with Crohn's disease. *Gastrointest Endosc* 2006; **63**: 433-464
- 17 Rutgeerts P, Feagan BG, Lichtenstein GR, Mayer LF, Schreiber S, Colombel JF, Rachmilewitz D, Wolf DC, Olson A, Bao W, Hanauer SB. Comparison of scheduled and episodic treatment strategies of infliximab in Crohn's disease. *Gastroenterology* 2004; **126**: 402-413
- 18 Froslie KF, Jahnsen J, Moum BA, Vatn MH. Mucosal healing in inflammatory bowel disease: results from a Norwegian population-based cohort. *Gastroenterology* 2007; **133**: 412-422
- 19 Malchow H, Ewe K, Brandes JW, Goebell H, Ehms H, Sommer H, Jesdinsky H. European Cooperative Crohn's Disease Study (ECCDS): results of drug treatment. *Gastroenterology* 1984; **86**: 249-266
- 20 Sandborn WJ, Feagan BG, Lichtenstein GR. Medical management of mild to moderate Crohn's disease: evidence-

- based treatment algorithms for induction and maintenance of remission. *Aliment Pharmacol Ther* 2007; **26**: 987-1003
- 21 **Greenberg GR**, Feagan BG, Martin F, Sutherland LR, Thomson AB, Williams CN, Nilsson LG, Persson T. Oral budesonide for active Crohn's disease. Canadian Inflammatory Bowel Disease Study Group. *N Engl J Med* 1994; **331**: 836-841
  - 22 **Sandborn WJ**, Lofberg R, Feagan BG, Hanauer SB, Campieri M, Greenberg GR. Budesonide for maintenance of remission in patients with Crohn's disease in medically induced remission: a predetermined pooled analysis of four randomized, double-blind, placebo-controlled trials. *Am J Gastroenterol* 2005; **100**: 1780-1787
  - 23 **Travis SP**, Stange EF, Lemann M, Oresland T, Chowers Y, Forbes A, D'Haens G, Kitis G, Cortot A, Prantera C, Marteau P, Colombel JF, Gionchetti P, Bouhnik Y, Turet E, Kroesen J, Starlinger M, Mortensen NJ. European evidence based consensus on the diagnosis and management of Crohn's disease: current management. *Gut* 2006; **55** Suppl 1: i16-i35
  - 24 **Modigliani R**, Mary JY, Simon JF, Cortot A, Soule JC, Gendre JP, Rene E. Clinical, biological, and endoscopic picture of attacks of Crohn's disease. Evolution on prednisolone. Groupe d'Etude Therapeutique des Affections Inflammatoires Digestives. *Gastroenterology* 1990; **98**: 811-818
  - 25 **Agrawal A**, Durrani S, Leiper K, Ellis A, Morris AI, Rhodes JM. Effect of systemic corticosteroid therapy on risk for intra-abdominal or pelvic abscess in non-operated Crohn's disease. *Clin Gastroenterol Hepatol* 2005; **3**: 1215-1220
  - 26 **Baumgart DC**, Sandborn WJ. Inflammatory bowel disease: clinical aspects and established and evolving therapies. *Lancet* 2007; **369**: 1641-1657
  - 27 **Candy S**, Wright J, Gerber M, Adams G, Gerig M, Goodman R. A controlled double blind study of azathioprine in the management of Crohn's disease. *Gut* 1995; **37**: 674-678
  - 28 **D'Haens G**, Geboes K, Rutgeerts P. Endoscopic and histologic healing of Crohn's (ileo-) colitis with azathioprine. *Gastrointest Endosc* 1999; **50**: 667-671
  - 29 **Cosnes J**, Nion-Larmurier I, Beaugerie L, Afchain P, Turet E, Gendre JP. Impact of the increasing use of immunosuppressants in Crohn's disease on the need for intestinal surgery. *Gut* 2005; **54**: 237-241
  - 30 **Markowitz J**, Grancher K, Kohn N, Lesser M, Daum F. A multicenter trial of 6-mercaptopurine and prednisone in children with newly diagnosed Crohn's disease. *Gastroenterology* 2000; **119**: 895-902
  - 31 **Derijks LJ**, Gilissen LP, Hooymans PM, Hommes DW. Review article: thiopurines in inflammatory bowel disease. *Aliment Pharmacol Ther* 2006; **24**: 715-729
  - 32 **Kandiel A**, Fraser AG, Korelitz BI, Brensinger C, Lewis JD. Increased risk of lymphoma among inflammatory bowel disease patients treated with azathioprine and 6-mercaptopurine. *Gut* 2005; **54**: 1121-1125
  - 33 **Mackey AC**, Green L, Liang LC, Dinndorf P, Avigan M. Hepatosplenic T cell lymphoma associated with infliximab use in young patients treated for inflammatory bowel disease. *J Pediatr Gastroenterol Nutr* 2007; **44**: 265-267
  - 34 **Inadomi JM**, Terdiman J. Exploring utilities and outcomes with infliximab therapy. *Clin Gastroenterol Hepatol* 2006; **4**: 976-978
  - 35 **Kugathasan S**, Werlin SL, Martinez A, Rivera MT, Heikenen JB, Binion DG. Prolonged duration of response to infliximab in early but not late pediatric Crohn's disease. *Am J Gastroenterol* 2000; **95**: 3189-3194
  - 36 **Hyams J**, Crandall W, Kugathasan S, Griffiths A, Olson A, Johanns J, Liu G, Travers S, Heuschkel R, Markowitz J, Cohen S, Winter H, Veereman-Wauters G, Ferry G, Baldassano R. Induction and maintenance infliximab therapy for the treatment of moderate-to-severe Crohn's disease in children. *Gastroenterology* 2007; **132**: 863-873; quiz 1165-1166
  - 37 **Sandborn WJ**, Colombel JF, Panes J, Scholmerich J, McColm J, Schreiber S. Higher remission and maintenance of response rates with subcutaneous monthly Certolizumab Pegol in patients with recent-onset Crohn's Disease: Data from PRECISE 2. *Am J Gastroenterol* 2006; **101** Suppl 2: S394
  - 38 **Schreiber S**, Reinisch W, Colombel JF, Sandborn WJ, Hommes DW, Li J, Kent JD, Pollack PF. Early Crohn's Disease shows high levels of remission to therapy with Adalimumab: Sub-analysis of Charm. *Gastroenterology* 2007; **132**: A-147
  - 39 **D'Haens G**, Hommes DW, Baert F, De Vos M, Caenepeel F, Van Assche G, Lambrecht G, Coche JC, Vermeire S, Van Camp M. A combined regimen of Infliximab and Azathioprine induces better endoscopic mucosal healing than classic step-up therapy in newly diagnosed Crohn's Disease. *Gastroenterology* 2006; **130**: A-110
  - 40 **Hommes DW**, Baert F, Van Assche G, Caenepeel F, Vergauwe P, Tuynman H, De Vos M, Van Deventer S, Stitt L, Rutgeerts P, Feagan BC, D'Haens G. The ideal management of Crohn's Disease: Top Down versus Step Up Strategies, a randomized controlled trial. *Gastroenterology* 2006; **130**: A-108
  - 41 **Lowenberg M**, Peppelenbosch M, Hommes D. Biological therapy in the management of recent-onset Crohn's disease: why, when and how? *Drugs* 2006; **66**: 1431-1439
  - 42 **Lemann M**, Mary JY, Duclos B, Veyrac M, Dupas JL, Delchier JC, Laharie D, Moreau J, Cadiot G, Picon L, Bourreille A, Sobahni I, Colombel JF. Infliximab plus azathioprine for steroid-dependent Crohn's disease patients: a randomized placebo-controlled trial. *Gastroenterology* 2006; **130**: 1054-1061
  - 43 **Van Assche G**, Painsaud G, Magdelaine C, D'Haens G, Baert F, Vermeire S, Noman M, Temant D, Watier H, Rutgeerts P. Concomitant immunosuppression does not impact on the outcome of maintenance Infliximab therapy in Crohn's Disease: Final results of the IMID trial. *Gastroenterology* 2007; **132**: A-103
  - 44 **Colombel JF**, Sandborn WJ, Rutgeerts P, Enns R, Hanauer SB, Panaccione R, Schreiber S, Byczkowski D, Li J, Kent JD, Pollack PF. Adalimumab for maintenance of clinical response and remission in patients with Crohn's disease: the CHARM trial. *Gastroenterology* 2007; **132**: 52-65
  - 45 **Lichtenstein GR**, Diamond RH, Wagner C, Olson A, Hegedus R, Bala M, Sandborn WJ. Infliximab administered as 3-dose induction followed by scheduled maintenance therapy in IBD: comparable clinical outcomes with or without concomitant immunomodulators. *Gastroenterology* 2007; **132**: A-146
  - 46 **Beaugerie L**, Seksik P, Nion-Larmurier I, Gendre JP, Cosnes J. Predictors of Crohn's disease. *Gastroenterology* 2006; **130**: 650-656
  - 47 **Sands BE**, Arsenault JE, Rosen MJ, Alsahli M, Bailen L, Banks P, Bensen S, Bousvaros A, Cave D, Cooley JS, Cooper HL, Edwards ST, Farrell RJ, Griffin MJ, Hay DW, John A, Lidofsky S, Olans LB, Peppercorn MA, Rothstein RI, Roy MA, Saletta MJ, Shah SA, Warner AS, Wolf JL, Vecchio J, Winter HS, Zawacki JK. Risk of early surgery for Crohn's disease: implications for early treatment strategies. *Am J Gastroenterol* 2003; **98**: 2712-2718
  - 48 **Dubinsky MC**, Lin YC, Dutridge D, Picornell Y, Landers CJ, Farrior S, Wrobel I, Quiros A, Vasiliauskas EA, Grill B, Israel D, Bahar R, Christie D, Wahbeh G, Silber G, Dallazadeh S, Shah P, Thomas D, Kelts D, Hershsberg RM, Elson CO, Targan SR, Taylor KD, Rotter JI, Yang H. Serum immune responses predict rapid disease progression among children with Crohn's disease: immune responses predict disease progression. *Am J Gastroenterol* 2006; **101**: 360-367
  - 49 **Vermeire S**. Review article: genetic susceptibility and application of genetic testing in clinical management of inflammatory bowel disease. *Aliment Pharmacol Ther* 2006; **24** Suppl 3: 2-10



## TOPIC HIGHLIGHT

Lynne V McFarland, PhD, Series Editor

# Surgery for inflammatory bowel disease

John M Hwang, Madhulika G Varma

John M Hwang, Center for Colorectal Surgery, Department of Surgery, University of California, San Francisco, 2330 Post St. Suite 260, San Francisco 94115, United States

Madhulika G Varma, Center for Colorectal Surgery, Department of Surgery, University of California, San Francisco, 2330 Post St. Suite 260, San Francisco 94115, United States

**Author contributions:** Hwang JM performed literature review and wrote manuscript. Varma MG conceived of organization of manuscript and extensively edited and revised paper.

**Correspondence to:** Madhulika G Varma, MD, Assistant Professor of Surgery, Director, Center for Pelvic Physiology, Department of Surgery, University of California, 2330 Post St. Suite 260, San Francisco 94115, United States. [varmam@surgery.ucsf.edu](mailto:varmam@surgery.ucsf.edu)  
Telephone: +1-415-8853611

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## Abstract

Despite the new and ever expanding array of medications for the treatment of inflammatory bowel disease (IBD), there are still clear indications for operative management of IBD and its complications. We present an overview of indications, procedures, considerations, and controversies in the surgical therapy of IBD.

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**Key words:** Crohn's disease; Ulcerative colitis; Operation; Surgical treatment; Ileal pouch

**Peer reviewer:** Elias A Kouroumalis, Professor, Department of Gastroenterology, University of Crete, Medical School, Department of Gastroenterology, University Hospital, PO Box 1352, Heraklion, Crete 71110, Greece

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## ULCERATIVE COLITIS

Approximately 25%-35% of ulcerative colitis patients will ultimately require surgery for either a complication of the disease or inadequate control of symptoms<sup>[1,2]</sup>. While most of these surgeries can be done in the setting of an elective operation, a minority will require emergent or urgent treatment. A number of options are available in the

surgical management of ulcerative colitis, many of which may require more than one operation. Each procedure is associated with its own benefits and drawbacks, so a thorough understanding of each procedure, and its indications, is important.

## EMERGENT OR URGENT OPERATION FOR ULCERATIVE COLITIS

### Indications

Worsening signs and symptoms of colitis, including numerous bloody stools per day, fever, elevated heart rate, anemia, elevated sedimentation rate, radiographic evidence of colonic distension, and abdominal distension with tenderness on exam<sup>[3]</sup>, can predict the need for surgery. In most cases, acute flares of severe colitis respond to medical therapy. Up to 80% of patients with severe colitis will avoid an operation<sup>[4,5]</sup>. Failure to improve within a few days following an initial stabilization period, or worsening colitis should trigger consideration for operative intervention. Most studies define 48-96 h as an adequate trial of medical therapy in which significant improvement should occur<sup>[6,7]</sup>. One study of 49 patients with severe colitis cites an 85% colectomy rate if patients are still having 8 bowel movements per day, or 3-8 bowel movements per day with a C-reactive protein of > 45 mg/mL, despite 3 d of medical therapy<sup>[7]</sup>. Further, patients with an incomplete response within 7 d of medical management (as defined by four or more bowel movements per day or visible blood in the stools) had a 60% chance of continuing colitis symptoms and a 40% chance of colectomy within 1 year<sup>[7]</sup>.

The diagnosis of toxic megacolon, defined by a transverse colon diameter exceeding 6 centimeters, in ulcerative colitis has been reported in 7%-17% of patients requiring hospitalization and is thought to have a lifetime incidence of 0.5%-2.5%<sup>[8]</sup>. A European study looking at 796 patients showed that the mortality rate from toxic megacolon was only 0.2% in ulcerative colitis patients over a 4-year follow-up<sup>[9]</sup>. However, this accounted for 50% of all ulcerative colitis patient deaths over the same period. As such, most surgeons consider the development of toxic megacolon in ulcerative colitis, particularly in the context of perforation, progressive colonic dilatation, massive hemorrhage or hemodynamic instability, to be an indication for emergent colectomy<sup>[8,9]</sup>. However, severely ill patients can have toxic symptoms without dilatation that still warrants urgent surgical intervention. Greenstein has published a series of 7 ulcerative colitis patients diagnosed



with colonic perforation in the absence of colonic dilatation<sup>[10]</sup>. Six of them did not display classic signs of perforation including peritonitis and rebound tenderness. Impending perforation is extremely difficult to predict. The authors speculated that this lack of symptoms may have been due to the use of high-dose steroids. Patients with signs of actual or impending perforation should receive emergent surgery. The associated mortality rate of perforation in ulcerative colitis patients is between 27% and 57%<sup>[10]</sup>.

As might be predicted, the development of hemodynamic instability or multi-organ failure is also predictive of a poor outcome and warrants resuscitation and emergent operation. Caprilli cited a 72.7% mortality rate in ulcerative colitis patients developing multi-organ dysfunction<sup>[11]</sup>. They suggest that early signs of multi-organ dysfunction, including disproportionate tachycardia, tachypnea, oliguria, jaundice, hypoxemia and mental confusion, should prompt an aggressive therapeutic approach including ICU admission and early colectomy.

### **Surgical options**

Surgical management of ulcerative colitis in the emergent setting is aimed toward removing the inflamed bowel while minimizing morbidity, and a total abdominal colectomy and end ileostomy is the procedure of choice<sup>[12]</sup>. This procedure removes the majority of diseased bowel while avoiding the complications associated with both pelvic dissection and an enteric anastomosis.

A number of studies have shown that total abdominal colectomy with end ileostomy is a safe procedure in the emergent setting with a post-operative complication rate of 23%-33% and low mortality in the absence of a perforation (0%-4%)<sup>[13,14]</sup>. Some debate has centered on the management of the rectal stump. One study suggested that exteriorization of the stump, either by bringing it up into the subcutaneous tissues or by creating a formal mucous fistula, may decrease the incidence of pelvic septic complications and facilitate future pelvic dissection<sup>[15]</sup>. They found a 12% rate of pelvic abscess with an intraperitoneal rectal stump compared to a 4%-7% abscess rate when the stump was exteriorized. However, Karch and colleagues published a pelvic sepsis rate of only 2.6% (3 of 114 patients) in patients with an intraperitoneal stump<sup>[16]</sup>. One of these three patients responded well to transanal drainage prompting the authors to suggest that routine transanal drainage of the rectal stump may be warranted. Forty-one consecutive patients in this series underwent routine transanal drainage with no incidence of pelvic sepsis peri-operatively<sup>[16]</sup>.

Another advantage of this procedure is that after the abdominal colon is resected, the specimen undergoes histopathological assessment to confirm the diagnosis of ulcerative colitis. Surprisingly, the number of patients found to have Crohn's disease instead of ulcerative colitis after this procedure is appreciable. In a recent review of patients undergoing emergent total abdominal colectomy, of the 52 patients with ulcerative colitis, 13% had their diagnosis altered post-operatively (5 diagnosed with Crohn's disease and 1 with indeterminate colitis)<sup>[14]</sup>. Thus, in a

patient with indeterminate colitis, this may be the preferred surgical option to further define the diagnosis.

More extensive and definitive procedures for ulcerative colitis are often technically feasible at the time of an emergent operation. However, proctectomy is rarely required for symptomatic relief at the time of an emergent surgery and residual rectal inflammation can be treated medically<sup>[17]</sup>. Furthermore, these patients are often nutritionally deplete, anemic, and on high-dose steroids which increases the risk for an anastomotic complication. As such, we believe the safest approach is to salvage the patient and avoid proctectomy in the emergent setting. Interestingly, a significant proportion of patients, particularly in the elderly, may elect not to reverse their ileostomy. In Hyman's series of 52 ulcerative colitis patients undergoing emergent surgery, 20 patients elected not to reverse the ileostomy, choosing either completion proctectomy or no further surgery<sup>[14]</sup>.

## **ELECTIVE OPERATION FOR ULCERATIVE COLITIS**

### **Indications**

Failure of medical management remains the most frequent indication for elective surgery in ulcerative colitis<sup>[12]</sup>. Intractability may be defined by inadequate control of symptoms despite optimal medical management, chronic disability due to disease, or control of symptoms with therapy associated with high probability of long-term morbidity such as steroids. Growth failure in the pediatric population may be considered a consequence of intractable disease and is an indication for surgery<sup>[18]</sup>.

Risk of malignancy is also an indication for elective operation. A meta-analysis by Eaden cites an overall cancer incidence of 3.7% in ulcerative colitis patients, increasing to 5.4% in patients with pancolitis. This incidence rises with a longer duration of disease symptoms. Cancer risk in ulcerative colitis patients is 2% at 10 years, 8% at 20 years and 18% at 30 years<sup>[19]</sup>. As such, surveillance colonoscopy has been suggested in ulcerative colitis patients despite a paucity of clear evidence that this process increases survival. At present, practice guidelines of the American Society of Colon and Rectal Surgeons<sup>[12]</sup> suggest that patients with pancolitis undergo surveillance endoscopy after 8 years of disease symptoms and those with left-sided disease undergo surveillance endoscopy after 15 years of symptoms. The consensus of experts is that 4 quadrant biopsies should be taken every 10 cm<sup>[20]</sup>.

While an established carcinoma is an absolute indication for surgery, the management of dysplasia is somewhat more controversial. High grade dysplasia and dysplasia-associated lesions or masses (DALM) have a high incidence of synchronous cancer and are generally considered indications for surgery. A systemic review by Bernstein reported that 42% of patients with high-grade dysplasia and 43% of patients with DALM had synchronous cancers at the time of immediate colectomy<sup>[21]</sup>. However, a high rate of inter-observer variability in the pre-operative diagnosis of dysplastic lesions in ulcerative colitis patients has cast some doubt on this data. Variability in the diagnosis of low

or high-grade dysplasia may be as high as 60% even among experts<sup>[22]</sup>. It has been noted that pathologists may alter their diagnosis over time with experience or with new knowledge in the field<sup>[21]</sup>.

Several studies have suggested that adenoma-like DALMs may not be associated with as aggressive a natural history as previously believed and can be treated with endoscopic resection. Odze and colleagues published a series of 34 ulcerative colitis patients found to have either adenoma-like DALM or sporadic adenoma who were compared to 49 non-ulcerative colitis patients with sporadic adenoma. Their findings suggested that the rates of recurrent adenoma after endoscopic resection were similar in both groups and that ongoing surveillance endoscopy was adequate<sup>[23]</sup>.

The treatment of low-grade dysplasia in the absence of a DALM is even more ambiguous as the natural history of these lesions is not well understood. Several studies have suggested a high incidence of synchronous cancers in the presence of low-grade dysplasia as well as a significant rate of progression to high-grade dysplasia<sup>[21]</sup>. As such, many colorectal surgeons advocate for colectomy with a diagnosis of low-grade dysplasia. However, Befrits has shown over an average endoscopic follow-up of 10 years that progression of low-grade dysplastic lesions to high grade dysplasia or cancer in the absence of a DALM did not occur in 60 patients<sup>[24]</sup>. Lim and colleagues have also published a series of 160 chronic ulcerative colitis patients of whom 40 were diagnosed with low-grade dysplasia on colonoscopy. Over a 10-year follow-up, 10% of low-grade dysplasia progressed to high-grade dysplasia or cancer. However this rate of high-grade dysplasia or cancer in previously diagnosed low-grade dysplasia patients was not found to be significantly different from ulcerative colitis patients without dysplasia. The authors suggested that surveillance endoscopy for low-grade dysplasia was a reasonable option. As stated above, part of the discrepancy in reported rates of progression to high-grade dysplasia or cancer may be due to the lack of standardized pathological definitions for low grade dysplasia in ulcerative colitis<sup>[22]</sup>.

The management of a stricture in the context of low-grade dysplasia is more clear cut as up to 20%-24% of strictures are malignant<sup>[25]</sup>. Biopsies of strictures are inadequate to rule out malignancy and, as such, strictures in general should be considered an indication for surgery<sup>[12]</sup>. Strictures in the setting of ulcerative colitis may also be indicative of Crohn's disease and thus, operative treatment should be adjusted accordingly.

Elective surgery should also be considered for treatment of severe extra-intestinal manifestations of ulcerative colitis. Monoarticular arthritis, uveitis, and iritis are often ameliorated by colectomy while primary sclerosing cholangitis, ankylosing spondylitis and sacroiliitis are not improved<sup>[26]</sup>. The utility of colectomy for control of cutaneous manifestations such as erythema nodosum and pyoderma gangrenosum is uncertain as the response to surgery is variable<sup>[26]</sup>.

### **Surgical options**

A number of surgical procedures may be considered for

the treatment of ulcerative colitis, each with its own set of benefits and drawbacks. When selecting a procedure, the surgeon must consider a number of factors including pre-existing bowel dysfunction, patient stability, the presence of cancer and other medical comorbidities. Primary goals include removal of all diseased colon and rectum, elimination of cancer risk and restoration of normal bowel function. However, accomplishment of these goals may result in increased morbidity and decreased quality of life. As such, selection of an appropriate procedure is largely dependent on patient expectations and requires an ongoing dialogue between the patient, gastroenterologist, and surgeon.

Total proctocolectomy with end ileostomy remains the operative standard against which all other resections for ulcerative colitis are compared<sup>[12]</sup>. This operation removes all disease and eliminates the risk of colorectal cancer. It also eliminates issues of bowel function such as defecatory frequency, urgency and night waking and is generally associated with few dietary restrictions. It is still the procedure of choice for those with impaired anal sphincter function, or a distal rectal cancer. Other candidates include those patients who do not wish to undergo a restorative procedure and hope to have a single operation<sup>[27]</sup>.

While total proctocolectomy with end ileostomy is generally a safe procedure, it is still associated with significant morbidity. The most common complications include stoma-related issues such as parastomal herniation, skin excoriation and stomal stenosis<sup>[28,29]</sup>. A prospective analysis of 104 ulcerative colitis patients with total proctocolectomy and end ileostomy cited a 24% rate of stoma revision over 8 years<sup>[29]</sup>. The majority of these revisions were for stenosis or regression of the stoma. Small bowel obstructions are also a frequent complication but can often be treated conservatively<sup>[28]</sup>. Other morbidities associated with this operation that are intrinsic to pelvic dissection include sexual dysfunction, infertility, altered bladder function, and delayed perineal wound healing<sup>[30]</sup>. Interestingly, despite the fact that these patients live with a permanent stoma, they have a remarkably similar quality of life to age- and sex-matched patients with restorative procedures<sup>[31]</sup>.

As a result of patient's dissatisfaction with having a permanent end ileostomy and need to wear an appliance, Kock advocated the continent ileostomy in the 1960s and 1970s<sup>[32]</sup>. The terminal ileum is intussuscepted within a proximal ileal pouch forming a continent nipple valve. This procedure is performed with decreasing frequency due to significant rates of failure of the continence mechanism as well as the success of restorative proctocolectomy. A study of 96 Kock pouches showed an overall failure rate (that is, conversion to conventional ileostomy or ileal pouch-anal anastomosis) of 29% at 29 years. Fifty-nine percent of patients required a revision of their Kock pouch with 19% requiring more than 1 revision. However, it was noted that the 71% long-term success rate of Kock pouch was similar to that of restorative proctocolectomy<sup>[33]</sup>.

Although it is considered an option for the elective treatment of ulcerative colitis in a certain selection of patients, total abdominal colectomy with ileorectal anastomosis is not a common procedure. It may be

appropriate in the context of minimal rectal involvement or indeterminant colitis. A Swedish study of 51 ulcerative colitis patients treated by total colectomy with ileorectal anastomosis demonstrated that 29 patients (57%) ultimately failed this procedure and required completion proctectomy or restorative proctocolectomy<sup>[34]</sup>. Most (23 of 29 patients) failed due to ongoing rectal inflammation and diarrhea, but dysplasia was detected in 3 patients necessitating proctectomy.

Currently, the most frequent elective procedure performed for ulcerative colitis is the restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA). Its greatest benefits are removal of disease up to the anal transition zone, maintenance of a normal pathway for defecation, and avoidance of a perineal wound and permanent stoma. Disadvantages of IPAA include the need for a second operation to close the diverting loop ileostomy, the need for continued surveillance of the residual anal transition zone<sup>[27]</sup>, and ongoing management of bowel function.

IPAA is an appropriate procedure for most patients with ulcerative colitis. Two large, retrospective cohort studies from the Cleveland Clinic and Mayo Clinic have shown that it is a relatively safe and durable procedure with a low perioperative mortality rate of 0.2%-1.0%<sup>[35,36]</sup>. However, the morbidity of this procedure is still considerable. In the peri-operative period, the incidence of anastomotic separation has been cited between 5% and 10%<sup>[35-37]</sup>. The need for aggressive therapy is often unnecessary in the immediate post-operative period as routine pelvic drainage and diverting ileostomy obviate the need for surgical or radiologic management. In this setting, reversal of the ileostomy should be delayed until there is both clinical and radiographic evidence of complete resolution of the anastomotic separation.

A relatively common complication often associated with anastomotic dehiscence is ileal pouch-vaginal fistulas which occur in 3%-16% of IPAA performed in women<sup>[38]</sup>. The spectrum of disease may range from relatively asymptomatic leakage of gas, to debilitating soilage and pelvic sepsis. Pelvic sepsis secondary to anastomotic dehiscence is thought to be the major contributor to the development of these fistulas. However, other causative factors may include anal cryptoglandular disease and vaginal injury during rectal dissection. Treatment is associated with a high failure rate. A recent study of 22 patients with pouch-vaginal fistula repairs cited a 50% recurrence rate after surgery. Combined abdominoperineal approaches were associated with a significantly higher success rate than local perineal repairs (52.9% *vs* 7.9%). Overall, 21% of patients required pouch excision for non-functional pouches and recurrent fistulas<sup>[39]</sup>.

The most frequent long-term complication of IPAA is pouchitis, a non-specific inflammation of the ileal pouch that occurs in 24%-48% of IPAA patients depending on the length of follow-up<sup>[35,36]</sup>. Presenting symptoms include cramping, fever, chills, perineal pain and an increase in stool frequency. The mainstay of treatment is antibiotics such as ciprofloxacin or metronidazole. Patients can develop antibiotic-dependent or antibiotic-resistant

pouchitis that requires escalation of treatment and a small fraction may need to be treated with pouch excision<sup>[40]</sup>.

Recent studies have suggested that fertility following IPAA is significantly decreased<sup>[41,42]</sup>. A Canadian study comparing 153 females with ulcerative colitis who had IPAA with 60 females who were medically managed showed an infertility rate of 38.6% which is 3 to 4 times higher than that in the normal population<sup>[41]</sup>. A history of post-operative small bowel obstruction, pelvic sepsis, or greater number of abdominal operations was not found to increase infertility. This is somewhat surprising as the etiology of decreased fertility following IPAA is suspected to be related to the presence of post-operative adhesions in the pelvis which may cause obstruction of the fallopian tube<sup>[42]</sup>.

The most common long-term complications of the pouch are related to altered bowel function<sup>[43,44]</sup>. A number of studies have suggested that IPAA patients have, on average 6 stools during the day with 1-2 stools overnight. Occasional daytime fecal incontinence may occur in 31%-45% of patients and this rate increases to 40%-59% at night. Up to 55% of patients use an undergarment pad to absorb seepage and 45% of patients will require bulking agents or anti-diarrheal medications to help regulate their bowels. A recent meta-analysis by Hueting and colleagues evaluated 43 observational studies encompassing 9317 patients. Severe and urge incontinence rates were reported at 3.7% and 7.3% respectively<sup>[45]</sup>. They found a pouch failure rate of 6.8% at a median follow-up of 36.7 mo increasing to 8.5% with follow-up over 60 mo.

Despite these morbidities, IPAA is an exceptionally durable and well tolerated procedure which results in markedly improved quality of life. A study of 1895 patients suggested that quality of life and quality of health following IPAA was similar that of the general population<sup>[44]</sup>. In patients under 65, social, work and sexual restrictions were experienced in only 11%-17% of those who had an IPAA. Interestingly, 98% stated that they would have the surgery again or recommend it to someone else with ulcerative colitis<sup>[44]</sup>. In addition, health-related quality of life has been found to be closely linked to pouch function<sup>[46,47]</sup>. Carmon and colleagues also found that elderly patients tended to have worse quality of life as well as pouch function<sup>[47]</sup>.

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## CONTROVERSIES IN THE SURGICAL MANAGEMENT OF ULCERATIVE COLITIS

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### Age

A debatable issue is whether IPAA should be offered to elderly patients, mainly due to increased incidence of anal sphincter dysfunction and comorbidities in this cohort. Several groups have shown that IPAA is feasible in the elderly<sup>[44,48]</sup>. The Cleveland Clinic published their series of 17 IPAA patients over the age of 70 and found similar functional outcomes to younger patients<sup>[49]</sup>. However, while continence in the first 10 years of IPAA remains relatively stable, it is uncertain whether this deteriorates with time, particularly in the elderly. Delaney and colleagues have shown that incontinence and night

seepage in patients over 65 approach rates of 67% and 60%, respectively<sup>[44]</sup>. However, the authors are quick to note that these decreased levels of perfect continence do not seem to translate into a decreased quality of life or satisfaction with the surgical outcome. While it seems that chronologic age alone should not be a contraindication to IPAA, older patients must be appropriately motivated and well-informed regarding the potential problems of IPAA.

### **Hand sewn vs stapled anastomosis**

Another debate centers on the need to perform a mucosectomy to remove the last 1-2 cm of rectal mucosa and, thus, require a hand sewn ileal pouch anal anastomosis. This approach was the only technique available until the advent of the circular staplers in the early 1990s. Most surgeons quickly switched to using a double-stapled anastomosis for ileal pouches due to the ease of this technique. This involves leaving the distal 1-2 cm of rectal mucosa intact, transecting the rectum above the anorectal junction with a stapler, and performing the anastomosis with the circular stapler. Because this method avoids a mucosectomy, the abundant nerve supply to the anal transition zone is preserved and sphincter injury is minimized. Additionally, double-stapled techniques are associated with less tension on the anastomosis<sup>[12]</sup>. With increasing experience, it was felt that "mucosectomy" led to unnecessary anal sphincter and nerve damage with minimal benefit in terms of disease control. A meta-analysis of 4183 patients (2699 hand sewn with mucosectomy and 1484 with stapled IPAA) showed no significant difference in the rate of post-operative complications<sup>[50]</sup>. While the frequency of bowel movements and use of anti-diarrheal medications did not differ in the two groups, the incidence of nocturnal seepage and usage of pads favored stapled IPAA. Further, manometric measures suggested that resting and squeeze pressures were significantly reduced in the hand sewn population<sup>[50]</sup>.

Detractors of double-stapled approaches will cite the potential for ongoing rectal cuff inflammation and the consequent risk of malignancy. However, a recent review of the literature cites a total of only 17 reported cases of cancer in the pouch or anastomosis<sup>[51]</sup>. In 12 cases, dysplasia or cancer was present in the original resection specimen. All 17 patients had a diagnosis of ulcerative colitis for at least 10 years at the time of the IPAA cancer diagnosis. The authors recommended surveillance starting at 10 years after disease onset. In post-IPAA patients, they recommended more intensive surveillance with biopsies in patients with cancer or dysplasia in the original specimen<sup>[51]</sup>.

### **Routine proximal diversion**

Routine diversion of the fecal stream by loop ileostomy following IPAA is also a debated issue. Ileostomies are thought to minimize both postoperative septic complications and morbidity associated with anastomotic leak<sup>[52]</sup>. However, many surgeons believe that routine diverting loops ileostomy is counterproductive. First, loop ileostomies are associated with some morbidity including poor body image, leakage, and skin breakdown. A second

procedure to reverse the ileostomy, with its' associated cost and morbidity, is also required. It has been suggested that routine diversion is not needed in carefully selected patients depending on patient, disease, and operative factors<sup>[53]</sup>. A relatively healthy, well-nourished patient, who is not anemic, or on high dose steroids would be a candidate<sup>[53]</sup>. Intraoperative factors, such as healthy-appearing, well-vascularized small bowel, low blood loss, hemodynamic stability, and a tensionless anastomosis, may allow for omission of a diverting loop ileostomy<sup>[53]</sup>.

Using these criteria for omission of a diverting loop ileostomy, Remzi retrospectively studied 2002 IPAA patients (1725 diverted by ileostomy and 277 undiverted)<sup>[53]</sup>. While a single stage procedure was associated with a higher incidence of post-operative ileus, he found no significant differences in septic complications, quality of life or functional outcomes in the two groups. This contrasts an earlier report from the same institution that rates of septic complications are increased, particularly in patients taking high doses of steroids<sup>[52]</sup>. Another report has suggested that the routine omission of a diverting loops ileostomy is associated with an increased risk to life<sup>[54]</sup>. As such, Remzi and others have suggested that only carefully selected patients are suitable for one-stage restorative proctocolectomy.

## **LAPAROSCOPIC PROCEDURES FOR ULCERATIVE COLITIS**

All operations for ulcerative colitis can be performed laparoscopically, including subtotal colectomy, total proctocolectomy and restorative proctocolectomy. Earlier reports in the mid-1990's demonstrated significantly longer operative times without a benefit in post-operative ileus or length of stay (traditional rationales for minimally invasive procedures)<sup>[55]</sup>. Further, it was shown that postoperative morbidity and transfusion requirements were higher in laparoscopic procedures<sup>[56]</sup>.

With better experience and technique, the increased morbidity of laparoscopic procedures for ulcerative colitis has decreased<sup>[57]</sup>. Marcello and colleagues have published a series using only experienced surgeons who have performed more than 700 laparoscopic colorectal procedures of which at least 100 were total abdominal colectomies<sup>[58]</sup>. The study compared 20 consecutive laparoscopic restorative proctocolectomies with 20 conventional procedures. While a significant increase in operative time was noted in the laparoscopic group (100 min), many of the previously cited morbidities associated with laparoscopic procedures, including blood loss and post-operative infection, were not significantly different from the open group. The length of stay in hospital was also shorter (7 d *vs* 8 d). A few subsequent studies have confirmed Marcello's findings<sup>[59,60]</sup>. Further, recent analysis of functional outcomes including number of bowel movements, stool consistency, incontinence, need for anti-diarrheal medications, and dietary restrictions has not revealed a significant difference between laparoscopic and conventional IPAA<sup>[60]</sup>.

A randomized, prospective study by Milsom has shown that laparoscopic resection in ileocolic Crohn's disease is associated with less blood loss, fewer respiratory



complications, faster return of bowel function and shorter hospital stay<sup>[61]</sup>; no similar study has been performed for laparoscopic procedures in the treatment of ulcerative colitis. Part of the problem is the lack of agreement on the optimal technique for laparoscopic IPAA which makes a comparative study with the open procedure difficult<sup>[57]</sup>. As discussed, there is also a steep learning curve associated with laparoscopic IPAA; most recent published results involve very experienced surgeons. It may be difficult to attain this experience outside of dedicated colorectal centers with a high volume of laparoscopic inflammatory bowel disease cases. While the current data suggest that laparoscopic IPAA may be a safe and effective treatment in the hands of an experienced surgeon, there are insufficient studies to recommend its general use. Further, despite improvements in technology, increased operative times may pose a barrier in institutions where operating room time is at a premium<sup>[57]</sup>.

## CROHN'S DISEASE

While advances in the medical management of Crohn's disease have decreased the need for surgery, it is estimated that between 70% and 90% of Crohn's patients will need a surgical intervention at some point during their disease<sup>[62]</sup>. The panenteric nature of Crohn's disease results in a variety of presentations depending on both the location of the disease as well as the disease behavior. Nonetheless, as Crohn's disease cannot be cured, the indications for surgery remain relatively straight-forward: failure of medical management and complications of the disease process. The specific surgical managements available are often multiple and selection of the most appropriate modality is dependent on several patient and disease factors.

## CROHN'S DISEASE: SMALL BOWEL

### Indications

Failure of medical management remains the most common indication for surgery in most series of patients with small bowel Crohn's disease<sup>[63,64]</sup>. Failure is defined by (1) symptoms that cannot be controlled or progress with maximum medical therapy, (2) problems with treatment side effects, and (3) inability of patient to maintain compliance with a medical regimen. Extra-intestinal manifestations (EIM) of disease are another indication for surgery and may occur in as many as 25% of Crohn's patients<sup>[65]</sup>. Disorders of the skin, mouth, eye and joints are common in colonic disease and tend to parallel disease activity. As such, surgical resection of diseased bowel tends to ameliorate these EIMs. Hepatic, vascular, hematologic, pulmonary, cardiac and neurologic system EIMs tend to act independently of intestinal disease. Other disorders such as nephrolithiasis and cholelithiasis are actually complications of disease secondary to altered intestinal absorption. As such, surgical resection may improve these conditions.

Intestinal obstruction represents a frequent complication of small bowel Crohn's disease<sup>[63,64]</sup>. Acute obstruction can occur due to a primary stricture or series of strictures.

Healthy, non-diseased, bowel may be mechanically obstructed as part of an inflammatory mass or fistula. Acute obstructions are more likely to be the result of active inflammation and will often resolve with medical management<sup>[66]</sup>. Conversely, chronic obstruction, which is usually the result of a fixed fibrostenotic lesion, tends to require surgical management<sup>[66]</sup>. Surgery usually involves a resection of the diseased segment, but other options include intestinal bypass, creation of ileostomy, or stricturoplasty (discussed below).

Fistulas with associated abscess or stricture are another common complications of small bowel Crohn's that requires surgery<sup>[63,64]</sup>. In a study of 1379 patients, Michelassi found fistulas in 35% of surgically managed patients. However, fistula was the primary surgical indication in only 6.3% of patients<sup>[67]</sup>. Enterointestinal fistulas are the most common type of fistula and may be relatively asymptomatic unless a large segment of intestine is bypassed or complications of obstruction arise. Broe and colleagues published that 40% of patients with fistula initially managed non-operatively eventually required surgery within 1 year, usually secondary to medical intractability<sup>[68]</sup>. The surgical management of enterointestinal fistulas generally involves resection of the primary site that has active disease and simple debridement and primary closure of the secondary site that is usually normal. Enteral fistulas to the vagina and urinary bladder often require surgery and treatment adheres to similar principles of primary site resection with repair of the secondary site. Ileal-sigmoid fistulas may represent an exception to this rule. Fazio and colleagues have shown that primary repair of a sigmoid defect is vulnerable to breakdown, particularly when the sigmoid is involved in a phlegmon<sup>[69]</sup>. They suggest that a minimal sigmoid resection can be performed with minimal morbidity<sup>[69]</sup>.

Enterocutaneous fistulas deserve special mention as these fistulas will occasionally respond to medical therapy. The most promising trials to date have evaluated the use of infliximab. The ACCENT-1 trial suggested a rapid response to 3 infusions of infliximab in 46% of patients, but the duration of effect was short-lived (only 3-4 mo)<sup>[70]</sup>. The follow-up ACCENT-2 trial showed that the duration of effect could be sustained in 46% of patients with maintenance doses of infliximab every 8 wk<sup>[71]</sup>. Surgical principles for the management of enterocutaneous fistulas parallel that for other types of fistulas. Those patients with short fistula tracts, exposed bowel mucosa, and high outputs will generally require operative intervention, but this should be delayed until the patient's health and nutritional status have been optimized.

The life-time risk for developing an abscess in Crohn's patients is estimated to be around 25%<sup>[72]</sup>. Using radiographic techniques, abscesses can often be drained percutaneously. However, some controversy exists as to whether abscesses must eventually be followed up with surgical resection of the associated diseased bowel. One study has shown that abscesses recur more frequently when they are percutaneously drained compared to surgically drained abscesses (56% *vs* 12% respectively)<sup>[73]</sup>. However, Gutierrez and colleagues have shown in a study of 66 patients only one third of patients who were



percutaneously drained required surgery at 1 year follow-up<sup>[74]</sup>. Certainly, if an abscess contains enteric contents, it is less likely to resolve without surgical resection<sup>[62]</sup>.

Other less common indications for surgery in small bowel include perforation, bleeding and cancer. Free perforation is associated with a high mortality if not treated and surgical management should involve resection rather than repair<sup>[75]</sup>. Massive hemorrhage due to Crohn's is a rare indication for surgery<sup>[76]</sup>. Other more common sources of bleeding such as peptic ulcer disease or diverticulitis must be actively ruled out. Adenocarcinoma of the small intestine, while rare, is increased 12-fold to 60-fold compared to the general population<sup>[77]</sup>. Prognosis is generally poor due to the often advanced stage of disease at the time of diagnosis. Mortality rates at 1 year or 2 years have been reported to be 30%-60%<sup>[77]</sup>.

### Surgical options

Resection is the most commonly performed surgical procedure for small bowel Crohn's disease. In general, the panenteric nature of Crohn's disease has resulted in a surgical philosophy of conservatism. Recurrence rates tend to increase with the passage of time<sup>[78]</sup> and Crohn's disease patients may eventually require multiple resections, each increasing the risk of short-bowel syndrome and its associated metabolic morbidities. Of note, Glehen and colleagues have reported that Crohn's disease patients start out with shorter bowel than the normal population<sup>[79]</sup>.

A number of studies have investigated whether certain technical factors, including resection margin and configuration of anastomosis, influence the rate of recurrence. Two retrospective Swedish studies suggested that "radical" resection resulted in a much lower rate of resection and better quality of life in Crohn's patients. Krause and colleagues studied 186 patients with margins of uninvolved bowel of less than 10 cm or greater than 10 cm (a radical resection). They reported a 31% recurrence rate and better quality of life with radical resection compared to an 83% recurrence rate in the other group<sup>[80]</sup>. Softley and colleagues used a 4 cm histologic margin and found that impingement on this margin resulted in a ten-fold increase in recurrence<sup>[81]</sup>.

However, the best evidence available that large resection margins do not decrease the rate of recurrence is based on a study performed by Fazio<sup>[82]</sup> where 131 patients were randomized to resection with margins of either 2 cm of uninvolved bowel (75 patients) or 12 cm (56 patients). Although the rate of recurrence was lower in the group with more extensive resection (25% *vs* 18%), this difference did not achieve statistical significance. In Fazio's study, grossly normal resection margins were used. Hamilton and colleagues studied the role of frozen section examination of bowel wall at the resection margins during surgery and found there was no difference in reoperation or recurrence rates in Crohn's patients with disease-free margins that were detected histologically or grossly<sup>[82]</sup>.

The type of anastomosis performed in small bowel resections has also been speculated to affect recurrence rates. Since fibrostenotic disease is a described clinical phenotype of Crohn's disease by the Vienna Classification<sup>[83]</sup>, it was

thought that the larger lumen of a side-to-side anastomosis would be less likely to obstruct and require re-operation. Stapled anastomoses have been reported to have lower morbidity and recurrence rates<sup>[84,85]</sup>. Resegotti also reported lower anastomotic leak rates in stapled versus hand-sewn anastomosis<sup>[86]</sup>. However, it has been suggested that certain circumstances clearly favor hand-sewn anastomosis, particularly when joining thickened (but grossly disease-free) bowel which may exceed the specifications of a bowel stapler<sup>[62]</sup>.

Recurrence rates following resection remain high and although not all symptomatic recurrence requires surgery, it has been reported that surgical re-intervention occurs in 25%-35% of patients at 5 years and 40%-70% at 15 years<sup>[87]</sup>. However, several trials have been done to evaluate patient and disease factors as well as medication regimens which may decrease recurrence. Yamamoto has recently published a comprehensive systematic review of factors affecting Crohn's recurrence after surgery<sup>[88]</sup>. His review concludes that cessation of smoking seems to be the most consistent factor in reducing recurrence. While several other factors (including 5-ASA use, immunosuppressant drugs, wider anastomosis, and disease duration) may affect recurrence rates, further studies are still required<sup>[88]</sup>. Post-surgical recurrence in Crohn's disease is still largely unpredictable<sup>[89]</sup>.

When preservation of intestinal length is an issue, stricturoplasty represents an alternative to resection and can reduce the risk of short bowel syndrome. Indications for stricturoplasty include short fibrous strictures; diffuse involvement of the small bowel involving multiple strictures, a stricture in someone who has short bowel syndrome or a history of multiple prior small bowel resections, patients with a rapid recurrence of Crohn's disease manifested as obstruction, or patients with duodenal Crohn's disease. Stricturoplasties should not be done in patients with multiple strictures within a short segment, a long (< 20 cm) stricture, or stricture close to a site of resection<sup>[62]</sup>. Perforation, fistula and phlegmon at the site of stricture are also contraindications to stricturoplasty. The largest stricturoplasty experience published to date comes from the Cleveland Clinic in which 698 stricturoplasties were performed in 162 patients<sup>[90]</sup>. Their cited recurrence rate at 5 years was 28% which is similar to published rates of recurrence following resection. In addition to relieving obstruction, there have been reports of disease regression at sites of stricturoplasty<sup>[91]</sup>. Indeed, in the Cleveland Clinic series, documented recurrences only occurred at the previous stricturoplasty site in 5% of cases<sup>[90]</sup>. Fearnhead and colleagues have recently published a long-term follow-up of 479 stricturoplasties in 100 patients<sup>[92]</sup>. Over a mean follow-up of 85 mo, the overall morbidity rate was 22.6% with septic complications (leak, fistula or abscess) occurring in 11.3%. Obstruction occurred in 4.4% of patients while rate peri-operative mortality rate was 0.6%. Although bleeding from stricturoplasty sites has previously been cited as a potentially serious problem due to the presence of a suture line in diseased bowel, Fearnhead found a gastrointestinal hemorrhage incidence of only 3.8%<sup>[92]</sup>.

One disadvantage of performing a stricturoplasty is

that a malignancy of the bowel can be missed. Several case reports have been published of adenocarcinoma arising from Crohn's-related stricture sites. To avoid missing a cancer in a longstanding stricture, it has been suggested that full-thickness biopsies should be taken for frozen section to aid the surgeon in the decision to perform either stricturoplasty or resection<sup>[93]</sup>.

Other surgical options for the treatment of small bowel Crohn's disease include bypass operations or ileostomy formation. Bypass operations have largely been abandoned due to the risk of malignancy and continued disease activity<sup>[62]</sup>. A role for bypass may be for temporary relief of obstruction when a future resection is planned<sup>[62]</sup>. An ileostomy may be required in Crohn's patients when enteric anastomosis is unsafe (sepsis, unstable patient, severe malnutrition, chronic immunosuppression) or when small bowel resection is done in conjunction with colonic or rectal resection.

## CROHN'S DISEASE: COLON

### Indications

Crohn's colitis occurs in approximately one quarter of Crohn's patients although colonic disease is most frequently seen in conjunction with terminal ileal disease<sup>[62]</sup>. As with small bowel Crohn's disease, indications for surgery in colonic Crohn's disease can be grouped into complications of the disease and failure of medical therapy. Indications specific to colonic disease include the development of dysplasia or colorectal cancer and toxic colitis. The treatment of obstruction and fistula of the colon may differ from that in the small bowel.

Obstruction from colonic strictures may be present in as many as 17% of patients with Crohn's colitis<sup>[94]</sup>. There is some evidence to suggest that benign strictures may be adequately treated by endoscopic balloon dilatation<sup>[95,96]</sup>. A recent series of 16 patients by Nomura showed 75% had symptomatic relief after an initial dilation with one third of patients requiring further dilations within two years<sup>[96]</sup>. All colonic strictures should be endoscopically biopsied as 7%-10% may contain malignancy<sup>[94]</sup>.

Despite earlier studies suggesting that the risk of colorectal cancer was lower than that seen in ulcerative colitis, the current literature suggests that the risk is equivalent<sup>[97]</sup>. Maykel and colleagues have published a retrospective analysis of 222 patients who underwent resection for colonic Crohn's disease<sup>[98]</sup>. Five cases of dysplasia (2.3%) and 6 cases of adenocarcinoma (2.7%) were identified. Of note, in only 3 of the dysplasia cases and 1 of the adenocarcinoma cases was the abnormality identified preoperatively. Consistent with previous reports, Maykel further identified older age at diagnosis of Crohn's disease, duration of disease (greater than 8 years) and extent of disease (pancolitis) as risk factors for the development of dysplasia or cancer<sup>[98]</sup>.

As with ulcerative colitis, surgery is indicated for a proven malignancy, high-grade dysplasia, or DALM in colonic Crohn's disease and the diagnosis and management of low-grade dysplasia is still controversial<sup>[99]</sup>. A recent Cochrane review suggests that surveillance endoscopy does

not necessarily improve survival despite the earlier detection of cancers<sup>[100]</sup>. Nonetheless, current recommendations for surveillance in Crohn's colitis mirror those for ulcerative colitis<sup>[19]</sup>.

Fistulous disease can also occur with Crohn's colitis. However, it is important when assessing fistulas involving the colon to determine the primary site of the fistula. Endoscopic evaluation of the colonic mucosa should be performed. Enterocolonic fistulas in Crohn's patients are often a result of primary small bowel disease with the colon only secondarily involved. In these circumstances, it is generally preferable to debride the colonic side of the fistula and close the defect rather than perform a colonic resection<sup>[101]</sup>. Despite the introduction of new medical therapies, such as infliximab, 50%-75% of patients with colonic fistulas will require surgery<sup>[102]</sup>.

Toxic colitis in Crohn's disease has a similar presentation to that in ulcerative colitis. Operative indications include perforation, lack of improvement with medical management, fulminant colitis, massive hemorrhage and hemodynamic instability. As with ulcerative colitis, the most common procedure for toxic colitis in Crohn's disease patients is subtotal colectomy and end ileostomy<sup>[62]</sup>.

### Surgical options

The extent of resection in patients with only segmental colonic disease is a subject of some debate. A recent meta-analysis of six studies encompassing 488 patients suggests that there is no significant difference in overall recurrence rate, complications or need for permanent stoma between segmental colectomy and total abdominal colectomy with ileorectal anastomosis, but time to recurrence was longer in the total abdominal colectomy group by 4.4 years<sup>[103]</sup>. This is in contrast to previous studies which actually suggested the more extensive procedure (total abdominal colectomy) was associated with a higher recurrence. Bernell published a review of 833 patients and found the 10 year recurrence rate of total abdominal colectomy and ileorectal anastomosis to be 58% compared to 47% in the segmental colectomy group<sup>[87]</sup>. Another study found similar recurrence rates in both groups, but better functional outcomes in the segmental colectomy group<sup>[104]</sup>. In patients with diffuse colitis and proctitis, total proctocolectomy has been associated with less medication use 1 year after operation as well as increased time interval to first recurrence when compared to total abdominal colectomy or segmental colectomy<sup>[105]</sup>. Tekkis has suggested that more extensive resection such as total abdominal colectomy may be more appropriate when 2 or more segments of colon are diseased<sup>[103]</sup>.

Restorative proctocolectomy with ileal pouch-anal anastomosis (IPAA) for Crohn's disease has most often occurred in the setting of a changed diagnosis following IPAA for ulcerative colitis or indeterminate colitis<sup>[62]</sup>. A majority of studies suggest that IPAA in Crohn's disease is associated with a significantly higher rate of morbidity (including pouch failure, incontinence, and pouchitis) than in ulcerative colitis<sup>[106,107]</sup>. Although a few authors have suggested that IPAA may be appropriate in highly selected patients with Crohn's disease<sup>[108,109]</sup>, the current literature

certainly does not support its general use in the Crohn's population.

## ANORECTAL CROHN'S DISEASE

### *Indications for surgery*

About 10%-15% of Crohn's disease patients will have disease limited to the anorectal area but up to 90% of all patients have some manifestation of anorectal disease<sup>[110]</sup>. Perianal disease, including fissures, fistulas and abscesses, can have both typical and atypical presentations. Damage of even a small amount of sphincter muscle can lead to severe morbidity.

Anal skin tags and hemorrhoidal disease may present in up to 70% of patients with anorectal Crohn's<sup>[110]</sup>. These lesions are most often asymptomatic, but may present problems with hygiene, particularly in the context of a diarrheal illness. Patients being seen for potential surgical therapy of these problems should be counseled regarding the risk for non-healing wounds and loss of continence following surgery. Anal skin tags and hemorrhoidal disease are best treated conservatively<sup>[111]</sup>.

Anal fissures are a challenging problem in the Crohn's population. These fissures are often painless and do not necessarily present in the anterior or posterior midline. Often, there is concomitant disease or multiple fissures. While the initial therapy for fissures should be conservative (that is, fiber and fluid supplementation with local medications to aid in sphincter relaxation), some studies have shown that 50% of Crohn's patients will fail medical management<sup>[112]</sup>. Thus, if patients are suffering from significant symptoms of pain, these authors have advocated for a more aggressive approach including lateral sphincterotomy. Wolomir and colleagues published a series of 25 Crohn's patients who underwent sphincterotomy<sup>[113]</sup>. Twenty-two patients had completely healed their fissures within 2 mo and at an average follow-up of 7.5 years, there were no direct complications of sphincterotomy. However, given the risks of decreased continence with surgery, surgical management should be reserved for those with minimal active anorectal inflammation who have truly failed conservative management and have significant symptoms. Division of sphincter muscle should be minimal.

Anorectal abscesses and fistula-in-ano are common problems in the Crohn's population. Treatment of simple abscesses involves incision and drainage. It is important to place the incision as close to the anal verge as possible while still achieving adequate drainage as a fistula may form in the future. It is unclear whether fistulas from Crohn's disease are more common following abscesses or develop in the same way as those of a cryptoglandular origin. As such, Crohn's fistulas may erode deep into sphincter muscle or have complicated, blind tracts. Treatment of these fistulas is closely associated with the anatomy of the fistula, the amount of sphincter complex involved, as well as the status of the patient's fecal and gas continence. Furthermore, the surgeon must consider active disease elsewhere in the gastrointestinal tract and the potential for chronic diarrhea. Aggressive procedures which may

alter continence should be avoided. Treatment options include setons, fistula plugs and mucosal advancement flaps. While several authors have suggested that these procedures can be performed with similar success rates to identical procedures performed in non-Crohn's patients, these studies tend to lack appropriate follow-up<sup>[62]</sup>. There has been some suggestion that fecal diversion following a definitive procedure for fistula-in-ano may improve resolution rates<sup>[114]</sup>.

In rare cases, anorectal disease is insufficiently controlled by adequate drainage of infection and medical management. Assuming that Crohn's disease is still the primary diagnosis, these patients may be assessed for diversion with or without proctectomy<sup>[62]</sup>. While the vast majority of patients will respond to medical management or conservative surgical management such as seton placement, there is evidence that fecal diversion helps the resolution of peri-anal disease, at least temporarily<sup>[115]</sup>. As many as 80% of patients with peri-anal Crohn's manifestations will respond to diversion, but relapse may occur despite a stoma, and restoration of intestinal continuity occurs in a minority of patients<sup>[115]</sup>. A multivariate analysis performed by Galandiuk and colleagues suggest that colonic Crohn's disease and anal stenosis are risk factors for fecal diversion<sup>[116]</sup>.

## LAPAROSCOPIC PROCEDURES FOR CROHN'S DISEASE

The safety and feasibility of laparoscopic resections for treatment of Crohn's disease has been extensively studied<sup>[117]</sup>. Unlike ulcerative colitis, robust data is available for laparoscopic resection in Crohn's disease. Two randomized, controlled studies have been published suggesting that the laparoscopic ileocolic resection is associated with decreased morbidity and length of stay in the hospital<sup>[61,118]</sup>. Maartense further suggested that overall costs of laparoscopic resection were lower<sup>[118]</sup>. While operative times have decreased with increased experience, most studies still suggest significantly longer operative times when compared to open procedures.

## PREPARING THE IBD PATIENT FOR SURGERY

Preparation for surgery is a multidisciplinary approach. Surgery for inflammatory bowel disease differs from cancer surgery in several aspects. Surgery is generally elective and several treatment options are available. Medically intractability and unacceptable symptoms are subjective in nature. Pre-operatively, the patients may be severely malnourished or on high-dose immunosuppressants or steroids. Patients are generally younger and issues including future child-bearing, sexual function and body image have increased importance. Preparation for surgery requires close interaction between many health care team members (including surgeons, gastroenterologist, primary care physicians and enterostomal therapists) and the patient.

Counseling should include a discussion of the extent of disease, the specific indication for surgery,

and alternatives to surgery. A description of the surgical procedure should include risks and complications of the surgery, but also possible intra-operative findings and a discussion about how intra-operative decisions will be made. Particular attention should be made towards issues of bowel function and continence. It is important to assess the patient's expectations from surgery. As stated above, surgery is often elective, but still associated with significant morbidity. The specific surgical procedure or timing of the procedure should be modified, as possible, to reflect the needs of the patient.

Finally, we recommend that patients undergoing surgery for inflammatory bowel disease are referred to former patients who have had similar procedures. In our experience, previous patients are very willing to share their experiences, offer a different perspective and are usually helpful. If possible, we try to match patients according to sex, age and marital status.

## CONCLUSION

While the indications for surgery in IBD are relatively straight forward (intractability or complications), the precise timing and specific procedure(s) to be performed are often fraught with controversy. In ulcerative colitis, the correct diagnosis and management of low grade dysplasia is still a work in evolution. IPAA is the most common procedure performed, but may be contraindicated in certain populations such as the elderly. Other patient cohorts may be appropriate for single-stage IPAA. The performance of a mucosectomy (and its associated morbidity) may be indicated, particularly in those with rectal dysplasia or cancer. In Crohn's disease, the panenteric nature of the disease leads to significant recurrence rates. While surgical conservatism is the current general rule, several authors have advocated for a more aggressive approach in small bowel, colonic, and anorectal disease. In both ulcerative colitis and Crohn's disease, laparoscopic procedures seem to be associated with increased operative times, but could also result in decreased morbidity while providing similar functional results to open procedures.

## REFERENCES

- 1 Wexner SD, Rosen L, Lowry A, Roberts PL, Burnstein M, Hicks T, Kerner B, Oliver GC, Robertson HD, Robertson WG, Ross TM, Senatore PJ Jr, Simmang C, Smith C, Vernava AM 3rd, Wong WD. Practice parameters for the treatment of mucosal ulcerative colitis--supporting documentation. The Standards Practice Task Force. The American Society of Colon and Rectal Surgeons. *Dis Colon Rectum* 1997; **40**: 1277-1285
- 2 Berg DF, Bahadursingh AM, Kaminski DL, Longo WE. Acute surgical emergencies in inflammatory bowel disease. *Am J Surg* 2002; **184**: 45-51
- 3 Truelove SC, Witts LJ. Cortisone in ulcerative colitis; final report on a therapeutic trial. *Br Med J* 1955; **2**: 1041-1048
- 4 Katz JA. Medical and surgical management of severe colitis. *Semin Gastrointest Dis* 2000; **11**: 18-32
- 5 Jewell DP. How I do it. Medical management of severe ulcerative colitis. *Int J Colorectal Dis* 1988; **3**: 186-189
- 6 Greenstein AJ, Sachar DB, Gibas A, Schrag D, Heimann T, Janowitz HD, Aufses AH Jr. Outcome of toxic dilatation in ulcerative and Crohn's colitis. *J Clin Gastroenterol* 1985; **7**: 137-143
- 7 Travis SP, Farrant JM, Ricketts C, Nolan DJ, Mortensen NM, Kettlewell MG, Jewell DP. Predicting outcome in severe ulcerative colitis. *Gut* 1996; **38**: 905-910
- 8 Latella G, Vernia P, Viscido A, Frieri G, Cadau G, Cocco A, Cossu A, Tomei E, Caprilli R. GI distension in severe ulcerative colitis. *Am J Gastroenterol* 2002; **97**: 1169-1175
- 9 Witte J, Shivananda S, Lennard-Jones JE, Beltrami M, Politi P, Bonanomi A, Tsianos EV, Mouzas I, Schulz TB, Monteiro E, Clofent J, Odes S, Limonard CB, Stockbrugger RW, Russel MG. Disease outcome in inflammatory bowel disease: mortality, morbidity and therapeutic management of a 796-person inception cohort in the European Collaborative Study on Inflammatory Bowel Disease (EC-IBD). *Scand J Gastroenterol* 2000; **35**: 1272-1277
- 10 Greenstein AJ, Barth JA, Sachar DB, Aufses AH Jr. Free colonic perforation without dilatation in ulcerative colitis. *Am J Surg* 1986; **152**: 272-275
- 11 Caprilli R, Latella G, Vernia P, Frieri G. Multiple organ dysfunction in ulcerative colitis. *Am J Gastroenterol* 2000; **95**: 1258-1262
- 12 Cohen JL, Strong SA, Hyman NH, Buie WD, Dunn GD, Ko CY, Fleshner PR, Stahl TJ, Kim DG, Bastawrous AL, Perry WB, Cataldo PA, Rafferty JF, Ellis CN, Rakinic J, Gregorcyk S, Shellito PC, Kilkenny JW 3rd, Ternent CA, Koltun W, Tjandra JJ, Orsay CP, Whiteford MH, Penzer JR. Practice parameters for the surgical treatment of ulcerative colitis. *Dis Colon Rectum* 2005; **48**: 1997-2009
- 13 Alves A, Panis Y, Bouhnik Y, Maylin V, Lavergne-Slove A, Valleur P. Subtotal colectomy for severe acute colitis: a 20-year experience of a tertiary care center with an aggressive and early surgical policy. *J Am Coll Surg* 2003; **197**: 379-385
- 14 Hyman NH, Cataldo P, Osler T. Urgent subtotal colectomy for severe inflammatory bowel disease. *Dis Colon Rectum* 2005; **48**: 70-73
- 15 Carter FM, McLeod RS, Cohen Z. Subtotal colectomy for ulcerative colitis: complications related to the rectal remnant. *Dis Colon Rectum* 1991; **34**: 1005-1009
- 16 Karch LA, Bauer JJ, Gorfine SR, Gelernt IM. Subtotal colectomy with Hartmann's pouch for inflammatory bowel disease. *Dis Colon Rectum* 1995; **38**: 635-639
- 17 Khubchandani IT, Kontostolis SB. Outcome of ileorectal anastomosis in an inflammatory bowel disease surgery experience of three decades. *Arch Surg* 1994; **129**: 866-869
- 18 Berger M, Gribetz D, Korelitz BI. Growth retardation in children with ulcerative colitis: the effect of medical and surgical therapy. *Pediatrics* 1975; **55**: 459-467
- 19 Eaden JA, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001; **48**: 526-535
- 20 Winawer S, Fletcher R, Rex D, Bond J, Burt R, Ferrucci J, Ganiats T, Levin T, Woolf S, Johnson D, Kirk L, Litin S, Simmang C. Colorectal cancer screening and surveillance: clinical guidelines and rationale-Update based on new evidence. *Gastroenterology* 2003; **124**: 544-560
- 21 Bernstein CN, Shanahan F, Weinstein WM. Are we telling patients the truth about surveillance colonoscopy in ulcerative colitis? *Lancet* 1994; **343**: 71-74
- 22 Odze RD, Goldblum J, Noffsinger A, Alsaigh N, Rybicki LA, Fogt F. Interobserver variability in the diagnosis of ulcerative colitis-associated dysplasia by telepathology. *Mod Pathol* 2002; **15**: 379-386
- 23 Odze RD, Farraye FA, Hecht JL, Hornick JL. Long-term follow-up after polypectomy treatment for adenoma-like dysplastic lesions in ulcerative colitis. *Clin Gastroenterol Hepatol* 2004; **2**: 534-541
- 24 Befrits R, Ljung T, Jaramillo E, Rubio C. Low-grade dysplasia in extensive, long-standing inflammatory bowel disease: a follow-up study. *Dis Colon Rectum* 2002; **45**: 615-620
- 25 Gumaste V, Sachar DB, Greenstein AJ. Benign and malignant colorectal strictures in ulcerative colitis. *Gut* 1992; **33**: 938-941
- 26 Goudet P, Dozois RR, Kelly KA, Ilstrup DM, Phillips SF. Characteristics and evolution of extraintestinal manifestations



- associated with ulcerative colitis after proctocolectomy. *Dig Surg* 2001; **18**: 51-55
- 27 **Metcalf AM**. Elective and emergent operative management of ulcerative colitis. *Surg Clin North Am* 2007; **87**: 633-641
- 28 **Hulten L**. Proctocolectomy and ileostomy to pouch surgery for ulcerative colitis. *World J Surg* 1998; **22**: 335-341
- 29 **Carlstedt A**, Fasth S, Hulten L, Nordgren S, Palselius I. Long-term ileostomy complications in patients with ulcerative colitis and Crohn's disease. *Int J Colorectal Dis* 1987; **2**: 22-25
- 30 **Cornish JA**, Tan E, Teare J, Teoh TG, Rai R, Darzi AW, Paraskevas P, Clark SK, Tekkis PP. The effect of restorative proctocolectomy on sexual function, urinary function, fertility, pregnancy and delivery: a systematic review. *Dis Colon Rectum* 2007; **50**: 1128-1138
- 31 **Camilleri-Brennan J**, Munro A, Steele RJ. Does an ileoanal pouch offer a better quality of life than a permanent ileostomy for patients with ulcerative colitis? *J Gastrointest Surg* 2003; **7**: 814-819
- 32 **Kock NG**, Darle N, Hulten L, Kewenter J, Myrvold H, Philipson B. Ileostomy. *Curr Probl Surg* 1977; **14**: 1-52
- 33 **Lepisto AH**, Jarvinen HJ. Durability of Kock continent ileostomy. *Dis Colon Rectum* 2003; **46**: 925-928
- 34 **Leijonmarck CE**, Lofberg R, Ost A, Hellers G. Long-term results of ileorectal anastomosis in ulcerative colitis in Stockholm County. *Dis Colon Rectum* 1990; **33**: 195-200
- 35 **Fazio VW**, Ziv Y, Church JM, Oakley JR, Lavery IC, Milsom JW, Schroeder TK. Ileal pouch-anal anastomoses complications and function in 1005 patients. *Ann Surg* 1995; **222**: 120-127
- 36 **Meagher AP**, Farouk R, Dozois RR, Kelly KA, Pemberton JH. J ileal pouch-anal anastomosis for chronic ulcerative colitis: complications and long-term outcome in 1310 patients. *Br J Surg* 1998; **85**: 800-803
- 37 **Maartense S**, Dunker MS, Slors JF, Cuesta MA, Gouma DJ, van Deventer SJ, van Bodegraven AA, Bemelman WA. Hand-assisted laparoscopic versus open restorative proctocolectomy with ileal pouch anal anastomosis: a randomized trial. *Ann Surg* 2004; **240**: 984-991; discussion 991-992
- 38 **Shah NS**, Remzi F, Massmann A, Baixauli J, Fazio VW. Management and treatment outcome of pouch-vaginal fistulas following restorative proctocolectomy. *Dis Colon Rectum* 2003; **46**: 911-917
- 39 **Johnson LB**, Jorgensen LN, Adawi D, Blomqvist P, Asklof GB, Gottrup F, Jeppsson B. The effect of preoperative radiotherapy on systemic collagen deposition and postoperative infective complications in rectal cancer patients. *Dis Colon Rectum* 2005; **48**: 1573-1580
- 40 **Mahadevan U**, Sandborn WJ. Diagnosis and management of pouchitis. *Gastroenterology* 2003; **124**: 1636-1650
- 41 **Johnson P**, Richard C, Ravid A, Spencer L, Pinto E, Hanna M, Cohen Z, McLeod R. Female infertility after ileal pouch-anal anastomosis for ulcerative colitis. *Dis Colon Rectum* 2004; **47**: 1119-1126
- 42 **Gorgun E**, Remzi FH, Goldberg JM, Thornton J, Bast J, Hull TL, Loparo B, Fazio VW. Fertility is reduced after restorative proctocolectomy with ileal pouch anal anastomosis: a study of 300 patients. *Surgery* 2004; **136**: 795-803
- 43 **Michelassi F**, Lee J, Rubin M, Fichera A, Kasza K, Karrison T, Hurst RD. Long-term functional results after ileal pouch anal restorative proctocolectomy for ulcerative colitis: a prospective observational study. *Ann Surg* 2003; **238**: 433-441; discussion 442-445
- 44 **Delaney CP**, Fazio VW, Remzi FH, Hammel J, Church JM, Hull TL, Senagore AJ, Strong SA, Lavery IC. Prospective, age-related analysis of surgical results, functional outcome, and quality of life after ileal pouch-anal anastomosis. *Ann Surg* 2003; **238**: 221-228
- 45 **Huetting WE**, Buskens E, van der Tweel I, Gooszen HG, van Laarhoven CJ. Results and complications after ileal pouch anal anastomosis: a meta-analysis of 43 observational studies comprising 9,317 patients. *Dig Surg* 2005; **22**: 69-79
- 46 **Scarpa M**, Angriman I, Ruffolo C, Ferronato A, Polese L, Barollo M, Martin A, Sturniolo GC, D'Amico DF. Health-related quality of life after restorative proctocolectomy for ulcerative colitis: long-term results. *World J Surg* 2004; **28**: 124-129
- 47 **Carmon E**, Keidar A, Ravid A, Goldman G, Rabau M. The correlation between quality of life and functional outcome in ulcerative colitis patients after proctocolectomy ileal pouch anal anastomosis. *Colorectal Dis* 2003; **5**: 228-232
- 48 **Takao Y**, Gilliland R, Nogueras JJ, Weiss EG, Wexner SD. Is age relevant to functional outcome after restorative proctocolectomy for ulcerative colitis?: prospective assessment of 122 cases. *Ann Surg* 1998; **227**: 187-194
- 49 **Ho KS**, Chang CC, Baig MK, Borjesson L, Nogueras JJ, Efron J, Weiss EG, Sands D, Vernava AM 3rd, Wexner SD. Ileal pouch anal anastomosis for ulcerative colitis is feasible for septuagenarians. *Colorectal Dis* 2006; **8**: 235-238
- 50 **Lovegrove RE**, Constantinides VA, Heriot AG, Athanasiou T, Darzi A, Remzi FH, Nicholls RJ, Fazio VW, Tekkis PP. A comparison of hand-sewn versus stapled ileal pouch anal anastomosis (IPAA) following proctocolectomy: a meta-analysis of 4183 patients. *Ann Surg* 2006; **244**: 18-26
- 51 **Das P**, Johnson MW, Tekkis PP, Nicholls RJ. Risk of dysplasia and adenocarcinoma following restorative proctocolectomy for ulcerative colitis. *Colorectal Dis* 2007; **9**: 15-27
- 52 **Tjandra JJ**, Fazio VW, Milsom JW, Lavery IC, Oakley JR, Fabre JM. Omission of temporary diversion in restorative proctocolectomy--is it safe? *Dis Colon Rectum* 1993; **36**: 1007-1014
- 53 **Remzi FH**, Fazio VW, Gorgun E, Ooi BS, Hammel J, Preen M, Church JM, Madbouly K, Lavery IC. The outcome after restorative proctocolectomy with or without defunctioning ileostomy. *Dis Colon Rectum* 2006; **49**: 470-477
- 54 **Williamson ME**, Lewis WG, Sagar PM, Holdsworth PJ, Johnston D. One-stage restorative proctocolectomy without temporary ileostomy for ulcerative colitis: a note of caution. *Dis Colon Rectum* 1997; **40**: 1019-1022
- 55 **Wexner SD**, Johansen OB, Nogueras JJ, Jagelman DG. Laparoscopic total abdominal colectomy. A prospective trial. *Dis Colon Rectum* 1992; **35**: 651-655
- 56 **Schmitt SL**, Cohen SM, Wexner SD, Nogueras JJ, Jagelman DG. Does laparoscopic-assisted ileal pouch anal anastomosis reduce the length of hospitalization? *Int J Colorectal Dis* 1994; **9**: 134-137
- 57 **Wexner SD**, Cera SM. Laparoscopic surgery for ulcerative colitis. *Surg Clin North Am* 2005; **85**: 35-47, viii
- 58 **Marcello PW**, Milsom JW, Wong SK, Hammerhofer KA, Goormastic M, Church JM, Fazio VW. Laparoscopic restorative proctocolectomy: case-matched comparative study with open restorative proctocolectomy. *Dis Colon Rectum* 2000; **43**: 604-608
- 59 **Hashimoto A**, Funayama Y, Naito H, Fukushima K, Shibata C, Naitoh T, Shibuya K, Koyama K, Takahashi K, Ogawa H, Satoh S, Ueno T, Kitayama T, Matsuno S, Sasaki I. Laparoscope-assisted versus conventional restorative proctocolectomy with rectal mucosectomy. *Surg Today* 2001; **31**: 210-214
- 60 **Dunker MS**, Bemelman WA, Slors JF, van Duijvendijk P, Gouma DJ. Functional outcome, quality of life, body image, and cosmesis in patients after laparoscopic-assisted and conventional restorative proctocolectomy: a comparative study. *Dis Colon Rectum* 2001; **44**: 1800-1807
- 61 **Milsom JW**, Hammerhofer KA, Bohm B, Marcello P, Elson P, Fazio VW. Prospective, randomized trial comparing laparoscopic vs. conventional surgery for refractory ileocolic Crohn's disease. *Dis Colon Rectum* 2001; **44**: 1-8; discussion 8-9
- 62 **Gardiner KR**, Dasari BV. Operative management of small bowel Crohn's disease. *Surg Clin North Am* 2007; **87**: 587-610
- 63 **Michelassi F**, Balestracci T, Chappell R, Block GE. Primary and recurrent Crohn's disease. Experience with 1379 patients. *Ann Surg* 1991; **214**: 230-238; discussion 238-240
- 64 **Hurst RD**, Molinari M, Chung TP, Rubin M, Michelassi F. Prospective study of the features, indications, and surgical treatment in 513 consecutive patients affected by Crohn's disease. *Surgery* 1997; **122**: 661-667; discussion 667-668

- 65 **Bernstein CN**, Blanchard JF, Rawsthorne P, Yu N. The prevalence of extraintestinal diseases in inflammatory bowel disease: a population-based study. *Am J Gastroenterol* 2001; **96**: 1116-1122
- 66 **Prantera C**. Indications for surgery in Crohn's disease. *Am J Gastroenterol* 1990; **85**: 900-901
- 67 **Michelassi F**, Stella M, Balestracci T, Giuliani F, Marogna P, Block GE. Incidence, diagnosis, and treatment of enteric and colorectal fistulae in patients with Crohn's disease. *Ann Surg* 1993; **218**: 660-666
- 68 **Broe PJ**, Bayless TM, Cameron JL. Crohn's disease: are enteroenteral fistulas an indication for surgery? *Surgery* 1982; **91**: 249-253
- 69 **Fazio VW**, Wilk P, Turnbull RB Jr, Jagelman DG. The dilemma of Crohn's disease: ileosigmoidal fistula complicating Crohn's disease. *Dis Colon Rectum* 1977; **20**: 381-386
- 70 **Present DH**, Rutgeerts P, Targan S, Hanauer SB, Mayer L, van Hogezaand RA, Podolsky DK, Sands BE, Braakman T, DeWoody KL, Schaible TF, van Deventer SJ. Infliximab for the treatment of fistulas in patients with Crohn's disease. *N Engl J Med* 1999; **340**: 1398-1405
- 71 **Sands BE**, Anderson FH, Bernstein CN, Chey WY, Feagan BG, Fedorak RN, Kamm MA, Korzenik JR, Lashner BA, Onken JE, Rachmilewitz D, Rutgeerts P, Wild G, Wolf DC, Marsters PA, Travers SB, Blank MA, van Deventer SJ. Infliximab maintenance therapy for fistulizing Crohn's disease. *N Engl J Med* 2004; **350**: 876-885
- 72 **Ribeiro MB**, Greenstein AJ, Yamazaki Y, Aufses AH Jr. Intra-abdominal abscess in regional enteritis. *Ann Surg* 1991; **213**: 32-36
- 73 **Garcia JC**, Persky SE, Bonis PA, Topazian M. Abscesses in Crohn's disease: outcome of medical versus surgical treatment. *J Clin Gastroenterol* 2001; **32**: 409-412
- 74 **Gutierrez A**, Lee H, Sands BE. Outcome of surgical versus percutaneous drainage of abdominal and pelvic abscesses in Crohn's disease. *Am J Gastroenterol* 2006; **101**: 2283-2289
- 75 **Greenstein AJ**, Sachar DB, Mann D, Lachman P, Heimann T, Aufses AH Jr. Spontaneous free perforation and perforated abscess in 30 patients with Crohn's disease. *Ann Surg* 1987; **205**: 72-76
- 76 **Ciocco WC**, Reilly JC, Rusin LC. Life-threatening hemorrhage and exsanguination from Crohn's disease. Report of four cases. *Dis Colon Rectum* 1995; **38**: 85-95
- 77 **Kronberger IE**, Graziadei IW, Vogel W. Small bowel adenocarcinoma in Crohn's disease: a case report and review of literature. *World J Gastroenterol* 2006; **12**: 1317-1320
- 78 **Williams JG**, Wong WD, Rothenberger DA, Goldberg SM. Recurrence of Crohn's disease after resection. *Br J Surg* 1991; **78**: 10-19
- 79 **Glehen O**, Lifante JC, Vignal J, Francois Y, Gilly FN, Flourie B, Descos L, Chung RJ, Mithieux F. Small bowel length in Crohn's disease. *Int J Colorectal Dis* 2003; **18**: 423-427
- 80 **Krause U**, Ejerblad S, Bergman L. Crohn's disease. A long-term study of the clinical course in 186 patients. *Scand J Gastroenterol* 1985; **20**: 516-524
- 81 **Softley A**, Myren J, Clamp SE, Bouchier IA, Watkinson G, de Dombal FT. Factors affecting recurrence after surgery for Crohn's disease. *Scand J Gastroenterol Suppl* 1988; **144**: 31-34
- 82 **Fazio VW**, Marchetti F, Church M, Goldblum JR, Lavery C, Hull TL, Milsom JW, Strong SA, Oakley JR, Secic M. Effect of resection margins on the recurrence of Crohn's disease in the small bowel. A randomized controlled trial. *Ann Surg* 1996; **224**: 563-571; discussion 571-573
- 83 **Gasche C**, Scholmerich J, Brynskov J, D'Haens G, Hanauer SB, Irvine EJ, Jewell DP, Rachmilewitz D, Sachar DB, Sandborn WJ, Sutherland LR. A simple classification of Crohn's disease: report of the Working Party for the World Congresses of Gastroenterology, Vienna 1998. *Inflamm Bowel Dis* 2000; **6**: 8-15
- 84 **Munoz-Juarez M**, Yamamoto T, Wolff BG, Keighley MR. Wide-lumen stapled anastomosis vs. conventional end-to-end anastomosis in the treatment of Crohn's disease. *Dis Colon Rectum* 2001; **44**: 20-25; discussion 25-26
- 85 **Tersigni R**, Alessandrini L, Barreca M, Piovanello P, Prantera C. Does stapled functional end-to-end anastomosis affect recurrence of Crohn's disease after ileocolonic resection? *Hepatogastroenterology* 2003; **50**: 1422-1425
- 86 **Resegotti A**, Astegiano M, Farina EC, Ciccone G, Avagnina G, Giustetto A, Campa D, Fronda GR. Side-to-side stapled anastomosis strongly reduces anastomotic leak rates in Crohn's disease surgery. *Dis Colon Rectum* 2005; **48**: 464-468
- 87 **Bernell O**, Lapidus A, Hellers G. Risk factors for surgery and postoperative recurrence in Crohn's disease. *Ann Surg* 2000; **231**: 38-45
- 88 **Yamamoto T**. Factors affecting recurrence after surgery for Crohn's disease. *World J Gastroenterol* 2005; **11**: 3971-3979
- 89 **Kurer MA**, Stamou KM, Wilson TR, Bradford IM, Leveson SH. Early symptomatic recurrence after intestinal resection in Crohn's disease is unpredictable. *Colorectal Dis* 2007; **9**: 567-571
- 90 **Ozuner G**, Fazio VW, Lavery IC, Church JM, Hull TL. How safe is strictureplasty in the management of Crohn's disease? *Am J Surg* 1996; **171**: 57-60; discussion 60-61
- 91 **Tichansky D**, Cagir B, Yoo E, Marcus SM, Fry RD. Strictureplasty for Crohn's disease: meta-analysis. *Dis Colon Rectum* 2000; **43**: 911-919
- 92 **Fearnhead NS**, Chowdhury R, Box B, George BD, Jewell DP, Mortensen NJ. Long-term follow-up of strictureplasty for Crohn's disease. *Br J Surg* 2006; **93**: 475-482
- 93 **Barwood N**, Platell C. Case report: adenocarcinoma arising in a Crohn's stricture of the jejunum. *J Gastroenterol Hepatol* 1999; **14**: 1132-1134
- 94 **Yamazaki Y**, Ribeiro MB, Sachar DB, Aufses AH Jr, Greenstein AJ. Malignant colorectal strictures in Crohn's disease. *Am J Gastroenterol* 1991; **86**: 882-885
- 95 **Singh VV**, Draganov P, Valentine J. Efficacy and safety of endoscopic balloon dilation of symptomatic upper and lower gastrointestinal Crohn's disease strictures. *J Clin Gastroenterol* 2005; **39**: 284-290
- 96 **Nomura E**, Takagi S, Kikuchi T, Negoro K, Takahashi S, Kinouchi Y, Hiwatashi N, Shimosegawa T. Efficacy and safety of endoscopic balloon dilation for Crohn's strictures. *Dis Colon Rectum* 2006; **49**: S59-S67
- 97 **Jess T**, Loftus EV Jr, Velayos FS, Harmsen WS, Zinsmeister AR, Smyrk TC, Schleck CD, Tremaine WJ, Melton LJ 3rd, Munkholm P, Sandborn WJ. Risk of intestinal cancer in inflammatory bowel disease: a population-based study from olmsted county, Minnesota. *Gastroenterology* 2006; **130**: 1039-1046
- 98 **Maykel JA**, Hagerman G, Mellgren AF, Li SY, Alavi K, Baxter NN, Madoff RD. Crohn's colitis: the incidence of dysplasia and adenocarcinoma in surgical patients. *Dis Colon Rectum* 2006; **49**: 950-957
- 99 **Itzkowitz SH**, Harpaz N. Diagnosis and management of dysplasia in patients with inflammatory bowel diseases. *Gastroenterology* 2004; **126**: 1634-1648
- 100 **Collins PD**, Mpofu C, Watson AJ, Rhodes JM. Strategies for detecting colon cancer and/or dysplasia in patients with inflammatory bowel disease. *Cochrane Database Syst Rev* 2006; CD000279
- 101 **Poritz LS**, Gagliano GA, McLeod RS, MacRae H, Cohen Z. Surgical management of entero and colocutaneous fistulae in Crohn's disease: 17 year's experience. *Int J Colorectal Dis* 2004; **19**: 481-485; discussion 486
- 102 **Poritz LS**. How should complex perianal Crohn's disease be treated in the Remicade era. *J Gastrointest Surg* 2006; **10**: 633-634
- 103 **Tekkis PP**, Purkayastha S, Lanitis S, Athanasiou T, Heriot AG, Orchard TR, Nicholls RJ, Darzi AW. A comparison of segmental vs subtotal/total colectomy for colonic Crohn's disease: a meta-analysis. *Colorectal Dis* 2006; **8**: 82-90
- 104 **Andersson P**, Olaison G, Hallbook O, Sjodahl R. Segmental resection or subtotal colectomy in Crohn's colitis? *Dis Colon Rectum* 2002; **45**: 47-53
- 105 **Fichera A**, McCormack R, Rubin MA, Hurst RD, Michelassi F. Long-term outcome of surgically treated Crohn's colitis: a prospective study. *Dis Colon Rectum* 2005; **48**: 963-969

- 106 **Brown CJ**, Maclean AR, Cohen Z, Macrae HM, O'Connor BI, McLeod RS. Crohn's disease and indeterminate colitis and the ileal pouch-anal anastomosis: outcomes and patterns of failure. *Dis Colon Rectum* 2005; **48**: 1542-1549
- 107 **Braveman JM**, Schoetz DJ Jr, Marcello PW, Roberts PL, Collier JA, Murray JJ, Rusin LC. The fate of the ileal pouch in patients developing Crohn's disease. *Dis Colon Rectum* 2004; **47**: 1613-1619
- 108 **Hartley JE**, Fazio VW, Remzi FH, Lavery IC, Church JM, Strong SA, Hull TL, Senagore AJ, Delaney CP. Analysis of the outcome of ileal pouch-anal anastomosis in patients with Crohn's disease. *Dis Colon Rectum* 2004; **47**: 1808-1815
- 109 **Reese GE**, Lovegrove RE, Tilney HS, Yamamoto T, Heriot AG, Fazio VW, Tekkis PP. The effect of Crohn's disease on outcomes after restorative proctocolectomy. *Dis Colon Rectum* 2007; **50**: 239-250
- 110 **Solomon MJ**. Fistulae and abscesses in symptomatic perianal Crohn's disease. *Int J Colorectal Dis* 1996; **11**: 222-226
- 111 **Bernard D**, Morgan S, Tasse D. Selective surgical management of Crohn's disease of the anus. *Can J Surg* 1986; **29**: 318-321
- 112 **Fleshner PR**, Schoetz DJ Jr, Roberts PL, Murray JJ, Collier JA, Veidenheimer MC. Anal fissure in Crohn's disease: a plea for aggressive management. *Dis Colon Rectum* 1995; **38**: 1137-1143
- 113 **Wolkomir AF**, Luchtefeld MA. Surgery for symptomatic hemorrhoids and anal fissures in Crohn's disease. *Dis Colon Rectum* 1993; **36**: 545-547
- 114 **Joo JS**, Weiss EG, Nogueras JJ, Wexner SD. Endorectal advancement flap in perianal Crohn's disease. *Am Surg* 1998; **64**: 147-150
- 115 **Yamamoto T**, Allan RN, Keighley MR. Effect of fecal diversion alone on perianal Crohn's disease. *World J Surg* 2000; **24**: 1258-1262; discussion 1262-1263
- 116 **Galandiuk S**, Kimberling J, Al-Mishlab TG, Stromberg AJ. Perianal Crohn disease: predictors of need for permanent diversion. *Ann Surg* 2005; **241**: 796-801; discussion 801-802
- 117 **Tan JJ**, Tjandra JJ. Laparoscopic surgery for Crohn's disease: a meta-analysis. *Dis Colon Rectum* 2007; **50**: 576-585
- 118 **Maartense S**, Dunker MS, Slors JF, Cuesta MA, Pierik EG, Gouma DJ, Hommes DW, Sprangers MA, Bemelman WA. Laparoscopic-assisted versus open ileocolic resection for Crohn's disease: a randomized trial. *Ann Surg* 2006; **243**: 143-149; discussion 150-153

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# Circulating lymphangiogenic growth factors in gastrointestinal solid tumors, could they be of any clinical significance?

Theodore D Tsirlis, George Papastratis, Kyriaki Masselou, Christos Tsigris, Antonis Papachristodoulou, Alkiviadis Kostakis, Nikolaos I Nikiteas

Theodore D Tsirlis, George Papastratis, Third Department of Surgery, General Hospital of Athens "G.Gennimatas", Athens 115 26, Greece

Kyriaki Masselou, Department of Immunology, National Tissue Typing Center, General Hospital of Athens "G.Gennimatas", Athens 115 26, Greece

Christos Tsigris, Antonis Papachristodoulou, Alkiviadis Kostakis, Nikolaos I Nikiteas, Second Department of Surgery, University of Athens Medical School, Laikon General Hospital, Athens 115 27, Greece

**Author contributions:** Tsirlis TD, Nikiteas NI contributed equally to conception and design of the review; Tsirlis TD wrote and revised the review; Papastratis G, Masselou K, Tsigris C, Papachristodoulou A and Kostakis A contributed equally to supportive work and supervision.

**Correspondence to:** Theodore D Tsirlis, Third Department of Surgery, General Hospital of Athens "G.Gennimatas", Psaron 20 str. Agia Paraskevi, Athens 153 43, Greece. [theotsir@med.uoa.gr](mailto:theotsir@med.uoa.gr)  
Telephone: +30-210-6016351 Fax: +30-210-6867191

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that quantification of VEGF-C and VEGF-D in blood samples could serve as lymph node metastasis predictive biomarkers and contribute to preoperative staging of gastrointestinal malignancies.

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**Peer reviewer:** Finlay A Macrae, MD, Professor, Royal Melbourne Hospital, Po Box 2010, Victoria 3050, Australia

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## Abstract

Metastasis is the principal cause of cancer mortality, with the lymphatic system being the first route of tumor dissemination. The glycoproteins VEGF-C and VEGF-D are members of the vascular endothelial growth factor (VEGF) family, whose role has been recently recognized as lymphatic system regulators during embryogenesis and in pathological processes such as inflammation, lymphatic system disorders and malignant tumor metastasis. They are ligands for the VEGFR-3 receptor on the membrane of the lymphatic endothelial cell, resulting in dilatation of existing lymphatic vessels as well as in vegetation of new ones (lymphangiogenesis). Their determination is feasible in the circulating blood by immunoabsorption and in the tissue specimen by immunohistochemistry and reverse transcription polymerase chain reaction (RT-PCR). Experimental and clinicopathological studies have linked the VEGF-C, VEGF-D/VEGFR3 axis to lymphatic spread as well as to the clinical outcome in several human solid tumors. The majority of these data are derived from surgical specimens and malignant cell series, rendering their clinical application questionable, due to subjectivity factors and post-treatment quantification. In an effort to overcome these drawbacks, an alternative method of immunodetection of the circulating levels of these molecules has been used in studies on gastric, esophageal and colorectal cancer. Their results denote

## INTRODUCTION

Lymph node metastasis (LNM) is a major prognostic factor for most human solid epithelial tumors.

Although the phenomenon of lymphatic spread of tumors is well recognized for over a century, many aspects of cancer cells entrance, survival and proliferation in the lymphatic system remain unclear<sup>[1,2]</sup>. To date, the experimental findings regarding active lymphangiogenesis in human solid tumors are contradictory<sup>[3,4]</sup>.

The molecular and functional mechanisms of lymphatic system regulation and cancerous involvement have only recently been recognized, mainly due to the discovery of lymphatic endothelial cells (LEC) specific markers (LYVE-1, Prox-1, podoplanin, VEGFR3) in the past decade<sup>[5,6]</sup>.

## THE VEGF-C, D/VEGFR-3 SYSTEM

VEGFR-3 (fms-like tyrosine kinase 4, Flt4) is one of the first LECs surface molecules to be identified. It is a member of the VEGFR family, also including VEGFR-1 (Flt-1), VEGFR-2 (KDR), and which belongs to the platelet derived growth factor receptor sub-family of receptor tyrosine kinases<sup>[7]</sup>. VEGFR-3 is present on all endothelia during



development, but in the adult its expression is restricted to LECs and certain fenestrated blood vascular ECs<sup>[8,9]</sup>.

The importance of VEGFR-3 for the development of the lymphatic vasculature has been shown recently, where early onset primary lymphedema was linked to the VEGFR3 locus in distal chromosome 5q<sup>[10,11]</sup>. It also protects LECs from serum deprivation-induced apoptosis, induces their growth and migration, while one study on a corneal model showed that it could play a role in adaptive immunity<sup>[12,13]</sup>. Nevertheless, this LEC specificity seems to be lacking in cancer cell types, an observation which contributes to the difficulty of defining molecular regulation of LNM<sup>[14]</sup>.

The vascular endothelial growth factor (VEGF) family of glycoproteins comprises the most crucial group of neovascularization regulators in development and disease (Table 1). Currently, it consists of 5 cytokines in mammals, VEGF, PlGF (platelet induced growth factor), VEGF-B, VEGF-C and VEGF-D, and in addition, *parapoxvirus* genome-encoded VEGF (viral VEGF, also denoted as VEGF-E) and snake venom-derived VEGF (also referred as VEGF-F)<sup>[17-19]</sup>.

VEGF-C and VEGF-D subtypes have been identified as the ligands for the lymphatic endothelial receptor VEGFR3, as well as for the blood vessels endothelial receptor VEGFR2, while VEGF-C also binds the LEC surface molecule NRP-2<sup>[20-23]</sup>.

Among the other family members, they share a central VEGF homology domain, but they differ because of the distinct presence of long N- and C-terminal propeptides. VEGF-C and VEGF-D are secreted as precursor proteins, which are cleaved to their VEGFR-specific active forms through a two-step proteolytic procedure<sup>[24-27]</sup>. The extent of the proteolytic process defines its receptor affinity and presumably its biological activity, although this connection is only partially understood. Both VEGF-C and VEGF-D are able to induce proliferation and migration of lymphatic endothelial cells *in vitro*<sup>[23]</sup>.

The correlation of VEGF-C, D/VEGFR3 axis to the lymphatic spread of tumors is documented in several experimental models and clinicopathological studies in a variety of human malignancies.

The findings derived from malignant cell line models provide the most “direct” evidence for the implication of lymphangiogenic growth factors in tumor lymphatic spread<sup>[28-32]</sup>. However, the observed correlation does not explain the underlying mechanisms, nor does it clarify the role of active tumor induced lymphangiogenesis in cancer metastasis. Nevertheless, and more importantly, the inhibition of the ligand-receptor axis raised interest in anti-lymphangiogenic targeting research, a potentially promising novel field of cancer treatment<sup>[33-35]</sup>.

Taking into account the rationale of VEGF-C and VEGF-D involvement in lymphatic system regulation, researchers throughout the world studied the expression of these growth factors in human tumors and their possible connection to metastatic potential<sup>[36,37]</sup>. The methodology used for “quantitative” determination was either immunohistochemistry (IHC), or RT-PCR to detect mRNA and subsequently the level of gene expression. These studies include a wide range of solid tumors (gastrointestinal, breast, genitourinary, melanoma, thyroid, head and neck),

**Table 1** Mammalian vascular endothelial growth factor family of ligands

	Receptor	Chromosomal location	Angiogenesis	Lymphangiogenesis
VEGF	VEGFR1, VEGFR2, NRP1	6p23.1	+	Conflicting data <sup>1</sup>
VEGF-B	VEGFR1, NRP1	11q13	Conflicting data	-
PlGF	VEGFR1 NRP1, NRP2	14q24	Modest	Not determined
VEGF-C	VEGFR2	4q34	Modest	+
VEGF-D	VEGFR2, VEGFR3	Xp22.31	Modest	+

<sup>1</sup>Indirect lymphangiogenic effect, by recruiting VEGF-C and VEGF-D producing macrophages<sup>[15]</sup>. Lymphangiogenesis *via* alternative, VEGFR3-independent pathway<sup>[16]</sup>.

in an attempt to relate VEGF-C and VEGF-D expression to clinicopathological parameters (lymph node involvement, lymphatic and vascular invasion, clinical outcome).

The majority of such studies confirmed a positive correlation between growth factor expression and adverse oncological features. Yet, the results are not always consistent<sup>[38-41]</sup>.

Conflicting results could be attributed to methodological considerations<sup>[42,43]</sup>: (1) Immunohistochemical quantification is a somehow subjective observer-dependant modality. Terms like “overexpression” are not always well defined, as they are based on variable scoring systems; (2) RT-PCR does not discriminate the location of mRNA expression among cancer cells, adjacent normal epithelium and stromal cells in a tissue specimen, nor does it necessarily reflect the actual protein level<sup>[44]</sup>.

Van der Auwera *et al*<sup>[45]</sup> proposed a composite method for lymphangiogenesis quantification in solid tumors, in order to establish standardization of immunohistochemical assessment.

## CIRCULATING VEGF-C AND VEGF-D IN HUMAN SOLID TUMORS

An alternative method of VEGF-C and VEGF-D quantification is indirect enzyme-linked immunoadsorption assay (ELISA), which measures the protein levels in peripheral circulation samples. This approach has been applied in selected studies during the last 5 years (Table 2).

The quantification of circulating cytokines has the advantage of being more objective approach, which lacks the drawback of interobserver variability. Moreover, as a preoperatively practicable modality, it exhibits a potential application as a readily available LNM marker and subsequently surgical decision making tool, particularly in cases such as: (1) Early malignant lesions, which bear a small, yet substantial risk of lymphatic dissemination and which, otherwise, could be treated with minimally invasive techniques; (2) Cancers which necessitate accurate preoperative staging, in order to employ stage-specific neoadjuvant therapy; (3) Cancers whose treatment approach relies on the presence or extent of lymph nodes metastasis.

**Table 2** Studies on circulating VEGF-C/D in human solid malignancies (clinicopathological association)

Tumor	Marker	Sample	Cases (n)	LNM	Prognostic impact	Ref.
Gastric cancer	VEGF-C	Serum	80	$P = 0.001$	$P = 0.001$	67
Esophageal cancer	VEGF-C	Serum	70	$P = 0.022$	ND	102
Esophageal cancer	VEGF-C	Serum	73	(+) <sup>1</sup>	ND	103
Colorectal cancer	VEGF-D	Plasma	59	(-)	ND	38
Colorectal cancer	VEGF-C	Plasma	41	(+) <sup>2</sup>	ND	144
Colorectal cancer	VEGF-C	Plasma	120	(-)	ND	145
	VEGF-D			(-)		
Colorectal cancer	VEGF-C	Serum	66	(+)	ND	146
Breast cancer	VEGF-D	Plasma	51	(-)	ND	46
Breast cancer	VEGF-C	Plasma	122	(-)	(-)	47
Breast cancer <sup>3</sup>	VEGF-D	Plasma	142			48
Nsc <sup>4</sup> lung cancer	VEGF-C	Serum	92	$P = 0.0260$	ND	49
Nsc lung cancer	VEGF-C	Serum	78	$P = 0.0004$	ND	50
Nsc lung cancer	VEGF-C	Serum	116	$P = 0.0007$	ND	51
Cervical cancer	VEGF-C	Serum	78	$P = 0.0001$	$P = 0.0112$	52
Cervical cancer	VEGF-C	Serum	205	(+) <sup>2</sup>	(+) <sup>2</sup>	53
Prostate cancer	VEGF-D	Plasma	30	$P = 0.0043$	ND	54
HNSCC <sup>5</sup>	VEGF-C	Plasma	46	(-)	(-)	55

ND: Not determined; <sup>1</sup>Indirect result; <sup>2</sup>None statistically significant; <sup>3</sup>Post-treatment study; <sup>4</sup>Non small cell; <sup>5</sup>Head and Neck Squamous Cell Carcinoma.

Circulating lymphangiogenic growth factors have been investigated in malignant tumors whose lymph node status detection is crucial in terms of treatment planning, including cancers of the gastrointestinal tract.

### Gastric cancer

Gastric cancer remains a leading cause of cancer mortality worldwide, despite its declining incidence in the West in the last decades, and lymph node metastasis is the most powerful prognostic factor in R0 resected cases.

Clinicopathological studies mainly from Japan, where gastric cancer is the most common malignancy, have correlated mRNA and immunohistochemical expression of VEGF-C and VEGF-D in gastric tumour cells with lymphatic invasion and lymph node metastasis<sup>[56-60]</sup>. Their quantitative expression has been reported as a prognostic factor<sup>[56,59]</sup>, while the experimental blocking of the VEGFR-3 signalling pathway is under investigation<sup>[61]</sup>. Nikiteas *et al.*<sup>[62]</sup> showed that VEGF is also implicated in lymphatic spread of gastric cancers, a finding reproduced in a Japanese population<sup>[63]</sup>.

Studies on early gastric adenocarcinoma (EGC) have been carried out as well. Kabashima *et al.*<sup>[64]</sup> found using immunohistochemistry that the incidence of positive expression of VEGF-C in lymphatic invasion-positive EGC (36%) was significantly higher than that in lymphatic invasion-negative EGC (14%). The incidence of positive expression of VEGF-C in nodes (+) or venous invasion-positive EGC tended to be higher than that in nodes (-) or venous invasion-negative EGC. Ishikawa *et al.*<sup>[65]</sup> studied the expression of VEGF-C and VEGF-D in resection specimens related to tumors differentiation, concluding that in EGC of histologically undifferentiated type with negative expression of VEGF-C and -D, limited surgery might be safely applied because the possibility of nodal metastasis is very low. Onogawa *et al.*<sup>[66]</sup> investigated whether expres-

sion of VEGF-C and/or VEGF-D correlates with clinicopathological features of submucosally invasive gastric carcinoma. VEGF-C immunoreactivity was associated with histological type, lymphatic invasion, lymph node metastasis, and microvessel density, while no association was identified between VEGF-D immunoreactivity and clinicopathological variables. Those studies suggest that the detection of VEGF-C and VEGF-D could play a role as an additional element of EGC local excision criteria.

One study on circulating VEGF-C in gastric cancer has been reported so far. Wang *et al.*<sup>[67]</sup> investigated whether serum VEGF-C and immunohistochemically determined VEGF-C expression and lymphatic vessel density (LVD) in tumor tissues are related to lymph node metastasis and prognosis in gastric cancer. LVD was determined based on brown staining of endothelial cells with podoplanin under a 200-fold light microscopic field.

The sVEGF-C level was significantly ( $P = 0.000$ ) higher in patients with gastric cancer ( $595.9 \pm 201.0$  ng/L) than in healthy donors ( $360.0 \pm 97.4$  ng/L). With a cut-off value for sVEGF-C of 367.5 ng/L, the sensitivity and specificity for diagnosis of gastric cancer patients was 85% and 80%, respectively ( $P = 0.000$ ). VEGF-C positive expression was significantly ( $P = 0.001$ ) higher in gastric cancer tissue (50/80) than in normal gastric tissue (4/20). There was significantly ( $P = 0.000$ ) more LVD in the experimental group ( $10.7 \pm 3.1/200$  HP) than in control subjects ( $4.9 \pm 1.3/200$  HP). The sVEGF-C level was significantly ( $P = 0.000$ ) higher in VEGF-C positive patients ( $675.4 \pm 153.9$  ng/L) than in negative patients ( $463.5 \pm 200.4$  ng/L). There was a positive correlation between sVEGF-C and LVD ( $r = 0.728$ ,  $P = 0.000$ ). LVD in VEGF-C positive and negative groups was  $12.2 \pm 2.8/200$  HP and  $8.3 \pm 2.0/200$  HP, respectively ( $P = 0.000$ ).

With respect to clinicopathological correlations, sVEGF-C was significantly ( $P = 0.001$ ) higher in differentiation degree G3 group, LNM (+) group, M (+) group and pTNM III-IV group. With a 3 year follow up, the mean survival of patients with high ( $> 595.9$  ng/L) sVEGF-C and low ( $< 595.9$  ng/L) sVEGF-C was  $29.1 \pm 13.3$  mo and  $44.0 \pm 4.6$  mo, respectively ( $P = 0.001$ ).

**Clinical relevance:** It is well known that a notable discrepancy exists between Japan and USA-Europe regarding gastric cancer, with respect to staging, surgical management and outcome<sup>[68-70]</sup>. The extent of lymph node dissection (D1 vs D2) is considered a major factor of curative outcome according to the Japanese. The reluctance of the Western surgical community to uniformly adopt this approach is supported with evidence derived by two large prospective randomized controlled studies conducted in Europe in the 1990s<sup>[71,72]</sup>. Both trials concluded that D2 dissection is followed by significantly higher morbidity and mortality, without an overall proven survival benefit. However, the same investigators revised their long term results<sup>[73-75]</sup> and acknowledged that the complications should be largely attributed to modifiable technical aspects, such as splenectomy and distal pancreatectomy and to limited experience, and most importantly, there is evidence that D2 dissection could be beneficial for a subgroup of patients, basically

those with stage II and IIIa according to TNM. Similar conclusions are derived from non-randomized and retrospective studies from selected centers<sup>[76-80]</sup>.

Taking into account the suggestion of stage-specific benefit, the next challenge would be to identify the candidates for extended lymph nodes dissection. Several modalities for preoperative staging have been studied; but, the results on nodal detection are insufficient to dictate an individualized surgical approach.

Imaging techniques are the most widely used, relying on morphologic criteria<sup>[81-85]</sup>. Abdominal CT scan is the most popular method, with poor specificity on detecting N status, as the sole factor is nodal size, and without capability for accurate number detection. Endoscopic ultrasound is considered more valuable for evaluating primary tumors; yet it is not proven superior to CT regarding nodal involvement evaluation. Positron emission tomography scan is helpful in detecting occult distant metastasis; but, has no role in regional nodal staging. Besides, modern imaging techniques bear a considerable cost and entail operator-dependant variables.

Invasive staging methods, such as laparoscopy, peritoneal cytology and intraoperative ultrasonography are certainly not "preoperative" and studies on sentinel lymph node biopsy using radiographic mapping seem promising in gastric cancer staging; yet, the latter necessitates trained personnel and special equipment and is not widely applicable<sup>[86,87]</sup>. Maruyama and co-workers developed a computerized database program to calculate the probability of individual lymph node station involvement, a model with limited clinical impact<sup>[88,89]</sup>.

The connection of VEGF-C and VEGF-D to lymphatic spread, as previously shown in clinicopathological studies, provides the rationale that preoperative quantification of these cytokines could yield additional information regarding lymph nodes involvement in gastric cancer patients.

Wang *et al*<sup>[67]</sup> study converges as to that point. This study indicates that preoperative serum VEGF-C level might be a useful biomarker for the presence of LNM in patients with gastric cancer and a prognostic parameter to identify patients with poor outcome. Nonetheless, the researchers provide no evidence with respect to the extent of lymph node dissection they employed, and so no correlation can be made regarding prognostic significance of VEGF-C and extent of surgery.

### Esophageal cancer

Cancer of the esophagus is a human malignancy with unfavorable prognosis, regardless histological type and despite the induction of multimodality approach in the treatment of this formidable disease. The prevalence of adenocarcinoma of the distal esophagus in particular, is reported to increase in Western populations<sup>[90]</sup> and due to its location, bilateral lymph node metastasis to thoracic and abdominal cavity occurs with adverse prognostic aftermath.

Clinicopathological and experimental studies on VEGF-C/D expression in squamous cell (escc)<sup>[41,91-94]</sup> as well in adenocarcinoma (ac)<sup>[94-96]</sup> specimens have been published. Studies on escc resulted on a relatively consistent correlation of growth factors expression to tumor

progression and lymphatic spread, while evidence for their role in ac is contradictory. Interestingly, all studies on adenocarcinoma come from the West.

It is worth noting that there is evidence which correlate lymphangiogenic growth factors to malignant potential of early or precancerous lesions. Auvinen *et al*<sup>[97]</sup> showed immunohistochemically that VEGF-C expression increases in Barrett's epithelium as it progresses through dysplasia to adenocarcinoma and that VEGFR3 parallels this increase. Additionally, tumor-induced lymphatics were detected which could provide the route for systemic cancer dissemination. Ishikawa *et al*<sup>[98]</sup> examined the expression of VEGF-C and -D in 26 esophageal carcinoma cases and 11 dysplasia cases using IHC and found that active production of VEGF-C and -D was observed, not only in esophageal carcinomas, but also in some dysplastic lesions and in none of the normal mucosa specimens, raising the possibility that VEGF-C and -D might play positive roles in the early stage of esophageal carcinogenesis. Matsumoto *et al*<sup>[99]</sup> examined VEGF-C expression and tumor microvessel density of the primary tumors in escc and analyzed relationships between VEGF-C expression and clinicopathological findings, including lymph node micrometastasis (LMM), in 87 submucosal esccs. The findings indicate that in escc with submucosal invasion, VEGF-C overexpression of the primary tumor is a strong high risk factor for lymph node metastasis, including LMM.

Two studies on serum VEGF-C as biological marker in escc have been reported, both from the same department<sup>[101,102]</sup>. Krzystek-Korpacka *et al*<sup>[100]</sup> examined serum concentrations of VEGF-C in 70 patients with escc and 47 healthy individuals. However, only 23 patients were subjected to surgery, due to advanced disease of the remainder, which were staged using endoscopy, imaging modalities and laparoscopy. Median serum VEGF-C level (sVEGF-C) in escc patients was significantly elevated in comparison to controls (17.40 ng/mL *vs* 10.57 ng/mL,  $P < 0.001$ ). Serum VEGF-C was significantly elevated when metastatic lymph nodes were present, as median sVEGF-C was 21.78 ng/mL in N0 *vs* 15.77 ng/mL in N1 cases ( $P = 0.022$ ). The authors also examined the dependence of sVEGF-C and combined TN status of the examined cancers and found no stage-specific correlation, presumably due to a small sample of patients. The optimal cut-off value for application of sVEGF-C as a marker of the disease presence was calculated 14.57 ng/mL (mean  $\pm$  SD), whereas 16.24 ng/mL (mean  $\pm$  1.5 SD) for detection of metastatic lymph nodes. The accuracy of sVEGF-C determination as a disease marker was 83.7% while 64.4% as a lymph node involvement marker. Moreover, in an effort to address the issue of tumor induced secretion, the authors correlated WBC and PLT count to TNM stage and concluded that WBCs parallel sVEGF-C levels, rather than contribute to their elevation.

The same team enrolled the former group of patients and controls in a study on circulating levels of midkine (sMK), a cytokine whose secretion found to be an escc marker and prognostic factor in Japanese populations<sup>[102,103]</sup>. Statistically higher sMK levels were found in cancer patients than in controls (1373 pg/mL *vs* 130 pg/mL) and in cases with lymph nodes metastasis (775 pg/mL in N0 *vs*

1893 pg/mL in N1). The utility of sMK as a LNM marker was calculated to have a 91.2% sensitivity and a 77.8% specificity. The best cut-off values calculated were 563 pg/mL for determination of the presence of disease and 937 pg/mL for evaluation of LNM. Correlations of sMK and TNM stage have been implied as well. Serum midkine levels correlated significantly with serum VEGF-C levels in N1 ( $P = 0.008$ ) and combined N + M ( $P = 0.001$ ) cases.

**Clinical relevance:** Surgical resection offers the only realistic chance for cure in patients with esophageal cancer and accurate preoperative staging is of outmost importance when surgery with curative intent is contemplated.

Esophagectomy procedures are associated with high morbidity and mortality, especially when performed in low volume centers<sup>[104,105]</sup>, and even if successful, they negatively impact quality of life over a considerable period of time<sup>[106]</sup>, so it is imperative to identify resectable cases among the patients. Lymph nodes involvement is a major determinant of resectability, as LNM beyond regional lymph nodes as defined by the American Joint Commission in Cancer<sup>[107]</sup> precludes surgical treatment, with a debatable exception of celiac axis involvement<sup>[108]</sup>.

Despite the importance of R0 resection, prognosis of esophageal cancer remains bleak, and a multimodality strategy has been introduced currently, in an effort to improve curative outcome. This approach includes the combination of surgery, chemotherapy and radiotherapy. Nevertheless, much controversy exists regarding the appropriate combination of these modalities and the determination of patient categories which will mostly benefit from multimodality treatment<sup>[109-112]</sup>. Although hard evidence is lacking, the trend is to treat patients with locally advanced esophageal cancer (stage III, T3-4, N1) with neoadjuvant chemoradiotherapy followed by surgery, a strategy which necessitates accurate preoperative staging.

Imaging techniques are again the mainstay of staging, including CT, EUS, EUS-FNA, FDG-PET and CT-PET, with variable sensitivity, specificity, and feasibility<sup>[113-117]</sup>. Moreover, the value of case volume has been reported to influence preoperative staging accuracy<sup>[118]</sup>. The fact that only a subgroup of patients within the same pathological stage benefit from neoadjuvant therapy raised the need to identify the cases with biological favourable tumors, so as to avoid unnecessary toxicity without concomitant survival benefit<sup>[109,119]</sup>. Imaging modalities in this setting are not sufficient, as they fall short of discriminating viable tumor from necrotic or scar tissue and to date there is no universally accepted morphological means of monitoring the response to neoadjuvant chemoradiotherapy<sup>[120-122]</sup>.

The most innovative alternative is the identification of genetic and molecular markers of response to neoadjuvant therapy<sup>[123-125]</sup>, including gene expression, genomic polymorphism, growth factors receptors, angiogenic factors, cell cycle regulators and apoptotic factors. Experimental studies provide promising data to incorporate such markers in multimodality and targeted treatment.

Krzystek-Korpaczka *et al.*<sup>[100]</sup> reported up-regulation of serum VEGF-C in esophageal squamous cell carcinoma, a finding which parallels VEGF-C expression in tissue specimens. They also correlated serum levels with the presence

of lymph node metastasis and concluded that sVEGF-C up-regulation did not arise from platelets or white blood cells. Their results show that serum VEGF-C levels can be considered a biomarker of esophageal squamous cell cancer and a predictive molecular marker of lymph nodes metastasis in particular. This remark indicates a potential utility of serum lymphangiogenic growth factors in escc as a tool for early detection and LNM evaluation.

### Colorectal cancer

LNM is a significant prognostic factor in colorectal cancer and a determinant of combined therapy regarding adjuvant as well as neoadjuvant treatment strategies.

Several clinicopathological studies on VEGF-C and VEGF-D tumoral expression have been reported, providing evidence that they correlate to LNM and prognosis<sup>[126-130]</sup>, although findings are not always consistent<sup>[138,131]</sup>. Furodoi *et al.*<sup>[131]</sup> detected VEGF-C expression at the deepest invasive site in 71 of 152 lesions (46.7%) and correlated it to histological grade, depth of invasion, lymph node metastasis, venous invasion, liver metastasis and Duke's stage. At the central portion and superficial part, there were no significant differences between VEGF-C expression and clinicopathological findings.

With respect to early lesions, Maeda *et al.*<sup>[132]</sup> examined 221 endoscopically biopsied specimens from patients with T1 colorectal carcinoma prior to operation using IHC and found that VEGF-C expression was more frequently observed in tumors with nodal metastasis than in those without metastasis. Moreover, a multivariate analysis indicated that VEGF-C expression is an independent predictor of lymph node metastasis in T1 colorectal carcinoma. Kojima *et al.*<sup>[133]</sup> investigated VEGF-C and VEGF expression at the invasive end of 65 T1 resected carcinomas and significantly correlated VEGF-C with the presence of LNM. Kazama *et al.*<sup>[134]</sup> examined VEGF-C and VEGF-D expression in submucosal colorectal cancers and concluded that VEGF-C overexpression correlated with lymphatic involvement ( $P = 0.01$ ) and lymph node metastasis ( $P = 0.02$ ), but VEGF-D overexpression did not correlate significantly.

Limited studies on circulating VEGF-C, D in colorectal cancer (CRC) yielded conflicting results.

George *et al.*<sup>[38]</sup> studied a sample of normal mucosa, adenomatous polyps and CRCs regarding IHC expression and RT-PCR mRNA expression of VEGF-C and VEGF-D and also plasma levels of VEGF-D. Plasma levels of VEGF-D were similar in normal controls, polyp patients, and CRC patients [median 494 (303-744) pg/mL, 416 (351-938) pg/mL, and 463 (291-745) pg/mL, respectively]. An interesting finding was an inverse balance of VEGF-C/VEGF-D mRNA expression in CRC samples, indicating that VEGF-D could act as a competitive antagonist to other family members.

Duff *et al.*<sup>[135]</sup> measured plasma VEGF-C in 41 CRC patients and 31 normal controls. Median plasma levels of VEGF-C were 35.0 U/mL in colorectal cancer patients compared to 11.5 U/mL in controls ( $P < 0.001$ ). VEGF-C levels tended to be elevated in patients with advanced disease (Dukes C and D) compared to early disease, but this was not statistically significant owing to a relatively



small number of patients in each group. Plasma levels of VEGF-C in their study may represent both partially processed and fully mature forms of the cytokine.

Nevertheless, another study by Duff *et al*<sup>[136]</sup>, including 120 CRC patients, failed to show significant differences in plasma VEGF-C or VEGF-D levels between patients subgrouped by clinicopathological variables. In particular, there were no differences in median plasma VEGF-C or VEGF-D level in patients with and without lymph-node involvement (VEGF-C: 11.2 U/mL *vs* 9.9 U/mL; *P* = 0.90; VEGF-D: 335 pg/mL *vs* 316.5 pg/mL; *P* = 0.68).

Finally, in a study from China<sup>[137]</sup> 66 CRC patients and 30 controls were enrolled in quantification of serum levels of VEGF-C and VEGF. Serum VEGF-C and VEGF levels were reported higher in patients with colorectal carcinoma than in healthy controls as well as in patients with lymph node metastasis than those without lymph node metastasis. Serum VEGF-C levels reached a sensitivity of 81% and a specificity of 76% with a cut-off value of 1438.0 pg/mL.

**Clinical relevance:** Rectal cancer is the type of large intestinal malignancy whose management is the most challenging regarding surgical resection and preoperative staging. With the introduction of total mesorectal excision (TME), optimal surgical technique is considered the most pivotal factor influencing curative outcome<sup>[138-140]</sup>.

However, major rectal surgery is technically challenging, related to increased risks and can not eliminate local recurrence rates. Additionally, oncological resections may lead to debilitating functional results and substantially influence quality of life. Currently, treatment of rectal cancer should be individualized and evaluation of the extent of primary cancer is essential for planning the appropriate therapy regimen, spanning from simple local excision to complex multimodality treatments.

Transanal excision is considered an acceptable alternative to radical resection when treating intramural cancer without distant spread (T1N0M0). This approach is followed even with curative intent in some centers when confronting low-risk tumors with highly favourable features<sup>[141]</sup>. Major advantages are low morbidity and mortality rates and excellent functional outcome. On the other hand, T1 tumors are related to up to 12% risk of LNM<sup>[142,143]</sup> and to recurrence rates of 10%-25% following local excision, with a fatal result for half of these patents<sup>[144-146]</sup>. The key for these unsatisfactory results could lie in imperfect preoperative staging and unrecognizable biological behaviour.

Neoadjuvant chemoradiotherapy (CRT) has been shown to significantly reduce local recurrence rates of locally advanced rectal cancer (T3-4, N0-1) without concomitant proven benefit regarding overall survival while its effect on sphincter preservation is controversial<sup>[140,147]</sup>. Nevertheless, preoperative radiation is not without of toxicity, postoperative complications and considerable cost<sup>[148,149]</sup>. Moreover, studies evaluating treatment outcome after neoadjuvant CRT have demonstrated improved survival in responding patients compared to partial or non-responders<sup>[150,151]</sup>. These findings highlight the need for accurate preoperative staging so as not to overtreat unsuit-

able patients, but also for identification of biologically favorable cancers in order to predict an optimal response.

Pre-treatment clinical staging of rectal cancer is based on integration of information obtained from digital examination, endoscopy and imaging modalities. Endoluminal imaging is considered the most valuable in locoregional staging. Endorectal ultrasonography is reported to have the best accuracy in nodal staging with a mean rate of 75%, although its performance may be overestimated in the literature due to publication bias<sup>[152,153]</sup>. MRI techniques with endorectal coil is the best means for evaluating T stage and circumferential resection margin, yet LNM detection remains problematic because it relies on non specific morphological criteria<sup>[154,155]</sup>.

To date, there are no clinically useful molecular predictors of response to preoperative CRT which could assist to better patient selection<sup>[156]</sup>. The clinicopathological correlations of VEGF-C and VEGF-D in colorectal cancer, including early lesions, provide evidence that these cytokines play a role in colorectal LNM. Studies on circulating levels are contradictory, yet they do not discriminate between colon and rectal cancers and as a consequence their results can not be clarified.

## CONCLUSION

The role of lymphangiogenic growth factors VEGF-C and VEGF-D in malignant tumors metastasis is a novel field of cancer research. The results of current studies on their tumoral expression are strongly indicative of an active involvement of these cytokines to lymphatic spread, although the experimental and clinicopathological findings are not always consistent. Determination of circulating levels in preoperative blood samples might be a useful marker of advanced disease and a predictive factor of lymph node metastasis in gastric, esophageal and colorectal cancer, providing an additional tool in pre-treatment planning. Available studies are currently scant, with limited sample size and inadequate to conclude more than a presumption of a potential application of VEGF-C and VEGF-D in diagnostic and therapeutic regimens. However, modern research on understanding the mechanisms of lymphangiogenesis in human solid tumors is intensive and further studies on circulating growth factors are both desirable and justifiable in order to refine their role as nodal status biomarkers in gastrointestinal malignancies.

## REFERENCES

- 1 **Pepper MS.** Lymphangiogenesis and tumor metastasis: myth or reality? *Clin Cancer Res* 2001; 7: 462-468
- 2 **McCarter MD, Clarke JH, Harken AH.** Lymphangiogenesis is pivotal to the trials of a successful cancer metastasis. *Surgery* 2004; 135: 121-124
- 3 **He Y, Rajantie I, Pajusola K, Jeltsch M, Holopainen T, Yla-Herttuala S, Harding T, Jooss K, Takahashi T, Alitalo K.** Vascular endothelial cell growth factor receptor 3-mediated activation of lymphatic endothelium is crucial for tumor cell entry and spread via lymphatic vessels. *Cancer Res* 2005; 65: 4739-4746
- 4 **Ji RC.** Lymphatic endothelial cells, tumor lymphangiogenesis and metastasis: New insights into intratumoral and

- peritumoral lymphatics. *Cancer Metastasis Rev* 2006; **25**: 677-694
- 5 **Alitalo K**, Tammela T, Petrova TV. Lymphangiogenesis in development and human disease. *Nature* 2005; **438**: 946-953
  - 6 **Saharinen P**, Tammela T, Karkkainen MJ, Alitalo K. Lymphatic vasculature: development, molecular regulation and role in tumor metastasis and inflammation. *Trends Immunol* 2004; **25**: 387-395
  - 7 **Karkkainen MJ**, Petrova TV. Vascular endothelial growth factor receptors in the regulation of angiogenesis and lymphangiogenesis. *Oncogene* 2000; **19**: 5598-5605
  - 8 **Kaipainen A**, Korhonen J, Mustonen T, van Hinsbergh VW, Fang GH, Dumont D, Breitman M, Alitalo K. Expression of the fms-like tyrosine kinase 4 gene becomes restricted to lymphatic endothelium during development. *Proc Natl Acad Sci USA* 1995; **92**: 3566-3570
  - 9 **Partanen TA**, Arola J, Saaristo A, Jussila L, Ora A, Miettinen M, Stacker SA, Achen MG, Alitalo K. VEGF-C and VEGF-D expression in neuroendocrine cells and their receptor, VEGFR-3, in fenestrated blood vessels in human tissues. *FASEB J* 2000; **14**: 2087-2096
  - 10 **Ferrell RE**, Levinson KL, Esman JH, Kimak MA, Lawrence EC, Barmada MM, Finegold DN. Hereditary lymphedema: evidence for linkage and genetic heterogeneity. *Hum Mol Genet* 1998; **7**: 2073-2078
  - 11 **Evans AL**, Brice G, Sotirova V, Mortimer P, Beninson J, Burnand K, Rosbotham J, Child A, Sarfarazi M. Mapping of primary congenital lymphedema to the 5q35.3 region. *Am J Hum Genet* 1999; **64**: 547-555
  - 12 **Makinen T**, Veikkola T, Mustjoki S, Karpanen T, Catimel B, Nice EC, Wise L, Mercer A, Kowalski H, Kerjaschki D, Stacker SA, Achen MG, Alitalo K. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J* 2001; **20**: 4762-4773
  - 13 **Chen L**, Hamrah P, Cursiefen C, Zhang Q, Pytowski B, Streilein JW, Dana MR. Vascular endothelial growth factor receptor-3 mediates induction of corneal alloimmunity. *Nat Med* 2004; **10**: 813-815
  - 14 **Partanen TA**, Alitalo K, Miettinen M. Lack of lymphatic vascular specificity of vascular endothelial growth factor receptor 3 in 185 vascular tumors. *Cancer* 1999; **86**: 2406-2412
  - 15 **Cursiefen C**, Chen L, Borges LP, Jackson D, Cao J, Radziejewski C, D'Amore PA, Dana MR, Wiegand SJ, Streilein JW. VEGF-A stimulates lymphangiogenesis and hemangiogenesis in inflammatory neovascularization via macrophage recruitment. *J Clin Invest* 2004; **113**: 1040-1050
  - 16 **Borndahl MA**, Cao R, Burton JB, Brakenhielm E, Religa P, Galter D, Wu L, Cao Y. Vascular endothelial growth factor-a promotes peritumoral lymphangiogenesis and lymphatic metastasis. *Cancer Res* 2005; **65**: 9261-9268
  - 17 **Tammela T**, Enholm B, Alitalo K, Paavonen K. The biology of vascular endothelial growth factors. *Cardiovasc Res* 2005; **65**: 550-563
  - 18 **Yamazaki Y**, Morita T. Molecular and functional diversity of vascular endothelial growth factors. *Mol Divers* 2006; **10**: 515-527
  - 19 **Takahashi H**, Shibuya M. The vascular endothelial growth factor (VEGF)/VEGF receptor system and its role under physiological and pathological conditions. *Clin Sci (Lond)* 2005; **109**: 227-241
  - 20 **Joukov V**, Pajusola K, Kaipainen A, Chilov D, Lahtinen I, Kukk E, Saksela O, Kalkkinen N, Alitalo K. A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases. *EMBO J* 1996; **15**: 290-298
  - 21 **Achen MG**, Jeltsch M, Kukk E, Makinen T, Vitali A, Wilks AF, Alitalo K, Stacker SA. Vascular endothelial growth factor D (VEGF-D) is a ligand for the tyrosine kinases VEGF receptor 2 (Flk1) and VEGF receptor 3 (Flt4). *Proc Natl Acad Sci USA* 1998; **95**: 548-553
  - 22 **Kukk E**, Lymboussaki A, Taira S, Kaipainen A, Jeltsch M, Joukov V, Alitalo K. VEGF-C receptor binding and pattern of expression with VEGFR-3 suggests a role in lymphatic vascular development. *Development* 1996; **122**: 3829-3837
  - 23 **Veikkola T**, Jussila L, Makinen T, Karpanen T, Jeltsch M, Petrova TV, Kubo H, Thurston G, McDonald DM, Achen MG, Stacker SA, Alitalo K. Signalling via vascular endothelial growth factor receptor-3 is sufficient for lymphangiogenesis in transgenic mice. *EMBO J* 2001; **20**: 1223-1231
  - 24 **Joukov V**, Sorsa T, Kumar V, Jeltsch M, Claesson-Welsh L, Cao Y, Saksela O, Kalkkinen N, Alitalo K. Proteolytic processing regulates receptor specificity and activity of VEGF-C. *EMBO J* 1997; **16**: 3898-3911
  - 25 **Stacker SA**, Stenvers K, Caesar C, Vitali A, Domagala T, Nice E, Roufail S, Simpson RJ, Moritz R, Karpanen T, Alitalo K, Achen MG. Biosynthesis of vascular endothelial growth factor-D involves proteolytic processing which generates non-covalent homodimers. *J Biol Chem* 1999; **274**: 32127-32136
  - 26 **McColl BK**, Baldwin ME, Roufail S, Freeman C, Moritz RL, Simpson RJ, Alitalo K, Stacker SA, Achen MG. Plasmin activates the lymphangiogenic growth factors VEGF-C and VEGF-D. *J Exp Med* 2003; **198**: 863-868
  - 27 **Siegfried G**, Basak A, Cromlish JA, Benjannet S, Marcinkiewicz J, Chretien M, Seidah NG, Khatib AM. The secretory proprotein convertases furin, PC5, and PC7 activate VEGF-C to induce tumorigenesis. *J Clin Invest* 2003; **111**: 1723-1732
  - 28 **Karpanen T**, Egeblad M, Karkkainen MJ, Kubo H, Yla-Herttuala S, Jaattela M, Alitalo K. Vascular endothelial growth factor C promotes tumor lymphangiogenesis and intralymphatic tumor growth. *Cancer Res* 2001; **61**: 1786-1790
  - 29 **Schoppmann SF**, Birner P, Stockl J, Kalt R, Ullrich R, Caucig C, Kriehuber E, Nagy K, Alitalo K, Kerjaschki D. Tumor-associated macrophages express lymphatic endothelial growth factors and are related to peritumoral lymphangiogenesis. *Am J Pathol* 2002; **161**: 947-956
  - 30 **He Y**, Rajantie I, Pajusola K, Jeltsch M, Holopainen T, Yla-Herttuala S, Harding T, Jooss K, Takahashi T, Alitalo K. Vascular endothelial cell growth factor receptor 3-mediated activation of lymphatic endothelium is crucial for tumor cell entry and spread via lymphatic vessels. *Cancer Res* 2005; **65**: 4739-4746
  - 31 **Stacker SA**, Caesar C, Baldwin ME, Thornton GE, Williams RA, Prevo R, Jackson DG, Nishikawa S, Kubo H, Achen MG. VEGF-D promotes the metastatic spread of tumor cells via the lymphatics. *Nat Med* 2001; **7**: 186-191
  - 32 **Mandriota SJ**, Jussila L, Jeltsch M, Compagni A, Baetens D, Prevo R, Banerji S, Huarte J, Montesano R, Jackson DG, Orci L, Alitalo K, Christofori G, Pepper MS. Vascular endothelial growth factor-C-mediated lymphangiogenesis promotes tumour metastasis. *EMBO J* 2001; **20**: 672-682
  - 33 **Achen MG**, Mann GB, Stacker SA. Targeting lymphangiogenesis to prevent tumour metastasis. *Br J Cancer* 2006; **94**: 1355-1360
  - 34 **Jain RK**, Padera TP. Prevention and treatment of lymphatic metastasis by antilymphangiogenic therapy. *J Natl Cancer Inst* 2002; **94**: 785-787
  - 35 **Kobayashi S**, Kishimoto T, Kamata S, Otsuka M, Miyazaki M, Ishikura H. Rapamycin, a specific inhibitor of the mammalian target of rapamycin, suppresses lymphangiogenesis and lymphatic metastasis. *Cancer Sci* 2007; **98**: 726-733
  - 36 **Stacker SA**, Williams RA, Achen MG. Lymphangiogenic growth factors as markers of tumor metastasis. *APMIS* 2004; **112**: 539-549
  - 37 **Stacker SA**, Baldwin ME, Achen MG. The role of tumor lymphangiogenesis in metastatic spread. *FASEB J* 2002; **16**: 922-934
  - 38 **George ML**, Tutton MG, Janssen F, Arnaout A, Abulafi AM, Eccles SA, Swift RI. VEGF-A, VEGF-C, and VEGF-D in colorectal cancer progression. *Neoplasia* 2001; **3**: 420-427
  - 39 **Sipos B**, Kojima M, Tiemann K, Klapper W, Kruse ML, Kalthoff H, Schniewind B, Tepel J, Weich H, Kerjaschki D, Kloppel G. Lymphatic spread of ductal pancreatic adenocarcinoma is independent of lymphangiogenesis. *J Pathol* 2005; **207**: 301-312
  - 40 **Watanabe O**, Kinoshita J, Shimizu T, Imamura H, Hirano A, Okabe T, Aiba M, Ogawa K. Expression of a CD44 variant and VEGF-C and the implications for lymphatic metastasis and

- long-term prognosis of human breast cancer. *J Exp Clin Cancer Res* 2005; **24**: 75-82
- 41 **Noguchi T**, Takeno S, Shibata T, Uchida Y, Yokoyama S, Muller W. VEGF-C expression correlates with histological differentiation and metastasis in squamous cell carcinoma of the esophagus. *Oncol Rep* 2002; **9**: 995-999
  - 42 **Duff SE**, Li C, Jeziorska M, Kumar S, Saunders MP, Sherlock D, O'Dwyer ST, Jayson GC. Vascular endothelial growth factors C and D and lymphangiogenesis in gastrointestinal tract malignancy. *Br J Cancer* 2003; **89**: 426-430
  - 43 **Roukos DH**, Liakakos T, Karatzas G, Kappas AM. Can VEGF-D and VEGFR-3 be used as biomarkers for therapeutic decisions in patients with gastric cancer? *Nat Clin Pract Oncol* 2006; **3**: 418-419
  - 44 **Houghton SG**, Cockerill FR 3rd. Real-time PCR: overview and applications. *Surgery* 2006; **139**: 1-5
  - 45 **Van der Auwera I**, Cao Y, Tille JC, Pepper MS, Jackson DG, Fox SB, Harris AL, Dirix LY, Vermeulen PB. First international consensus on the methodology of lymphangiogenesis quantification in solid human tumours. *Br J Cancer* 2006; **95**: 1611-1625
  - 46 **Hoar FJ**, Lip GY, Belgore F, Stonelake PS. Circulating levels of VEGF-A, VEGF-D and soluble VEGF-A receptor (sFlt-1) in human breast cancer. *Int J Biol Markers* 2004; **19**: 229-235
  - 47 **Al-Mowallad A**, Kirwan C, Byrne G, McDowell G, Li C, Stewart A, Al-Qouzi A, Kumar S. Vascular endothelial growth factor-C in patients with breast cancer. *In Vivo* 2007; **21**: 549-551
  - 48 **Kummel S**, Eggemann H, Luftner D, Thomas A, Jeschke S, Zerfel N, Heilmann V, Emons G, Zeiser T, Ulm K, Kobl M, Korlach S, Schmid P, Schouli J, Elling D, Blohmer JU. Changes in the circulating plasma levels of VEGF and VEGF-D after adjuvant chemotherapy in patients with breast cancer and 1 to 3 positive lymph nodes. *Anticancer Res* 2006; **26**: 1719-1726
  - 49 **Tamura M**, Ohta Y. Serum vascular endothelial growth factor-C level in patients with primary nonsmall cell lung carcinoma: a possible diagnostic tool for lymph node metastasis. *Cancer* 2003; **98**: 1217-1225
  - 50 **Tamura M**, Oda M, Matsumoto I, Tsunazuka Y, Kawakami K, Ohta Y, Watanabe G. The combination assay with circulating vascular endothelial growth factor (VEGF)-C, matrix metalloproteinase-9, and VEGF for diagnosing lymph node metastasis in patients with non-small cell lung cancer. *Ann Surg Oncol* 2004; **11**: 928-933
  - 51 **Tamura M**, Oda M, Tsunazuka Y, Matsumoto I, Kawakami K, Ohta Y, Watanabe G. Chest CT and serum vascular endothelial growth factor-C level to diagnose lymph node metastasis in patients with primary non-small cell lung cancer. *Chest* 2004; **126**: 342-346
  - 52 **Mitsuhashi A**, Suzuka K, Yamazawa K, Matsui H, Seki K, Sekiya S. Serum vascular endothelial growth factor (VEGF) and VEGF-C levels as tumor markers in patients with cervical carcinoma. *Cancer* 2005; **103**: 724-730
  - 53 **Mathur SP**, Mathur RS, Gray EA, Lane D, Underwood PG, Kohler M, Creasman WT. Serum vascular endothelial growth factor C (VEGF-C) as a specific biomarker for advanced cervical cancer: Relationship to insulin-like growth factor II (IGF-II), IGF binding protein 3 (IGF-BP3) and VEGF-A (corrected). *Gynecol Oncol* 2005; **98**: 467-483
  - 54 **Kaushal V**, Mukunyadzi P, Dennis RA, Siegel ER, Johnson DE, Kohli M. Stage-specific characterization of the vascular endothelial growth factor axis in prostate cancer: expression of lymphangiogenic markers is associated with advanced-stage disease. *Clin Cancer Res* 2005; **11**: 584-593
  - 55 **Strauss L**, Volland D, Kunkel M, Reichert TE. Dual role of VEGF family members in the pathogenesis of head and neck cancer (HNSCC): possible link between angiogenesis and immune tolerance. *Med Sci Monit* 2005; **11**: BR280-BR292
  - 56 **Yonemura Y**, Endo Y, Fujita H, Fushida S, Ninomiya I, Bandou E, Taniguchi K, Miwa K, Ohoyama S, Sugiyama K, Sasaki T. Role of vascular endothelial growth factor C expression in the development of lymph node metastasis in gastric cancer. *Clin Cancer Res* 1999; **5**: 1823-1829
  - 57 **Shida A**, Fujioka S, Kobayashi K, Ishibashi Y, Nimura H, Mitsumori N, Yanaga K. Expression of vascular endothelial growth factor (VEGF)-C and -D in gastric carcinoma. *Int J Clin Oncol* 2006; **11**: 38-43
  - 58 **Kitadai Y**, Kodama M, Cho S, Kuroda T, Ochiuni T, Kimura S, Tanaka S, Matsumura S, Yasui W, Chayama K. Quantitative analysis of lymphangiogenic markers for predicting metastasis of human gastric carcinoma to lymph nodes. *Int J Cancer* 2005; **115**: 388-392
  - 59 **Juttner S**, Wissmann C, Jons T, Vieth M, Hertel J, Gretschel S, Schlag PM, Kemmner W, Hocker M. Vascular endothelial growth factor-D and its receptor VEGFR-3: two novel independent prognostic markers in gastric adenocarcinoma. *J Clin Oncol* 2006; **24**: 228-240
  - 60 **Hachisuka T**, Narikiyo M, Yamada Y, Ishikawa H, Ueno M, Uchida H, Yoriki R, Ohigashi Y, Miki K, Tamaki H, Mizuno T, Nakajima Y. High lymphatic vessel density correlates with overexpression of VEGF-C in gastric cancer. *Oncol Rep* 2005; **13**: 733-737
  - 61 **Shimizu K**, Kubo H, Yamaguchi K, Kawashima K, Ueda Y, Matsuo K, Awane M, Shimahara Y, Takabayashi A, Yamaoka Y, Satoh S. Suppression of VEGFR-3 signaling inhibits lymph node metastasis in gastric cancer. *Cancer Sci* 2004; **95**: 328-333
  - 62 **Nikiteas NI**, Tzanakis N, Theodoropoulos G, Atsaves V, Christoni Z, Karakitsos P, Lazaris AC, Papachristodoulou A, Klonaris C, Gazouli M. Vascular endothelial growth factor and endoglin (CD-105) in gastric cancer. *Gastric Cancer* 2007; **10**: 12-17
  - 63 **Kondo K**, Kaneko T, Baba M, Konno H. VEGF-C and VEGF-A synergistically enhance lymph node metastasis of gastric cancer. *Biol Pharm Bull* 2007; **30**: 633-637
  - 64 **Kabashima A**, Maehara Y, Kakeji Y, Sugimachi K. Overexpression of vascular endothelial growth factor C is related to lymphogenous metastasis in early gastric carcinoma. *Oncology* 2001; **60**: 146-150
  - 65 **Ishikawa M**, Kitayama J, Kazama S, Nagawa H. Expression of vascular endothelial growth factor C and D (VEGF-C and -D) is an important risk factor for lymphatic metastasis in undifferentiated early gastric carcinoma. *Jpn J Clin Oncol* 2003; **33**: 21-27
  - 66 **Onogawa S**, Kitadai Y, Amioka T, Kodama M, Cho S, Kuroda T, Ochiuni T, Kimura S, Kuwai T, Tanaka S, Chayama K. Expression of vascular endothelial growth factor (VEGF)-C and VEGF-D in early gastric carcinoma: correlation with clinicopathological parameters. *Cancer Lett* 2005; **226**: 85-90
  - 67 **Wang TB**, Deng MH, Qiu WS, Dong WG. Association of serum vascular endothelial growth factor-C and lymphatic vessel density with lymph node metastasis and prognosis of patients with gastric cancer. *World J Gastroenterol* 2007; **13**: 1794-1797; discussion 1797-1798
  - 68 **Shimada Y**. JGCA (The Japan Gastric Cancer Association). Gastric cancer treatment guidelines. *Jpn J Clin Oncol* 2004; **34**: 58
  - 69 **Sayegh ME**, Sano T, Dexter S, Katai H, Fukagawa T, Sasako M. TNM and Japanese staging systems for gastric cancer: how do they coexist? *Gastric Cancer* 2004; **7**: 140-148
  - 70 **Kappas AM**, Fatouros M, Roukos DH. Is it time to change surgical strategy for gastric cancer in the United States? *Ann Surg Oncol* 2004; **11**: 727-730
  - 71 **Cuschieri A**, Fayers P, Fielding J, Craven J, Banciewicz J, Joypaul V, Cook P. Postoperative morbidity and mortality after D1 and D2 resections for gastric cancer: preliminary results of the MRC randomised controlled surgical trial. The Surgical Cooperative Group. *Lancet* 1996; **347**: 995-999
  - 72 **Bonenkamp JJ**, Songun I, Hermans J, Sasako M, Welvaart K, Plukker JT, van Elk P, Obertop H, Gouma DJ, Taat CW. Randomised comparison of morbidity after D1 and D2 dissection for gastric cancer in 996 Dutch patients. *Lancet* 1995; **345**: 745-748
  - 73 **Bonenkamp JJ**, Hermans J, Sasako M, van de Velde CJ, Welvaart K, Songun I, Meyer S, Plukker JT, Van Elk P, Obertop H, Gouma DJ, van Lanschot JJ, Taat CW, de Graaf PW, von Meyenfeldt MF, Tilanus H. Extended lymph-node dissection

- for gastric cancer. *N Engl J Med* 1999; **340**: 908-914
- 74 **Hartgrink HH**, van de Velde CJ, Putter H, Bonenkamp JJ, Klein Kranenbarg E, Songun I, Welvaart K, von Krieken JH, Meijer S, Plukker JT, van Elk PJ, Obertop H, Gouma DJ, van Lanschot JJ, Taat CW, de Graaf PW, von Meyenfeldt MF, Tilanus H, Sasako M. Extended lymph node dissection for gastric cancer: who may benefit? Final results of the randomized Dutch gastric cancer group trial. *J Clin Oncol* 2004; **22**: 2069-2077
  - 75 **Cuschieri A**, Weeden S, Fielding J, Bancewicz J, Craven J, Joypaul V, Sydes M, Fayers P. Patient survival after D1 and D2 resections for gastric cancer: long-term results of the MRC randomized surgical trial. Surgical Co-operative Group. *Br J Cancer* 1999; **79**: 1522-1530
  - 76 **Siewert JR**, Bottcher K, Stein HJ, Roder JD. Relevant prognostic factors in gastric cancer: ten-year results of the German Gastric Cancer Study. *Ann Surg* 1998; **228**: 449-461
  - 77 **Degiuli M**, Sasako M, Ponti A, Calvo F. Survival results of a multicentre phase II study to evaluate D2 gastrectomy for gastric cancer. *Br J Cancer* 2004; **90**: 1727-1732
  - 78 **Roukos DH**, Lorenz M, Encke A. Evidence of survival benefit of extended (D2) lymphadenectomy in western patients with gastric cancer based on a new concept: a prospective long-term follow-up study. *Surgery* 1998; **123**: 573-578
  - 79 **Sue-Ling HM**, Johnston D, Martin IG, Dixon MF, Lansdown MR, McMahon MJ, Axon AT. Gastric cancer: a curable disease in Britain. *BMJ* 1993; **307**: 591-596
  - 80 **Harrison LE**, Karpeh MS, Brennan MF. Extended lymphadenectomy is associated with a survival benefit for node-negative gastric cancer. *J Gastrointest Surg* 1998; **2**: 126-131
  - 81 **Habermann CR**, Weiss F, Riecken R, Honarpisheh H, Bohnacker S, Staedtler C, Dieckmann C, Schoder V, Adam G. Preoperative staging of gastric adenocarcinoma: comparison of helical CT and endoscopic US. *Radiology* 2004; **230**: 465-471
  - 82 **Kim AY**, Kim HJ, Ha HK. Gastric cancer by multidetector row CT: preoperative staging. *Abdom Imaging* 2005; **30**: 465-472
  - 83 **Ganpathi IS**, So JB, Ho KY. Endoscopic ultrasonography for gastric cancer: does it influence treatment? *Surg Endosc* 2006; **20**: 559-562
  - 84 **Bentrem D**, Gerdes H, Tang L, Brennan M, Coit D. Clinical correlation of endoscopic ultrasonography with pathologic stage and outcome in patients undergoing curative resection for gastric cancer. *Ann Surg Oncol* 2007; **14**: 1853-1859
  - 85 **Chen J**, Cheong JH, Yun MJ, Kim J, Lim JS, Hyung WJ, Noh SH. Improvement in preoperative staging of gastric adenocarcinoma with positron emission tomography. *Cancer* 2005; **103**: 2383-2390
  - 86 **Kim MC**, Kim HH, Jung GJ, Lee JH, Choi SR, Kang DY, Roh MS, Jeong JS. Lymphatic mapping and sentinel node biopsy using <sup>99m</sup>Tc tin colloid in gastric cancer. *Ann Surg* 2004; **239**: 383-387
  - 87 **Ichikura T**, Chochi K, Sugawara H, Yaguchi Y, Sakamoto N, Takahata R, Kosuda S, Mochizuki H. Individualized surgery for early gastric cancer guided by sentinel node biopsy. *Surgery* 2006; **139**: 501-507
  - 88 **Kampschoer GH**, Maruyama K, van de Velde CJ, Sasako M, Kinoshita T, Okabayashi K. Computer analysis in making preoperative decisions: a rational approach to lymph node dissection in gastric cancer patients. *Br J Surg* 1989; **76**: 905-908
  - 89 **Gretschel S**, Bembek A, Ulmer Ch, Hunerbein M, Markwardt J, Schneider U, Schlag PM. Prediction of gastric cancer lymph node status by sentinel lymph node biopsy and the Maruyama computer model. *Eur J Surg Oncol* 2005; **31**: 393-400
  - 90 **Koch TR**. The changing face of esophageal malignancy. *Curr Gastroenterol Rep* 2003; **5**: 187-191
  - 91 **Kitadai Y**, Amioka T, Haruma K, Tanaka S, Yoshihara M, Sumii K, Matsutani N, Yasui W, Chayama K. Clinicopathological significance of vascular endothelial growth factor (VEGF)-C in human esophageal squamous cell carcinomas. *Int J Cancer* 2001; **93**: 662-666
  - 92 **Kimura Y**, Watanabe M, Ohga T, Saeki H, Kakeji Y, Baba H, Maehara Y. Vascular endothelial growth factor C expression correlates with lymphatic involvement and poor prognosis in patients with esophageal squamous cell carcinoma. *Oncol Rep* 2003; **10**: 1747-1751
  - 93 **Katsuta M**, Miyashita M, Makino H, Nomura T, Shinji S, Yamashita K, Tajiri T, Kudo M, Ishiwata T, Naito Z. Correlation of hypoxia inducible factor-1alpha with lymphatic metastasis via vascular endothelial growth factor-C in human esophageal cancer. *Exp Mol Pathol* 2005; **78**: 123-130
  - 94 **Mobius C**, Freire J, Becker I, Feith M, Brucher BL, Hennig M, Siewert JR, Stein HJ. VEGF-C expression in squamous cell carcinoma and adenocarcinoma of the esophagus. *World J Surg* 2007; **31**: 1768-1772; discussion 1773-1774
  - 95 **Kleespies A**, Bruns CJ, Jauch KW. Clinical significance of VEGF-A, -C and -D expression in esophageal malignancies. *Onkologie* 2005; **28**: 281-288
  - 96 **von Rahden BH**, Stein HJ, Pühringer F, Koch I, Langer R, Piontek G, Siewert JR, Hofler H, Sarbia M. Coexpression of cyclooxygenases (COX-1, COX-2) and vascular endothelial growth factors (VEGF-A, VEGF-C) in esophageal adenocarcinoma. *Cancer Res* 2005; **65**: 5038-5044
  - 97 **Auvinen MI**, Sihvo EL, Ruohutala T, Salminen JT, Koivistoinen A, Siivola P, Ronnholm R, Ramo JO, Bergman M, Salo JA. Incipient angiogenesis in Barrett's epithelium and lymphangiogenesis in Barrett's adenocarcinoma. *J Clin Oncol* 2002; **20**: 2971-2979
  - 98 **Ishikawa M**, Kitayama J, Kazama S, Nagawa H. The expression pattern of vascular endothelial growth factor C and D in human esophageal normal mucosa, dysplasia and neoplasia. *Hepatogastroenterology* 2004; **51**: 1319-1322
  - 99 **Matsumoto M**, Natsugoe S, Okumura H, Arima H, Yanagita S, Uchikado Y, Yokomakura N, Setoyama T, Ishigami S, Takao S, Aikou T. Overexpression of vascular endothelial growth factor-C correlates with lymph node micrometastasis in submucosal esophageal cancer. *J Gastrointest Surg* 2006; **10**: 1016-1022
  - 100 **Krzystek-Korpaczka M**, Matusiewicz M, Diakowska D, Grabowski K, Blachut K, Banas T. Up-regulation of VEGF-C secreted by cancer cells and not VEGF-A correlates with clinical evaluation of lymph node metastasis in esophageal squamous cell carcinoma (ESCC). *Cancer Lett* 2007; **249**: 171-177
  - 101 **Krzystek-Korpaczka M**, Matusiewicz M, Diakowska D, Grabowski K, Blachut K, Kustrzeba-Wojcicka I, Banas T. Serum midkine depends on lymph node involvement and correlates with circulating VEGF-C in oesophageal squamous cell carcinoma. *Biomarkers* 2007; **12**: 403-413
  - 102 **Shimada H**, Nabeya Y, Okazumi S, Matsubara H, Kadomatsu K, Muramatsu T, Ikematsu S, Sakuma S, Ochiai T. Increased serum midkine concentration as a possible tumor marker in patients with superficial esophageal cancer. *Oncol Rep* 2003; **10**: 411-414
  - 103 **Shimada H**, Nabeya Y, Tagawa M, Okazumi S, Matsubara H, Kadomatsu K, Muramatsu T, Ikematsu S, Sakuma S, Ochiai T. Preoperative serum midkine concentration is a prognostic marker for esophageal squamous cell carcinoma. *Cancer Sci* 2003; **94**: 628-632
  - 104 **Pal N**, Axisa B, Yusof S, Newcombe RG, Wemyss-Holden S, Rhodes M, Lewis MP. Volume and Outcome for Major Upper GI Surgery in England. *J Gastrointest Surg* 2008; **12**: 353-357
  - 105 **Birkmeyer JD**, Dimick JB, Staiger DO. Operative mortality and procedure volume as predictors of subsequent hospital performance. *Ann Surg* 2006; **243**: 411-417
  - 106 **de Boer AG**, van Lanschot JJ, van Sandick JW, Hulscher JB, Stalmeier PF, de Haes JC, Tilanus HW, Obertop H, Sprangers MA. Quality of life after transhiatal compared with extended transthoracic resection for adenocarcinoma of the esophagus. *J Clin Oncol* 2004; **22**: 4202-4208
  - 107 **Enzinger PZ**, Page DL, Fleming ID. AJCC Cancer Staging Manual. 6th ed. New York: New York Springer, 2002: 200
  - 108 **Eloubeidi MA**, Wallace MB, Hoffman BJ, Leveen MB, Van Velse A, Hawes RH, Reed CE. Predictors of survival for esophageal cancer patients with and without celiac axis lymphadenopathy: impact of staging endosonography. *Ann Thorac Surg* 2001; **72**: 212-219; discussion 219-220



- 109 **Thomas CR Jr.** Current and ongoing progress in the therapy for resectable esophageal cancer. *Dis Esophagus* 2005; **18**: 211-214
- 110 **McKian KP**, Miller RC, Cassivi SD, Jatoi A. Curing patients with locally advanced esophageal cancer: an update on multimodality therapy. *Dis Esophagus* 2006; **19**: 448-453
- 111 **Mariette C**, Piessen G, Triboulet JP. Therapeutic strategies in oesophageal carcinoma: role of surgery and other modalities. *Lancet Oncol* 2007; **8**: 545-553
- 112 **Kaklamanos IG**, Walker GR, Ferry K, Franceschi D, Livingstone AS. Neoadjuvant treatment for resectable cancer of the esophagus and the gastroesophageal junction: a meta-analysis of randomized clinical trials. *Ann Surg Oncol* 2003; **10**: 754-761
- 113 **Vazquez-Sequeiros E**, Norton ID, Clain JE, Wang KK, Affi A, Allen M, Deschamps C, Miller D, Salomao D, Wiersema MJ. Impact of EUS-guided fine-needle aspiration on lymph node staging in patients with esophageal carcinoma. *Gastrointest Endosc* 2001; **53**: 751-757
- 114 **Abdalla EK**, Pisters PW. Staging and preoperative evaluation of upper gastrointestinal malignancies. *Semin Oncol* 2004; **31**: 513-529
- 115 **Lerut T**, Flamen P, Ectors N, Van Cutsem E, Peeters M, Hiele M, De Wever W, Coosemans W, Decker G, De Leyn P, Deneffe G, Van Raemdonck D, Mortelmans L. Histopathologic validation of lymph node staging with FDG-PET scan in cancer of the esophagus and gastroesophageal junction: A prospective study based on primary surgery with extensive lymphadenectomy. *Ann Surg* 2000; **232**: 743-752
- 116 **Rasanen JV**, Sihvo EI, Knuuti MJ, Minn HR, Luostarinen ME, Laippala P, Viljanen T, Salo JA. Prospective analysis of accuracy of positron emission tomography, computed tomography, and endoscopic ultrasonography in staging of adenocarcinoma of the esophagus and the esophagogastric junction. *Ann Surg Oncol* 2003; **10**: 954-960
- 117 **Catalano ME**, Alcocer E, Chak A, Nguyen CC, Raijman I, Geenen JE, Lahoti S, Sivak MV Jr. Evaluation of metastatic celiac axis lymph nodes in patients with esophageal carcinoma: accuracy of EUS. *Gastrointest Endosc* 1999; **50**: 352-356
- 118 **van Vliet EP**, Eijkemans MJ, Kuipers EJ, Hermans JJ, Steyerberg EW, Tilanus HW, van der Gaast A, Siersema PD. A comparison between low-volume referring regional centers and a high-volume referral center in quality of preoperative metastasis detection in esophageal carcinoma. *Am J Gastroenterol* 2006; **101**: 234-242
- 119 **Forshaw MJ**, Gossage JA, Mason RC. Neoadjuvant chemotherapy for oesophageal cancer: the need for accurate response prediction and evaluation. *Surgeon* 2005; **3**: 373-382, 422
- 120 **Westerterp M**, van Westreenen HL, Reitsma JB, Hoekstra OS, Stoker J, Fockens P, Jager PL, Van Eck-Smit BL, Plukker JT, van Lanschot JJ, Sloof GW. Esophageal cancer: CT, endoscopic US, and FDG PET for assessment of response to neoadjuvant therapy--systematic review. *Radiology* 2005; **236**: 841-851
- 121 **Sloof GW**. Response monitoring of neoadjuvant therapy using CT, EUS, and FDG-PET. *Best Pract Res Clin Gastroenterol* 2006; **20**: 941-957
- 122 **Cerfolio RJ**, Bryant AS, Ohja B, Bartolucci AA, Eloubeidi MA. The accuracy of endoscopic ultrasonography with fine-needle aspiration, integrated positron emission tomography with computed tomography, and computed tomography in restaging patients with esophageal cancer after neoadjuvant chemoradiotherapy. *J Thorac Cardiovasc Surg* 2005; **129**: 1232-1241
- 123 **Vallbohmer D**, Lenz HJ. Predictive and prognostic molecular markers in outcome of esophageal cancer. *Dis Esophagus* 2006; **19**: 425-432
- 124 **Duong C**, Greenawalt DM, Kowalczyk A, Ciavarella ML, Raskutti G, Murray WK, Phillips WA, Thomas RJ. Pretreatment gene expression profiles can be used to predict response to neoadjuvant chemoradiotherapy in esophageal cancer. *Ann Surg Oncol* 2007; **14**: 3602-3609
- 125 **Hofler H**, Langer R, Ott K, Keller G. Prediction of response to neoadjuvant chemotherapy in carcinomas of the upper gastrointestinal tract. *Recent Results Cancer Res* 2007; **176**: 33-36
- 126 **Akagi K**, Ikeda Y, Miyazaki M, Abe T, Kinoshita J, Maehara Y, Sugimachi K. Vascular endothelial growth factor-C (VEGF-C) expression in human colorectal cancer tissues. *Br J Cancer* 2000; **83**: 887-891
- 127 **White JD**, Hewett PW, Kosuge D, McCulloch T, Enholm BC, Carmichael J, Murray JC. Vascular endothelial growth factor-D expression is an independent prognostic marker for survival in colorectal carcinoma. *Cancer Res* 2002; **62**: 1669-1675
- 128 **Jia YT**, Li ZX, He YT, Liang W, Yang HC, Ma HJ. Expression of vascular endothelial growth factor-C and the relationship between lymphangiogenesis and lymphatic metastasis in colorectal cancer. *World J Gastroenterol* 2004; **10**: 3261-3263
- 129 **Hu WG**, Li JW, Feng B, Beveridge M, Yue F, Lu AG, Ma JJ, Wang ML, Guo Y, Jin XL, Zheng MH. Vascular endothelial growth factors C and D represent novel prognostic markers in colorectal carcinoma using quantitative image analysis. *Eur Surg Res* 2007; **39**: 229-238
- 130 **Duff SE**, Jeziorska M, Rosa DD, Kumar S, Haboubi N, Sherlock D, O'Dwyer ST, Jayson GC. Vascular endothelial growth factors and receptors in colorectal cancer: implications for anti-angiogenic therapy. *Eur J Cancer* 2006; **42**: 112-117
- 131 **Furudoi A**, Tanaka S, Haruma K, Kitadai Y, Yoshihara M, Chayama K, Shimamoto F. Clinical significance of vascular endothelial growth factor C expression and angiogenesis at the deepest invasive site of advanced colorectal carcinoma. *Oncology* 2002; **62**: 157-166
- 132 **Maeda K**, Yashiro M, Nishihara T, Nishiguchi Y, Sawai M, Uchima K, Onoda N, Ohira M, Ishikawa T, Hirakawa K. Correlation between vascular endothelial growth factor C expression and lymph node metastasis in T1 carcinoma of the colon and rectum. *Surg Today* 2003; **33**: 736-739
- 133 **Kojima M**, Shiokawa A, Ohike N, Ohta Y, Kato H, Iwaku K, Hayasi R, Morohoshi T. Clinical significance of nuclear morphometry at the invasive front of T1 colorectal cancer and relation to expression of VEGF-A and VEGF-C. *Oncology* 2005; **68**: 230-238
- 134 **Kazama S**, Watanabe T, Kanazawa T, Hatano K, Nagawa H. Vascular endothelial growth factor-C (VEGF-C) is a more specific risk factor for lymph node metastasis than VEGF-D in submucosal colorectal cancer. *Hepatogastroenterology* 2007; **54**: 71-76
- 135 **Duff SE**, Li C, Renehan A, O'Dwyer ST, Kumar S. Immunodetection and molecular forms of plasma vascular endothelial growth factor-C. *Int J Oncol* 2003; **22**: 339-343
- 136 **Duff SE**, Saunders M, McCredie V, Kumar S, O'Dwyer ST, Jayson GC. Pre-operative plasma levels of vascular endothelial growth factor A, C and D in patients with colorectal cancer. *Clin Oncol (R Coll Radiol)* 2005; **17**: 367-371
- 137 **Xu T**, Chen D. Serum vascular endothelial growth factor-C and vascular endothelial growth factor level in patients with colorectal carcinoma and clinical significance. *J Huazhong Univ Sci Technolog Med Sci* 2006; **26**: 329-331, 355
- 138 **Heald RJ**. The 'Holy Plane' of rectal surgery. *J R Soc Med* 1988; **81**: 503-508
- 139 **Enker WE**. Total mesorectal excision--the new golden standard of surgery for rectal cancer. *Ann Med* 1997; **29**: 127-133
- 140 **Daniels IR**, Fisher SE, Heald RJ, Moran BJ. Accurate staging, selective preoperative therapy and optimal surgery improves outcome in rectal cancer: a review of the recent evidence. *Colorectal Dis* 2007; **9**: 290-301
- 141 **Bretagnol F**, Rullier E, George B, Warren BF, Mortensen NJ. Local therapy for rectal cancer: still controversial? *Dis Colon Rectum* 2007; **50**: 523-533
- 142 **Nascimbeni R**, Burgart LJ, Nivatvongs S, Larson DR. Risk of lymph node metastasis in T1 carcinoma of the colon and rectum. *Dis Colon Rectum* 2002; **45**: 200-206
- 143 **Sengupta S**, Tjandra JJ. Local excision of rectal cancer: what is the evidence? *Dis Colon Rectum* 2001; **44**: 1345-1361
- 144 **Madbouly KM**, Remzi FH, Erkek BA, Senagore AJ, Baeslach CM, Khandwala F, Fazio VW, Lavery IC. Recurrence after transanal excision of T1 rectal cancer: should we be concerned? *Dis Colon Rectum* 2005; **48**: 711-719; discussion 719-721
- 145 **Bentrem DJ**, Okabe S, Wong WD, Guillem JG, Weiser MR, Temple LK, Ben-Porat LS, Minsky BD, Cohen AM, Paty PB. T1

- adenocarcinoma of the rectum: transanal excision or radical surgery? *Ann Surg* 2005; **242**: 472-477; discussion 477-479
- 146 **Baron PL**, Enker WE, Zakowski MF, Urmacher C. Immediate vs. salvage resection after local treatment for early rectal cancer. *Dis Colon Rectum* 1995; **38**: 177-181
- 147 **Wallace MH**, Glynne-Jones R. Saving the sphincter in rectal cancer: are we prepared to change practice? *Colorectal Dis* 2007; **9**: 302-308; discussion 308-309
- 148 **Chessin DB**, Enker W, Cohen AM, Paty PB, Weiser MR, Saltz L, Minsky BD, Wong WD, Guillem JG. Complications after preoperative combined modality therapy and radical resection of locally advanced rectal cancer: a 14-year experience from a specialty service. *J Am Coll Surg* 2005; **200**: 876-882; discussion 882-884
- 149 **Guren MG**, Dueland S, Skovlund E, Fossa SD, Poulsen JP, Tveit KM. Quality of life during radiotherapy for rectal cancer. *Eur J Cancer* 2003; **39**: 587-594
- 150 **Chen ET**, Mohiuddin M, Brodovsky H, Fishbein G, Marks G. Downstaging of advanced rectal cancer following combined preoperative chemotherapy and high dose radiation. *Int J Radiat Oncol Biol Phys* 1994; **30**: 169-175
- 151 **Guillem JG**, Chessin DB, Cohen AM, Shia J, Mazumdar M, Enker W, Paty PB, Weiser MR, Klimstra D, Saltz L, Minsky BD, Wong WD. Long-term oncologic outcome following preoperative combined modality therapy and total mesorectal excision of locally advanced rectal cancer. *Ann Surg* 2005; **241**: 829-836; discussion 836-838
- 152 **Hunerbein M**. Endorectal ultrasound in rectal cancer. *Colorectal Dis* 2003; **5**: 402-405
- 153 **Harewood GC**. Assessment of publication bias in the reporting of EUS performance in staging rectal cancer. *Am J Gastroenterol* 2005; **100**: 808-816
- 154 **Beets-Tan RG**, Beets GL. Rectal cancer: review with emphasis on MR imaging. *Radiology* 2004; **232**: 335-346
- 155 **Kim JH**, Beets GL, Kim MJ, Kessels AG, Beets-Tan RG. High-resolution MR imaging for nodal staging in rectal cancer: are there any criteria in addition to the size? *Eur J Radiol* 2004; **52**: 78-83
- 156 **Smith FM**, Reynolds JV, Miller N, Stephens RB, Kennedy MJ. Pathological and molecular predictors of the response of rectal cancer to neoadjuvant radiochemotherapy. *Eur J Surg Oncol* 2006; **32**: 55-64

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## GASTRIC CANCER

# Transcriptional silencing of Dickkopf gene family by CpG island hypermethylation in human gastrointestinal cancer

Tadateru Maehata, Hiroaki Taniguchi, Hiroyuki Yamamoto, Katsuhiko Nosho, Yasushi Adachi, Nobuki Miyamoto, Chie Miyamoto, Noriyuki Akutsu, Satoshi Yamaoka, Fumio Itoh

Tadateru Maehata, Fumio Itoh, Department of Gastroenterology and Hepatology, St. Marianna University School of Medicine, Kanagawa 216-8511, Japan

Hiroaki Taniguchi, Hiroyuki Yamamoto, Katsuhiko Nosho, Yasushi Adachi, Nobuki Miyamoto, Chie Miyamoto, Noriyuki Akutsu, Satoshi Yamaoka, First Department of Internal Medicine, Sapporo Medical University, Sapporo 060-8543, Japan  
Author contributions: Maehata T, Taniguchi H, Yamamoto H designed research; Maehata T, Taniguchi H, Yamamoto H, Nosho K, Adachi Y, Akutsu N, and Yamaoka S performed research; Maehata T, Taniguchi H, Yamamoto H, Miyamoto N, and Miyamoto C analyzed data; and Maehata T, Taniguchi H, Yamamoto H, and Itoh F wrote the paper.

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Correspondence to: Hiroyuki Yamamoto, MD, FJSM, PhD, First Department of Internal Medicine, Sapporo Medical University, S.-1, W.-16, Chuo-ku, Sapporo 060-8543, Japan. [h-yama@sapmed.ac.jp](mailto:h-yama@sapmed.ac.jp)

Telephone: +81-11-611-2111 Fax: +81-11-611-2282

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**CONCLUSION:** Down-regulation of the Dkks associated to promoter hypermethylation appears to be frequently involved in gastrointestinal tumorigenesis.

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**Key words:** Dickkopf genes; Kremen2 gene; Methylation; Wnt signaling; Gastrointestinal cancer

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## Abstract

**AIM:** To clarify alterations of Dickkopfs (Dkks) and Kremen2 (Krm2) in gastrointestinal cancer.

**METHODS:** We investigated the expression profiles and epigenetic alterations of Dkks and Krm2 genes in gastrointestinal cancer using RT-PCR, tissue microarray analysis, and methylation specific PCR (MSP). Cancer cells were treated with the demethylating agent and/or histone deacetylase inhibitor. WST-8 assays and *in vitro* invasion assays after treatment with specific siRNA for those genes were performed.

**RESULTS:** Dkks and Krm2 expression levels were reduced in a certain subset of the gastrointestinal cancer cell lines and cancer tissues. This was correlated with promoter hypermethylation. There were significant correlations between Dkks over-expression levels and beta-catenin over-expression in colorectal cancer. In colorectal cancers with beta-catenin over-expression, Dkk-1 expression levels were significantly lower in those with lymph node metastases than in those without. Down-regulation of Dkks expression by siRNA resulted in a significant increase in cancer cell growth and invasiveness *in vitro*.

## INTRODUCTION

Wnt proteins influence many aspects such as embryonic development and tumorigenesis, and their activities are regulated by several secreted antagonists<sup>[1,2]</sup>. Canonical Wnt signaling *via* the beta-catenin pathway is transduced by two receptor families. Frizzled proteins and lipoprotein-receptor-related proteins 5 and 6 (LRP5/6) bind Wnts and transmit their signals by stabilizing intracellular beta-catenin. Dickkopfs (Dkks) are secreted antagonists of Wnt signaling and are important for induction of head formation<sup>[3]</sup>. Dkks contain two distinct cysteine-rich domains in which the positions of 10 cysteine residues are highly conserved between family members. Secreted Dkk-2 and Dkk-4 undergo proteolytic processing that results in cleavage of the second cysteine-rich domain from the full-length protein<sup>[4]</sup>. Members of the human Dkk-related family differ not only in their structures and expression patterns, but also in their abilities to inhibit Wnt signaling.

The human Dkk-1 (chromosome 10q11.2) gene encodes a powerful inhibitor of the Wnt signaling pathway by binding to and antagonizing LRP5/6<sup>[5]</sup>. Dkk-1 is a transcriptional target of TCF and p53<sup>[4,5]</sup>. Many chemotherapeutic agents that induce DNA adducts significantly enhanced the expression of Dkk-1 in human tumor cell lines, irrespective of their p53 gene

status<sup>[6]</sup>. Dkk-1 expression level was decreased in human colon cancers, mesothelioma, and malignant melanoma, suggesting that Dkk-1 acts as a tumor suppressor gene in these neoplasms<sup>[5,7-9]</sup>. The Wnt/beta-catenin pathway was down-regulated by the induction of Dkk-1 expression, a mechanism that is lost in a subset of colon cancers<sup>[5]</sup>. The Dkk-1 gene was silenced by CpG island promoter hypermethylation in colon cancer cell lines and in 9 (17%) of 54 primary colorectal cancers, especially in advanced Dukes' C and D colorectal cancers<sup>[7]</sup>. Dkk-1 showed suppressive effects on tumor growth through beta-catenin-independent non-canonical pathways (i.e., Wnt/JNK pathways) in human mesothelioma<sup>[8]</sup>. Kremen2 (Krm2) forms a ternary complex with Dkk-1 and LRP6 and induces rapid endocytosis and removal of the Wnt receptor LRP6 from the plasma membrane<sup>[10]</sup>.

Dkk-2 (chromosome 4q25) activates rather than inhibits the Wnt/beta-catenin signaling pathway in *Xenopus* embryos. Co-transfection of Krm2 blocked the ability of Dkk-2 to activate LRP6 and enhanced inhibition of Wnt/Frizzled signaling in human 293 fibroblasts. Dkk-2 expression was down-regulated in malignant melanoma cell lines and in most tumor samples<sup>[9]</sup>. Krm2 also cooperates with Dkk-4, but not with Dkk-3, to inhibit Wnt signaling. Krm2 can function as a switch that changes Dkk-2 from an activator to an inhibitor of Wnt/LRP6 signaling<sup>[11]</sup>.

Dkk-3/REIC gene (chromosome 11p15.2) expression level was reported to be decreased in non-small cell lung cancer<sup>[12,13]</sup>, renal clear cell carcinoma, urinary bladder cancer, prostate cancer, pancreatic carcinoma, hepatoma, malignant melanoma, and acute lymphocytic leukemia (ALL)<sup>[9,14-16]</sup>. Dkk-3 methylation occurred at an early stage in ALL pathogenesis and also influenced the prognosis<sup>[15]</sup>. Transfection of Dkk-3 into HeLa and Hep3B cells significantly reduced invasion capacity, cell motility, and tumor growth rate in inoculated athymic nude mice<sup>[16]</sup>.

Dkk-4 (chromosome 8p11.2-p11.1) mRNA was expressed in human embryonic stem cells differentiated to an early endodermal cell type, in breast cancer, and in diffuse-type gastric cancer. Dkk-4 is thought to be involved in the negative feedback mechanism of the canonical WNT/beta-catenin signaling pathway<sup>[17]</sup>.

Therefore, altered expression of Dkks and Krm2 appear to play important roles in tumor development and progression. Activated Wnt signal pathway, characterized by the stabilization of beta-catenin, plays an important role in most gastrointestinal cancers. We investigated the expression profiles and epigenetic alterations of the Dkks and Krm2 genes in gastrointestinal cancer in which the Wnt signaling plays an important role.

## MATERIALS AND METHODS

### Cell lines and tissue samples

Human esophageal cancer cell lines (TE 1, 3, 5, 6, 8-11, 14 and 15) were provided by the Cell Resource Center for Biomedical Research Institute of Development, Aging and Cancer, Tohoku University. Other esophageal cancer (T.T and T.Tn), gastric adenocarcinoma cell lines (AZ521, NUGC3, NUGC4, MKN28, MKN45, MKN74, KATOIII,

SNU1, SNU638, HGC27, and GC1Y), colorectal adenocarcinoma cell lines (Colo320DM, DLD1, HCT8, HCT15, HCT116, LoVo, LS123, LS174T, LS180, SKCO1, SW48, SW480, SW620, SW1116, SW1417 and WiDr), hepatocellular carcinoma cell line (CHC4, CHC32, Hep3B, HLF, HuH7 and PLC/PRF), biliary tract adenocarcinoma cell lines (TGBC1TKB and TGBC2TKB), pancreatic adenocarcinoma cell lines (BxPC3, Kp4, MiaPaCa, Panc1 and SuSu86) were purchased from the Japanese Cancer Research Resources Bank (Tokyo, Japan), Riken Cell Bank (Tokyo), or the American Type Culture Collection (Manassas, VA). Cells were cultured in DMEM or RPMI1640 supplemented with 10% fetal bovine serum. Paired specimens of tumor and adjacent non-tumor tissues of the esophagus ( $n = 10$ ), stomach ( $n = 24$ ), colorectum ( $n = 30$ ) and pancreas ( $n = 7$ ) were purchased from Genomics Collaborative (Laurel, MD). For RT-PCR, tumor cellularity is important. Only specimens containing more than 80% tumor cells were used for analysis.

### Semi-quantitative RT-PCR

Semi-quantitative reverse transcriptase-PCR was done as described previously<sup>[18,19]</sup>. Primer sequences were 5'-TCCGAGGAGAAATTGAGGAA-3' and 5'-CTGAGGCACAGTCTGATGA-3' for the Dkk-1 gene, 5'-AGTACCCGCTGCAATAATGG-3' and 5'-GAAATGACGAGCACAGCAAA-3' for the Dkk-2 gene, 5'-CTGGGAGCTAGAGCCTGATG-3' and 5'-TCA-TACTCATCGGGGACCTC-3' for the Dkk-3 gene, 5'-AGCTCTGGTCCTGGA CTTC-3' and 5'-CAACCCACGACATGTAGCAC-3' for the Dkk-4 gene, and 5'-ACGC AGCAACACAGCTACAG-3' and 5'-ATGTGACAGGAGGGGATGTC-3' for the Krm2 gene. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control of the reaction. All reactions were done at least in duplicate. The levels of gene transcripts were quantified as the ratio of the intensity of the Dkk or Krm2 gene to the intensity of GAPDH. To perform semi-quantitative RT-PCR, the ranges of linear amplification for the Dkk or Krm2 gene and for the GAPDH gene were studied by using standard curves. Underexpression or overexpression was judged when target gene expression in the tumor sample was at least four times lower or higher than that in the corresponding normal sample.

### Methylation-specific PCR (MSP)

Bisulfite modification of genomic DNA and MSP analysis were performed as described previously<sup>[20]</sup> using primers corresponding to the Dkk promoter region sequences. Sequences of methylation-specific primers were 5'-CGTTCGTTGGTAGTTTATTTATTCGA-3' and 5'-GCGACTACCTTTATACCGCGAA-3' for the Dkk-1 gene, 5'-GAGTAGAGAGA GAGAAA GCGGGAGTTC-3' and 5'-GTTATCCCCTAACTCA CAAAAACAACG-3' for the Dkk-2 gene, 5'-CGG TTTTTCGTTTTCGGG-3' and 5'-CAAACC GCTACATCTCC GCT-3' for the Dkk-3 gene, 5'-AGAAAAGTAGTGATAAATAGACGACGT-3' and 5'-CAACACTATACGTCACCAAAACGAA-3' for the Dkk-4



gene, and 5'-CGAGGCGGGTAG GAGTTAGTTC-3' and 5'-CGAAAAAATCTAACCGAAAAACGTT-3' for the Krm2 gene. Sequences of unmethylation-specific primers were 5'-TGTTTGTTGGTAGTTTTTATTTTG A-3' and 5'-ACCACAACCTTTATACCACAAA-3' for the Dkk-1 gene, 5'-AGAGAG TAGAGAGAG AGAAAGTGGGAGTTT-3' and 5'-ATTATCCCC TAACTCACAAAAAAC AAAAA-3' for the Dkk-2 gene, 5'-TTTTGGTTTTTTTTTTGTTTTTTGGG-3' and 5'-CCAA ACCACTACATCTCCACT-3' for the Dkk-3 gene, 5'-AGAAAAAGTAGTGATAAATAGA TGATGT-3' and 5'-CAACACTATACATCA CCAAAACAAA-3' for the Dkk-4 gene, and 5'-GTG AGGTGGGTAGGAGTTAGTTTGT-3' and 5'-AAA AAAAATCTAACCAAAAAACATT-3' for the Krm2 gene.

### Treatment of cancer cells

Cancer cells were treated with 2  $\mu$ mol/L or 5  $\mu$ mol/L of 5-aza-2'-deoxycytidine (5-aza-dC) (Sigma-Aldrich, MO, USA) for 72 h or with 600 nmol/L of trichostatin A (TSA) (ICN Biomedicals) for 24 h. Cells were also treated with 2  $\mu$ mol/L of 5-aza-dC for 72 h followed by 600 nmol/L of TSA for an additional 24 h. The timing and sequencing of 5-aza-dC and/or TSA was based on similar preliminary studies as well as published studies<sup>[21]</sup>. Immediately after completion of treatments, cells were harvested for RNA purification. RT-PCR was performed after 5-aza-dC and/or TSA treatment.

### Immunohistochemistry on tissue microarray

A tissue microarray (SuperBioChips Laboratories, Seoul, Korea) was used for immunohistochemistry. Immunohistochemistry was performed as described previously<sup>[22,23]</sup>. The antibodies used were as follows: goat anti-human Dkk-1 and Dkk-3 (ABCAM, Cambridge, UK), rabbit anti-human Dkk-2 and Krm (ABGENT, San Diego, CA), rabbit anti-human Dkk-4 (Santa Cruz, CA, USA), mouse anti-human Rac (Sigma-Aldrich), rabbit anti-human phospho-Rac (Cell Signaling, MA), rabbit anti-human Ca<sup>2+</sup>/calmodulin-dependent protein kinaseII (CaMKII) (Santa Cruz), and rabbit anti-human phospho-CaMKII (EPITOMICS, CA, USA). Normal rabbit or mouse immunoglobulins were substituted for each primary antibody as negative controls. The results were analyzed based on the intensity of the expression in the tumors; negative (no staining at high magnification), weak (only visible at high magnification), moderate (readily visible at low magnification) and strong (strikingly positive at low magnification).

### Western blot analysis

Western blot analysis was performed as described previously<sup>[18]</sup>. The protein signals were visualized by an enhanced chemiluminescence using ECL Western blotting detection reagents (Amersham, IL, USA).

### siRNA preparation and in vitro transfection

siRNA preparation and *in vitro* transfection were performed as described previously<sup>[22]</sup>. Levels of mRNA and protein inhibition were analyzed by RT-PCR and

Western blot analysis. siRNA-transfected cells were used for WST-8 and cell invasion assays.

### WST-8 assay

Tumor cell growth was evaluated using the tetrazolium compound WST-8 (Cell Counting Kit-8 Dojindo Laboratories, Kumamoto, Japan) as described previously<sup>[22]</sup>. Cell viability was determined according to the manufacturer's instructions.

### In vitro invasion assay

Assays were performed by the modified Boyden Chamber method as described previously<sup>[24]</sup>. The results were presented as mean  $\pm$  SD for each sample.

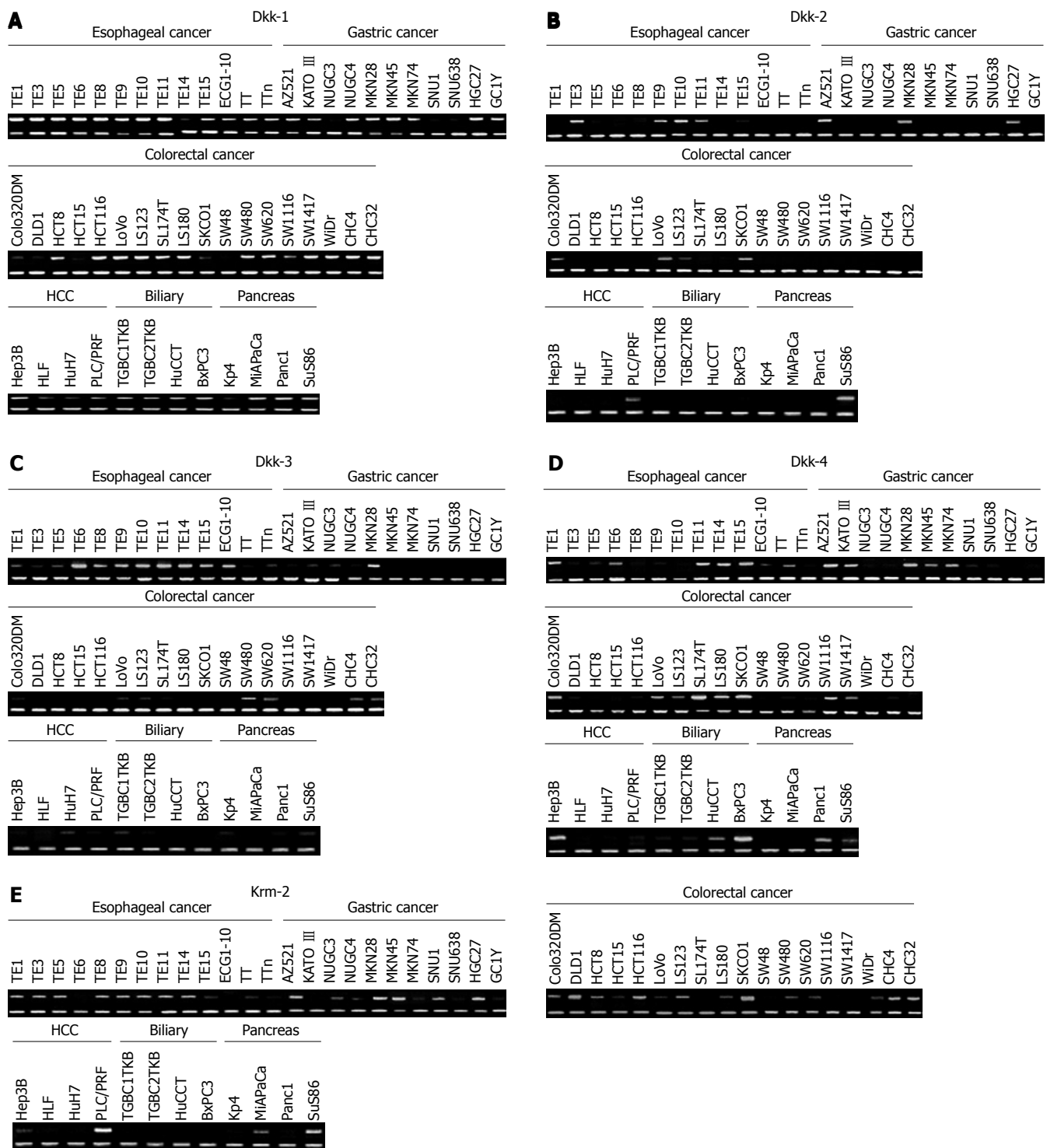
### Statistical analysis

Data were analyzed by using the computer software package SPSS for Windows 12.0. A *P* value less than 0.05 was considered statistically significant. The expression level of the Dkks and Krm2 genes was assessed for associations with clinicopathological characteristics using the following statistical tests: Student's *t*-test for age; the Mann-Whitney test for histological type, depth of invasion, pathological TNM stage; and the chi-square test or Fisher's exact test for the remaining parameters. For cell growth and invasion assay, all of the data were analyzed by one-way analysis of variance and the Bonferroni (Dunn) multiple-comparison method.

## RESULTS

### mRNA expression of the Dkk subfamily genes and Krm2 gene in gastrointestinal cancers

Expression levels of the Dkk genes and Krm2 gene in cancer cell lines and paired normal and cancer tissues were analyzed by using semi-quantitative RT-PCR and immunohistochemistry. The mRNA expression levels of Dkks were reduced in a certain subset of the esophageal, gastric, colorectal, hepatocellular, and pancreatic cancer cell lines (Figure 1). Reduction in the mRNA expression levels of Dkk-1, Dkk-2, Dkk-3, Dkk-4, and Krm2 was observed in 2/10 (20.0%), 3/10 (30.0%), 3/10 (30.0%), 3/10 (30.0%), and 4/10 (40.0%) of esophageal cancer tissues, in 5/24 (20.8%), 2/24 (8.3%), 6/24 (25.0%), 8/24 (33.3%), and 21/24 (87.5%) of gastric cancer tissues, in 6/30 (20.0%), 8/30 (26.7%), 12/30 (40.0%), 4/30 (13.3%), and 0/30 (0%) of colorectal cancer tissues, and in 1/7 (14.3%), 1/7 (14.3%), 2/7 (28.6%), 1/7 (14.3%) and 0/7 (0%; no expression) of pancreatic cancer tissues, respectively (Figure 2 and data not shown). On the other hand, over-expression of Dkks and Krm2 mRNA was also found in these cancer tissues. Dkk-1, Dkk-2, Dkk-3, Dkk-4 and Krm2 mRNA over-expression was observed in 5/10 (50.0%), 3/10 (30.0%), 2/10 (20.0%), 4/10 (40.0%) and 4/10 (40.0%) of esophageal cancer tissues, in 9/24 (37.5%), 16/24 (66.7%), 9/24 (37.5%), 11/24 (45.8%), and 3/24 (12.5%) of gastric cancer tissues, in 15/30 (50.0%), 14/30 (46.7%), 7/30 (23.3%), 16/30 (53.3%) and 4/30 (13.3%) of colorectal cancer tissues, and in 3/7 (42.9%), 3/7 (42.9%), 2/7 (28.6%), 3/7 (42.9%) and 0/7 (0%;



**Figure 1** RT-PCR analysis of the Dkks and Krm2 genes in gastrointestinal cancer cell lines. RT-PCR was performed using cDNA from 13 esophageal, 11 gastric, 16 colorectal, 6 hepatocellular, 3 biliary tract and 5 pancreas cancer cell lines.

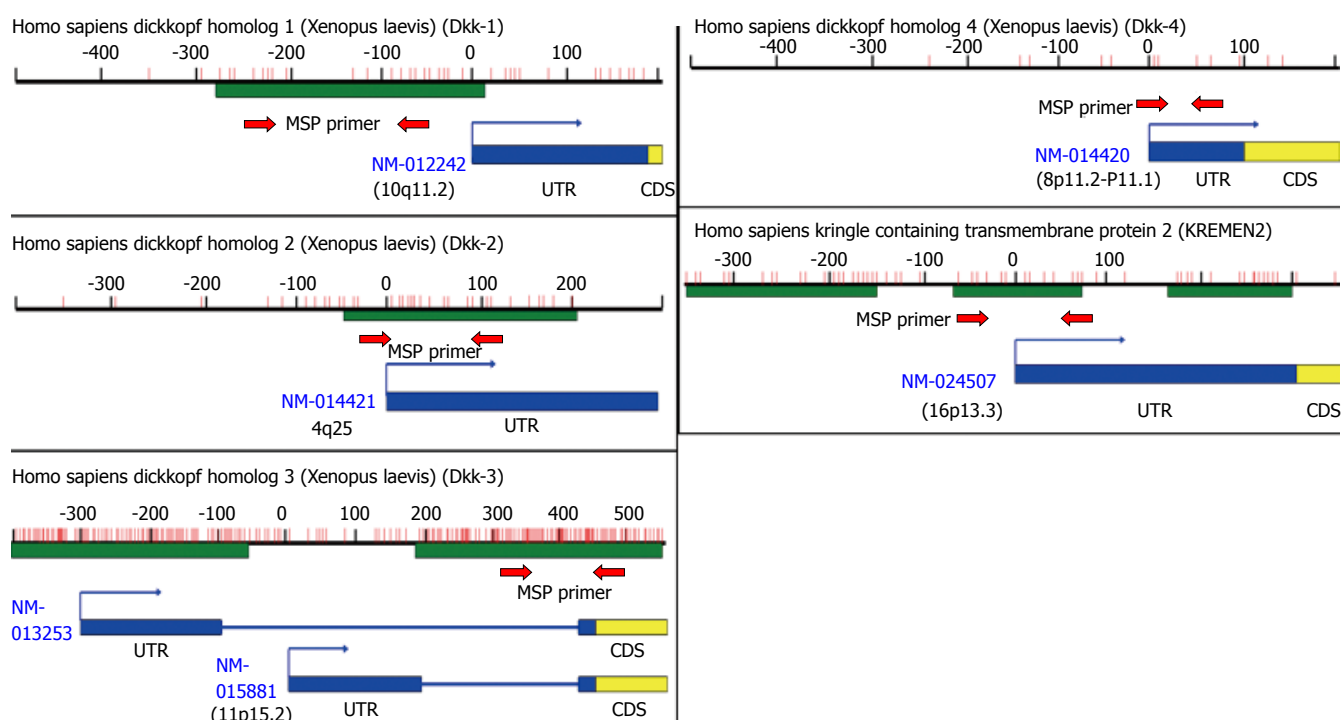
no expression) of pancreatic cancer tissues, respectively (Figure 2 and some data not shown).

### Protein expression of the Dkk subfamily genes and Krm2 gene in gastrointestinal cancers

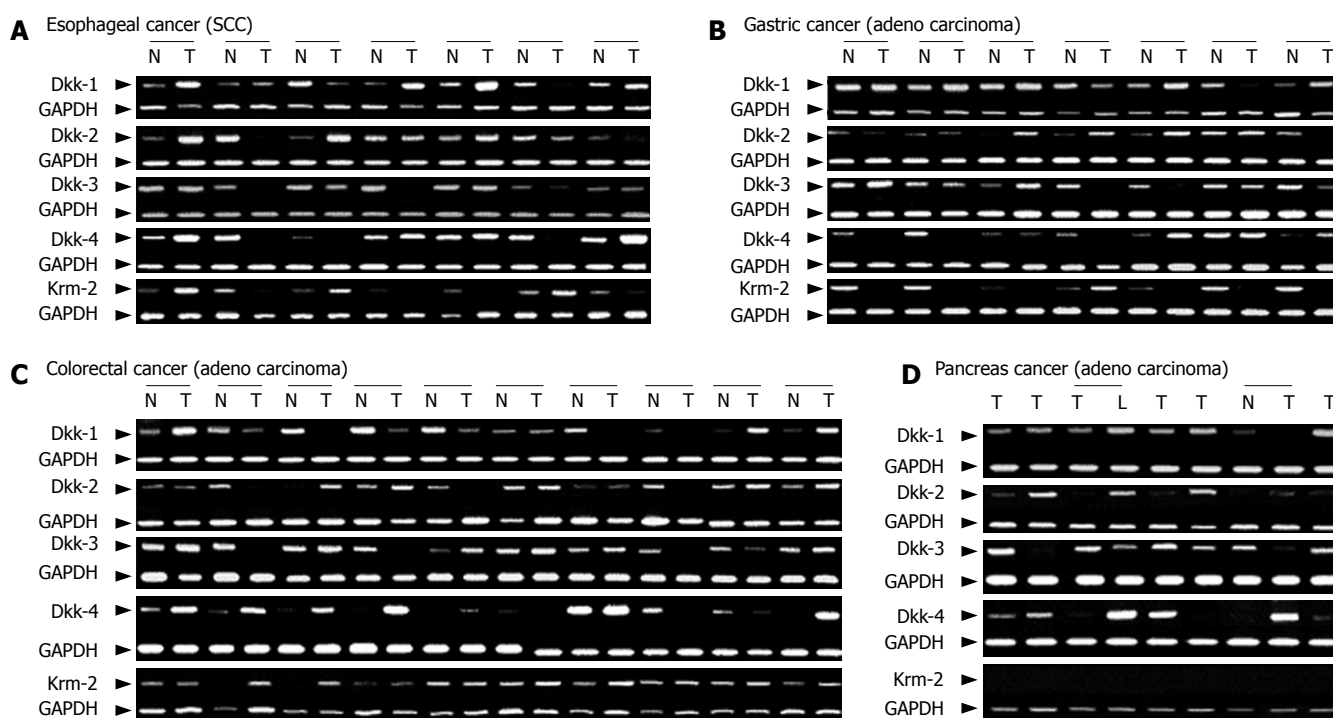
In normal tissues, the squamous epithelium in the esophagus expressed Dkks and Krm2 protein in the more keratinized cells of the epithelium (Figure 3A). Expression levels of Dkk-1, Dkk-2, Dkk-4, and Krm2 protein were strong in secreting cells of the gastric glands.

Expression level of Dkk-3 protein was weak in the gastric glands (Figure 3A). In the normal colorectal mucosa, Dkk-1, Dkk-3, Dkk-4, and Krm2 were localized in well-differentiated cells of crypts, whereas Dkk-2 was localized in secreting cells in proliferation layer in crypts (Figure 3A). These results may explain, at least in part, the variable levels of mRNA for these genes in normal tissues. There was no detectable immunoreactivity with the control normal rabbit or mouse immunoglobulins (data not shown).

The protein expression levels of Dkks and Krm2



**Figure 4** Scheme of the promoter regions of the Dkk genes and Krm2 gene. The 5' regions of the genes were identified by a BLAST search. We used a CpG island searcher that screens for CpG islands that meet the following criteria: CG percentage > 55% observed CpG/expected CpG > 0.65 length > 500 bp. CpG sites are represented by vertical bars. The arrows below CpG islands represent regions analyzed by MSP. The Dkk-1, Dkk-2, Dkk-3, and Krm2 genes contain CpG islands at their 5' ends and Dkk-4 has a few CpGs in the promoter region.

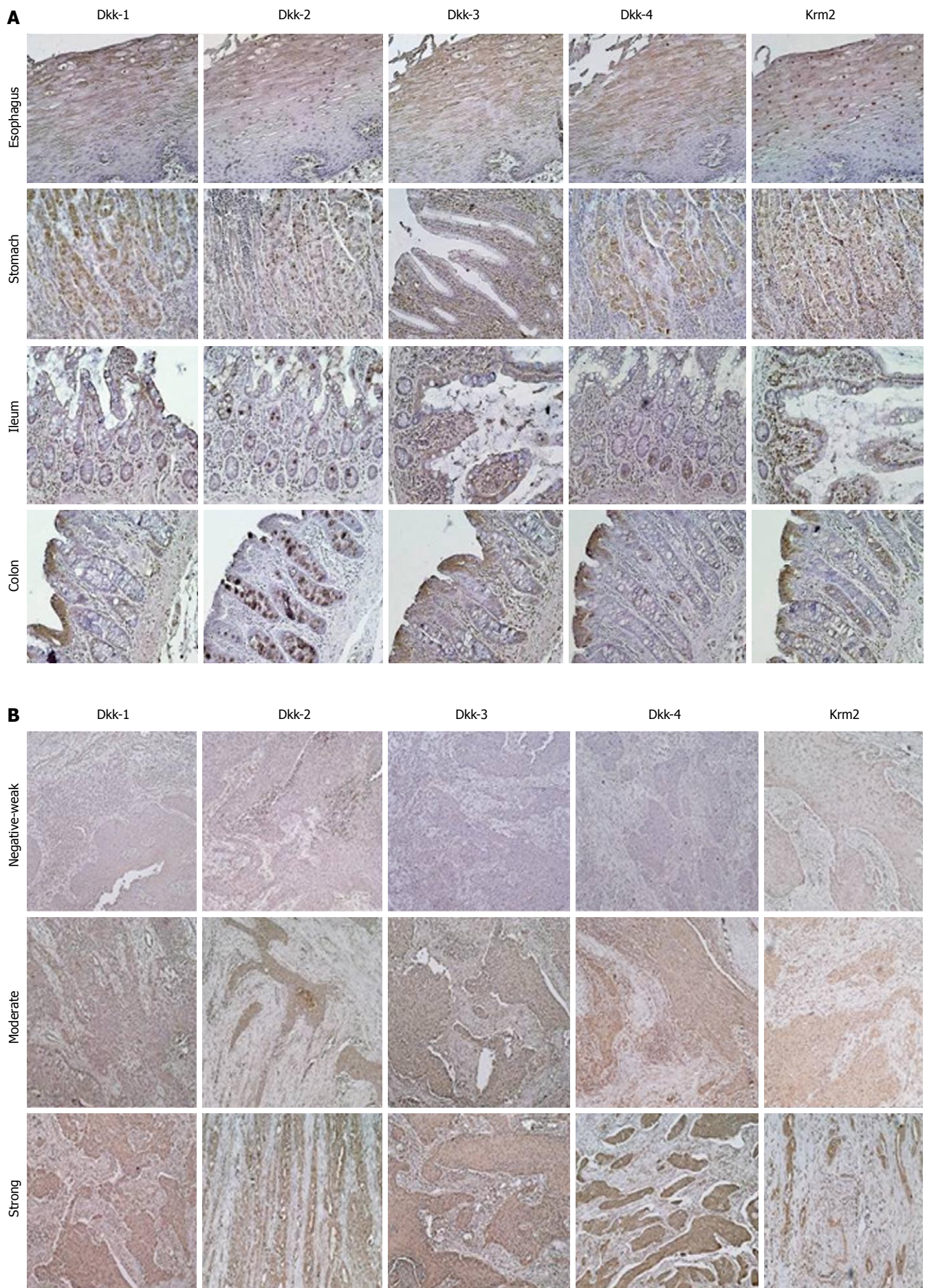


**Figure 2** RT-PCR analysis of the Dkks and Krm2 genes in gastrointestinal cancer tissues. Expression of GAPDH was examined to monitor cDNA integrity. N and T are matched samples from non-tumor and tumor tissue, respectively.

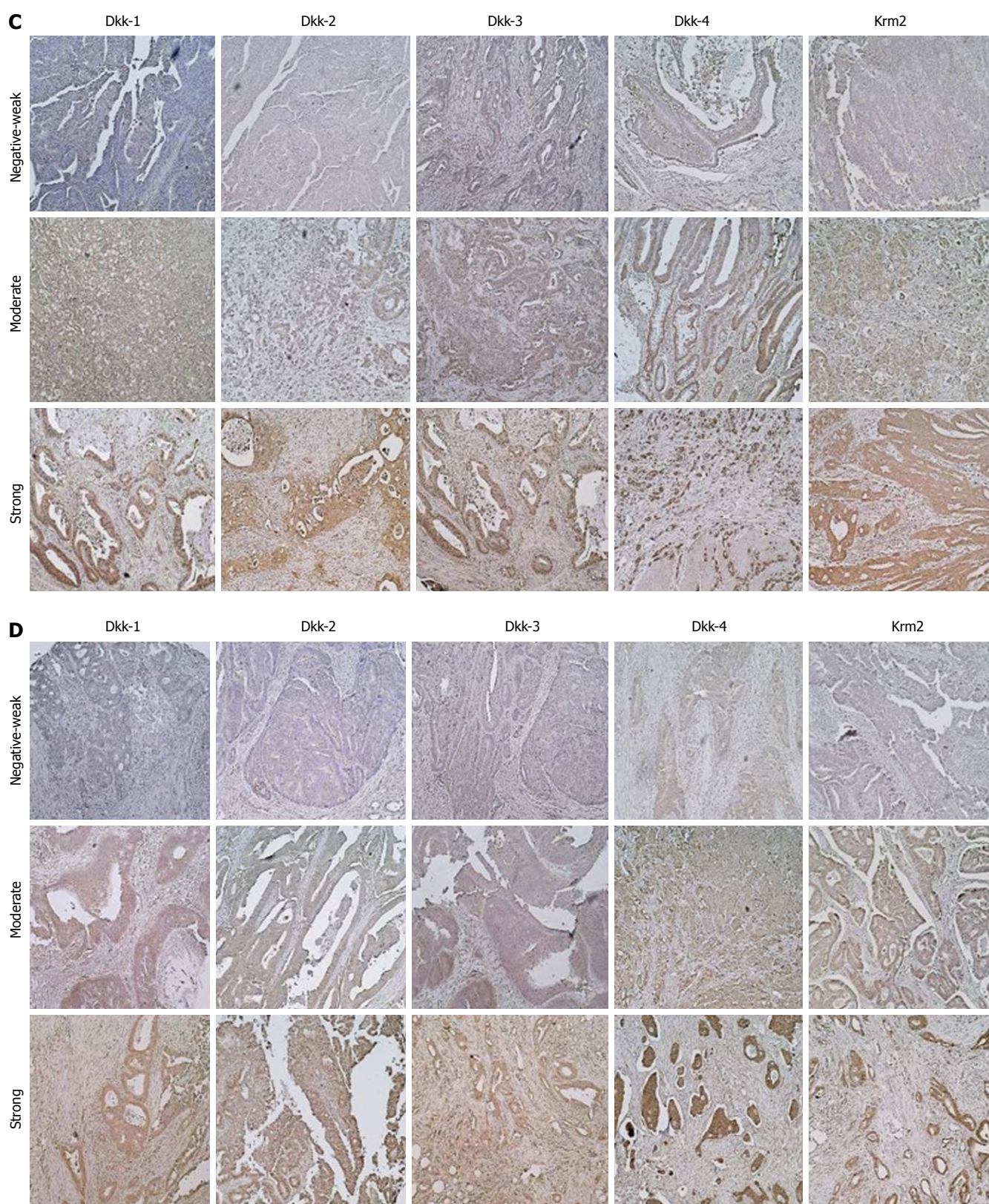
were reduced in a certain subset of the cancer tissues (Figure 3B-D). The protein expression levels of Dkk-1, Dkk-2, Dkk-3, Dkk-4, and Krm2 were negative or weak in 7/59 (11.9%), 33/59 (55.9%), 18/59 (30.5%), 7/59 (11.9%)

and 22/59 (37.3%) of esophageal cancer tissues, in 18/60 (30.0%), 19/60 (31.7%), 20/59 (33.9%), 6/59 (10.2%) and 21/60 (35.0%) of gastric cancer tissues, and in 21/60 (35.0%), 27/60 (45.0%), 27/60 (45.0%), 7/60 (11.7%) and





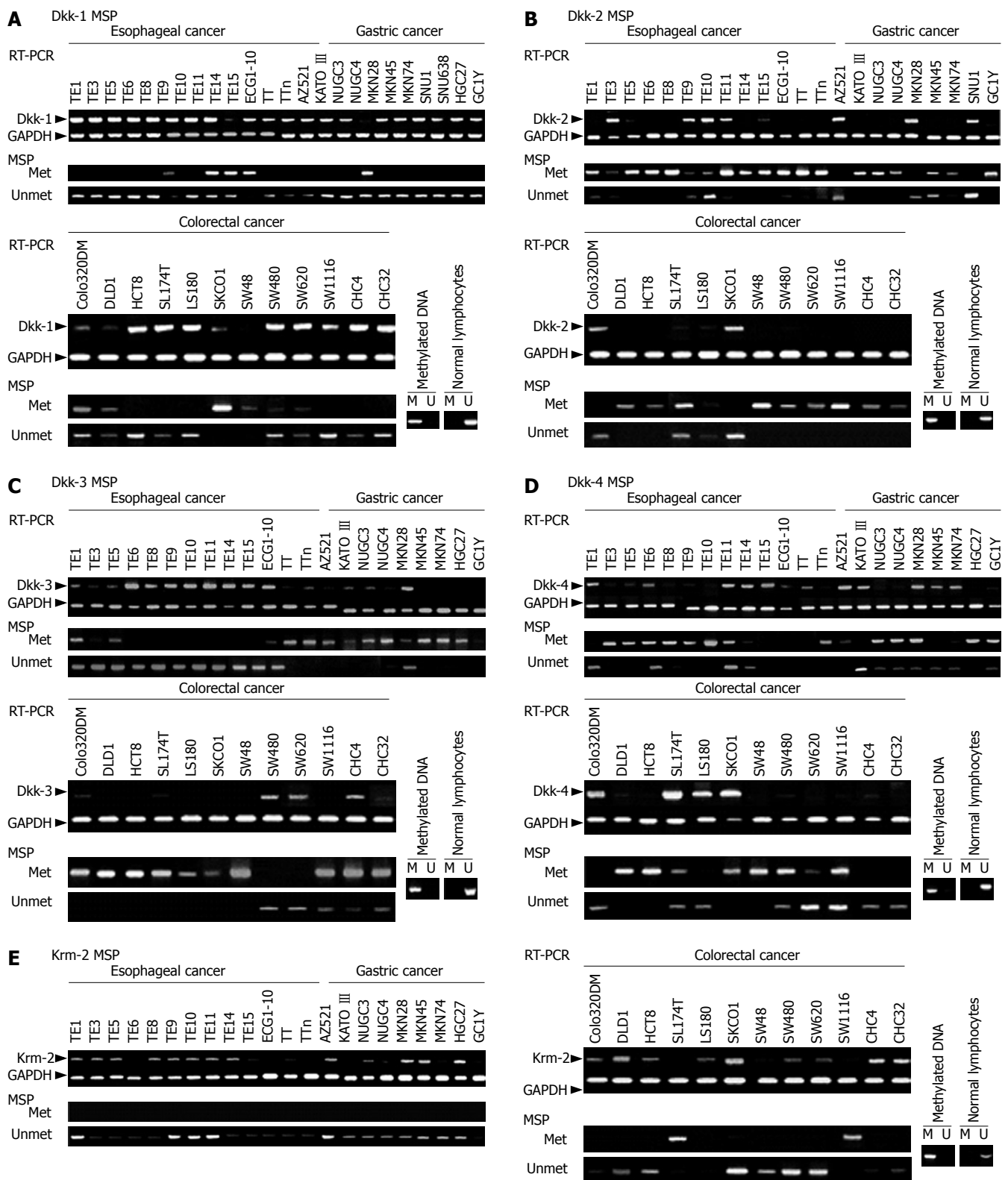




**Figure 3** Immunohistochemical expression of Dkks in gastrointestinal normal and cancer tissues. **A:** Normal tissues; **B:** Esophageal cancer tissues; **C:** Gastric cancer tissues; **D:** Colorectal cancer tissues ( $\times 200$ ).

6/60 (10.0%) of colorectal cancer tissues, respectively. On the other hand, strong staining of Dkk-1, Dkk-2, Dkk-3, Dkk-4, and Krm2 was found in 28/59 (47.5%), 9/59 (15.3%), 10/59 (16.9%), 28/59 (47.5%) and 11/59 (18.6%) of esophageal cancer tissues, in 15/60 (25.0%), 14/60

(23.3%), 22/59 (37.3%), 33/59 (55.9%) and 7/60 (11.7%) of gastric cancer tissues, and in 10/60 (16.7%), 12/60 (20.0%), 6/60 (10.0%), 35/60 (58.3%) and 28/60 (46.7%) of colorectal cancer tissues, respectively (Figure 3B-D and data not shown). Expression levels of Dkks and Krm2



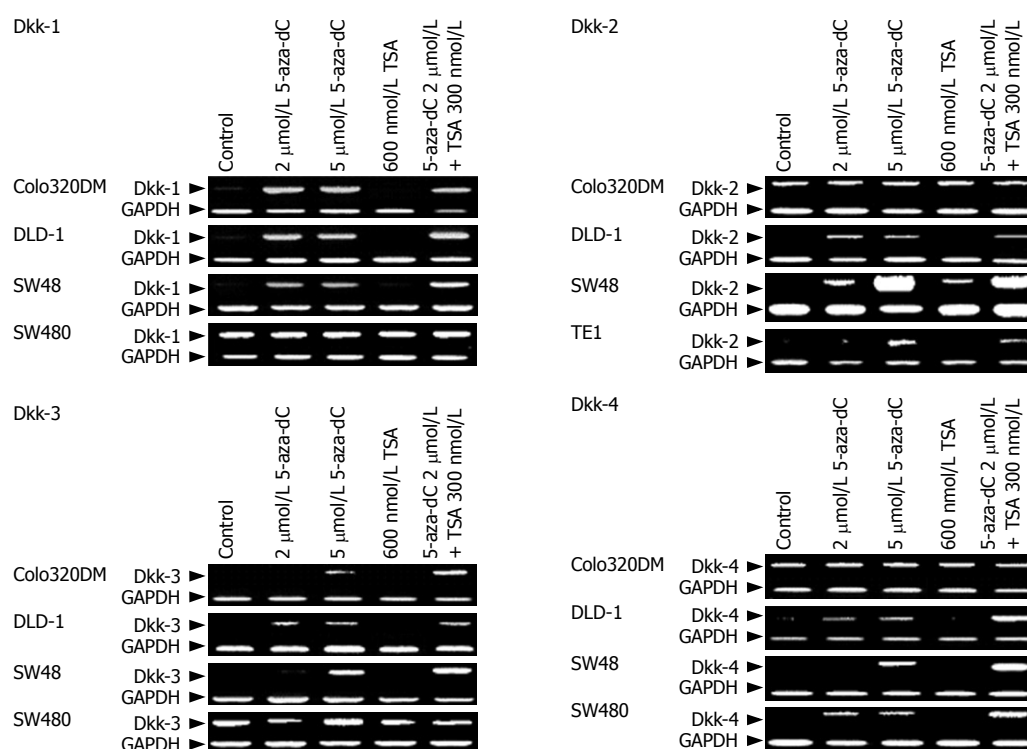
**Figure 5** Correlation of methylation in the promoter region with silencing of the Dkk genes in gastrointestinal cancer cell lines. RT-PCR and MSP were carried out using cDNA and genomic DNA from the indicated cancer cell lines, respectively. *In vitro* methylated DNA (CpG Genome Universal Methylated DNA from Chemicon International, Temecula, CA) was used as a positive control for methylated alleles, while DNA from normal lymphocytes was used as negative controls.

were not correlated with any of the clinicopathological characteristics. However, in colorectal cancers with beta-catenin over-expression, Dkk-1 expression levels were significantly lower in those with lymph node metastasis than in those without lymph node metastasis ( $P = 0.003$ ).

### CpG island hypermethylation of the Dkk subfamily genes in gastrointestinal cancer

Using Blast and CpG island searcher, we found that Dkk-1, Dkk-2, Dkk-3, and Krm2 genes contain CpG islands at their 5'ends and that Dkk-4 has a few CpGs in the





**Figure 6** Reactivation of Dkks expression by 5-aza-dC treatment in gastrointestinal cancer cell lines. Cells were treated indicated.

promoter region (Figure 4). Therefore, we analyzed the methylation status of the CpG islands of these genes in cancer cell lines and tissue samples using MSP. Methylation status was significantly associated with silencing of Dkks mRNA expression in cancer cell lines (Figure 5 and data not shown). Methylation status was associated with silencing of Krm2 mRNA expression in only some cancer cell lines, such as LS174T and SW1116 cells (Figure 5E). In cancer tissues, Dkk-1, Dkk-2, Dkk-3, and Dkk-4 were hypermethylated in 4/11 (36.4%), 1/11 (9.1%), 1/11 (9.1%) and 3/11 (27.3%) of esophageal cancer tissues, in 2/8 (25.0%), 2/8 (25.0%), 2/8 (25.0%) and 3/8 (37.5%) of gastric cancer tissues, and in 7/20 (35.0%), 13/20 (65.0%), 7/20 (35.0%) and 4/20 (20.0%) of colorectal cancer tissues, respectively (data not shown).

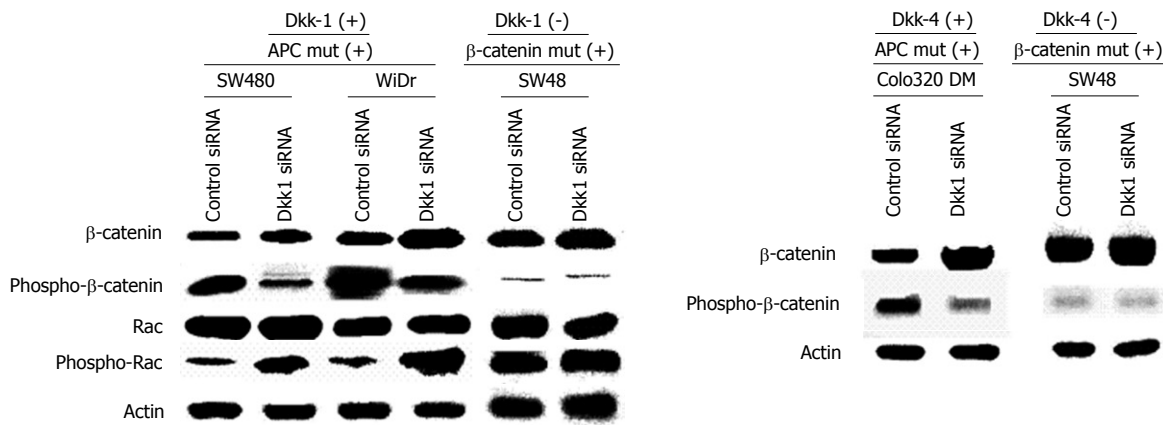
To confirm the role of epigenetic change in silencing of the Dkk genes, cell lines that lacked the Dkk genes expression were treated with 5-aza-dC and/or TSA. Treatment with 5-aza-dC resulted in restoration of Dkk-1 in colo320DM, DLD-1, and SW48 cells, restoration of Dkk-2 expression in DLD-1, SW48, and TE1 cells, restoration of Dkk-3 expression in colo320DM and DLD-1 cells, and restoration of Dkk-4 expression in DLD-1, SW48, and SW480 cells (Figure 6). However, treatment with TSA had no effect.

### **Correlations between expression levels of Dkks and those of canonical and non-canonical Wnt pathway signal genes in gastrointestinal cancer**

There were some binding motifs of beta-catenin (5'-YCTTTGWW-3') in the promoter regions of Dkk-1 and Dkk-4. Dkk-2, Dkk-3 and Krm2 did not have TCF-binding motifs in their promoter regions. There were significant correlations between levels of Dkk-1, Dkk-3, Dkk-4 and Krm2 and those of beta-catenin expression in

colorectal cancer tissues in tissue microarray data obtained by immunohistochemistry. Dkk-1 ( $r = 0.500$ ,  $P < 0.0001$ ), Dkk-3 ( $r = 0.326$ ,  $P = 0.0130$ ), Dkk-4 ( $r = 0.480$ ,  $P = 0.0003$ ) and Krm2 ( $r = 0.454$ ,  $P = 0.0005$ ) expression levels showed significant correlations with beta-catenin over-expression. However, no correlation was found between expression levels of these molecules and beta-catenin over-expression in gastric or esophageal cancer tissues (data not shown). We then analyzed expression levels of beta-catenin and phosphorylated beta-catenin in Dkk1 or Dkk4-specific siRNA-treated colon cancer cells with APC mutation (SW480, WiDr, and colo320DM cells) and SW48 cells with beta-catenin mutation using Western blot analysis. SW480 and WiDr cells treated with Dkk1-specific siRNA showed higher levels of beta-catenin expression and lower levels of phosphorylated beta-catenin compared with those of the control siRNA-treated cells (Figure 7). On the other hand, Dkk1-negative SW48 cells treated with Dkk1-specific siRNA showed no difference compared with the control cells. Colo320DM cells treated with Dkk4-specific siRNA showed higher levels of beta-catenin expression and lower levels of phosphorylated beta-catenin compared with those of the control siRNA-treated cells (Figure 7). On the other hand, Dkk4-negative SW48 cells treated with Dkk4-specific siRNA showed no difference compared with the control cells.

Next, we examined expression of Rac and phosphorylated Rac/CDC42, which Wnt4/5A/11 activates through Dvl, and CaMKII and phosphorylated CaMKII to investigate the effects on Wnt-PCP and Wnt-Ca<sup>2+</sup> signal transduction. Expression of Rac, phospho-Rac, CaMKII, and phospho-CaMKII was not correlated with any of the clinicopathological features. Rac activation ratio ((phospho-Rac) level/(Rac) level)/(Rac) level) was negatively correlated with Dkk-1 in colorectal cancer ( $r = -0.366$ ,  $P = 0.0053$ )



**Figure 7** Western blot analysis in cancer cells treated with Dkk-specific siRNA.

and with Dkk-2 ( $r = -0.290$ ,  $P = 0.0299$ ) and Dkk-3 ( $r = -0.3880$ ,  $P = 0.0037$ ) in esophageal cancer, but not in gastric cancer. On the other hand, CaMKII activation ratio was positively correlated with Dkk-2 ( $r = 0.285$ ,  $P = 0.0314$ ) and Krm2 ( $r = 0.3640$ ,  $P = 0.0075$ ) in esophageal cancer, but not in colorectal cancer or gastric cancer. We analyzed Rac expression level and the phosphorylation status of Rac in cells treated with Dkk1-specific siRNA using Western blot analysis. SW480 and WiDr cells treated with Dkk1-specific siRNA showed similar expression levels of Rac and higher levels of phosphorylated Rac compared with those of cells treated with the control siRNA (Figure 7). On the other hand, Dkk-1-negative SW48 cells treated with Dkk1-specific siRNA showed no difference compared with the control cells.

#### Enhancement of cancer cell growth and invasiveness by Dkk-1, Dkk-2, Dkk-3, or Dkk-4 siRNA treatment

WST-8 assays and *in vitro* invasion assays after treatment with specific siRNA for the Dkk genes were performed to assess the role of the expression of Dkks in cancer cell growth and invasiveness. Transfection with siRNA resulted in over 80% inhibition of mRNA and protein expression (data not shown). Transfection with Dkk1-specific siRNA enhanced the growth of SW480 and WiDr cells compared with control siRNA-transfected counterparts (Figure 8A;  $P < 0.01$ ). Transfection with Dkk2-, Dkk3- and Dkk4-specific siRNA enhanced the growth of TE9, SW480 and colo320DM cells compared with control siRNA-transfected counterparts, respectively (Figure 8A;  $P < 0.05$ ,  $P < 0.001$  and  $P < 0.001$ ). Transfection with Dkk-1, Dkk-3, or Dkk-4-specific siRNA enhanced the invasiveness of TE-1 cells compared with control siRNA-transfected counterparts (Figure 8B and C  $P < 0.01$ ). Transfection with Dkk-1 or Dkk-3-specific siRNA enhanced the invasiveness of TE-8 cells compared with control siRNA-transfected counterparts (Figure 8B and C,  $P < 0.01$ ). Transfection with Dkk-1-specific siRNA enhanced the invasiveness of SW480 cells compared with control siRNA-transfected counterparts (Figure 8B and C,  $P < 0.01$ ).

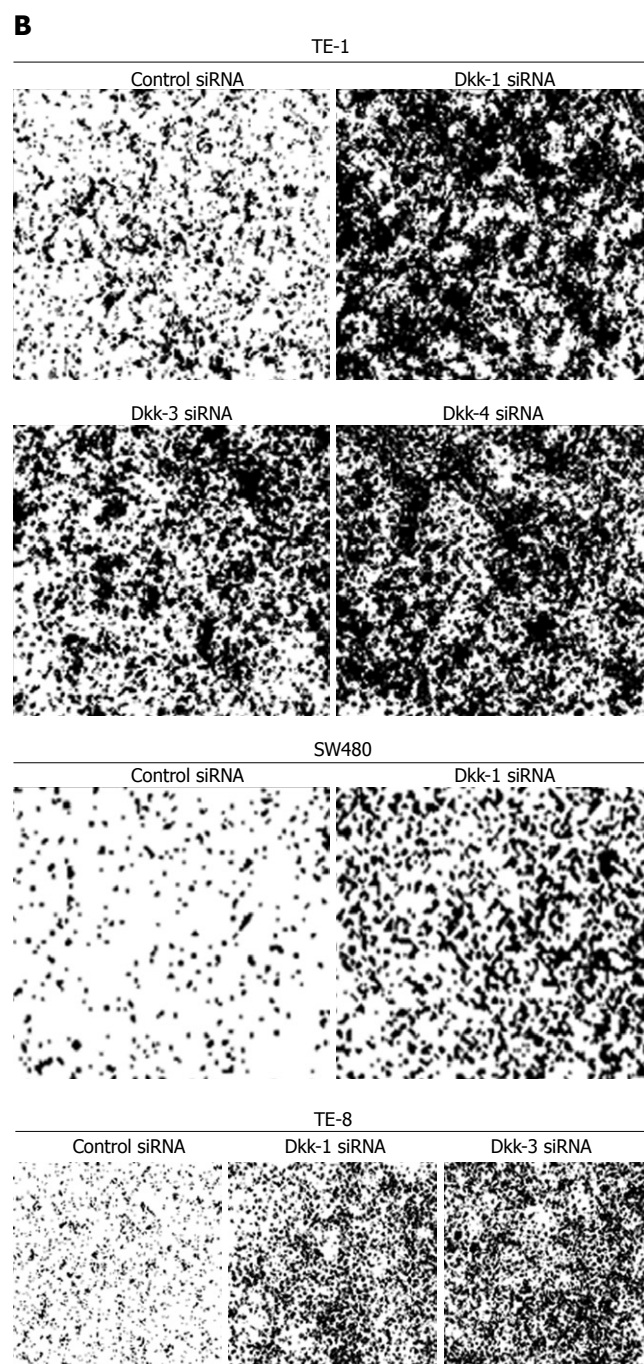
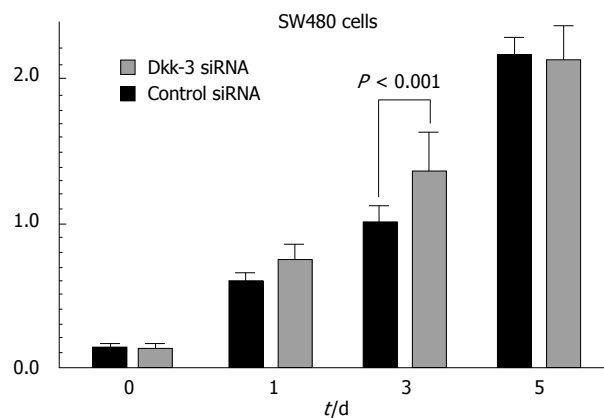
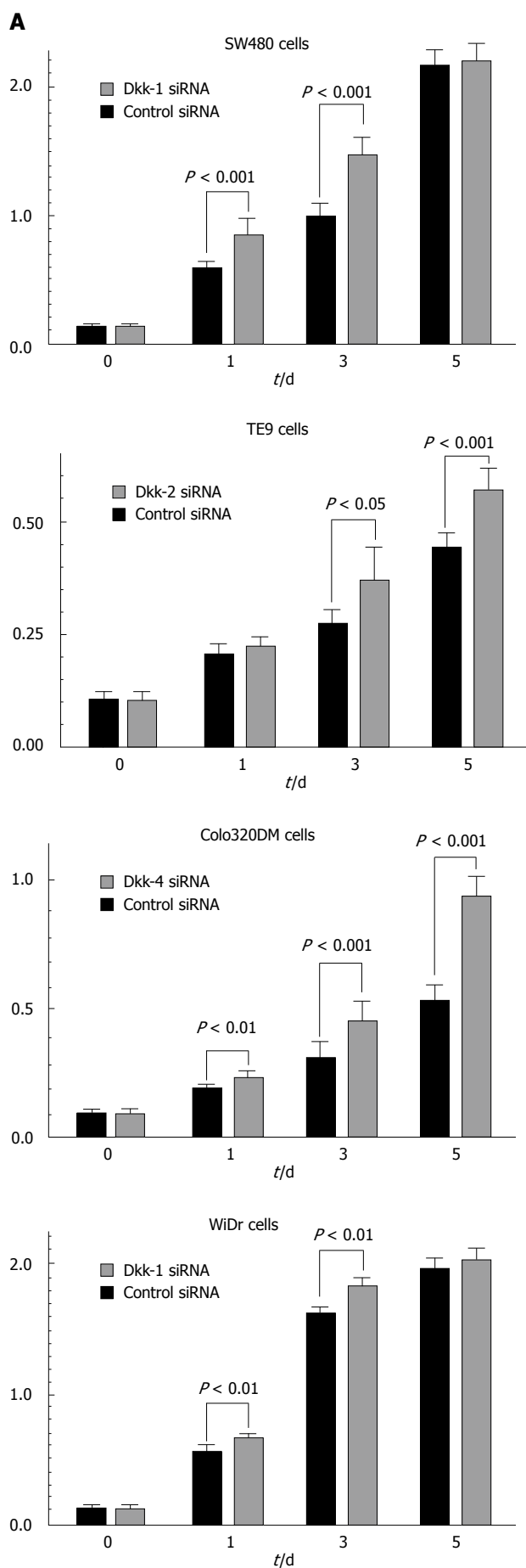
## DISCUSSION

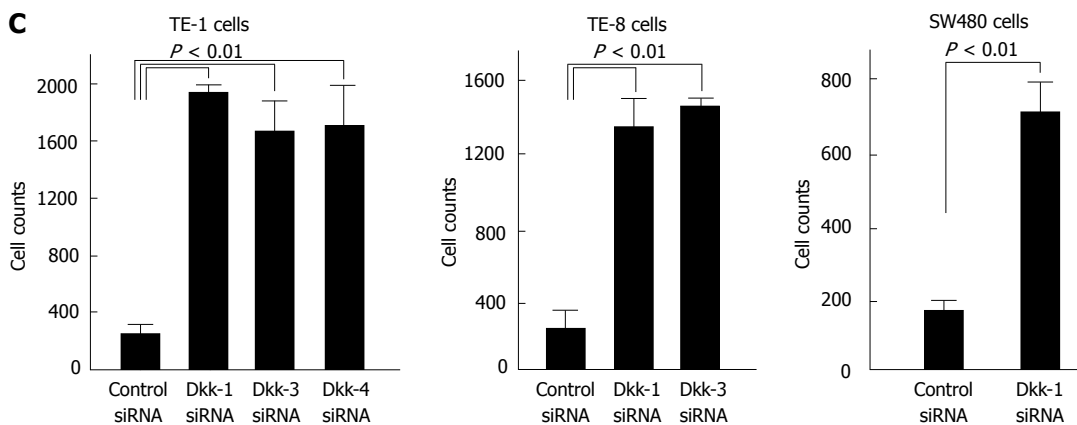
In the present study, we investigated the expression profiles

and epigenetic alterations of the Dkks and Krm2 genes in gastrointestinal cancer. Dkks and Krm2 expression levels were reduced in a certain subset of gastrointestinal cancer cell lines and cancer tissues. Methylation of Dkk-1, Dkk-2, Dkk-3 and Dkk-4 was significantly correlated with down-regulation of expression in gastrointestinal cancer cell lines and cancer tissues. Moreover, Dkk-1, Dkk-2, Dkk-3 and Dkk-4 mRNA expression was restored by treatment with the DNA-demethylating agent. These results suggest that promoter hypermethylation is an important mechanism of silencing of Dkk family in gastrointestinal cancer. Although there were only a few CpGs in the Dkk-4 promoter region, these CpG sites in cancer tissues were more methylated than in normal tissues. Similarly, the expression of rat placental lactogen-1 (rPL-I) has been reported to be controlled by DNA methylation although the gene has only 17 CpG sites in the 5'-flanking region<sup>[25]</sup>.

Over-expression of Dkk-1, Dkk-2, Dkk-3 and Dkk-4 was also found in a subset of gastrointestinal cancer tissues. This is not a surprising result, because of the following reasons. There are binding motifs of beta-catenin in the promoter regions of Dkk-1 and Dkk-4. Multiple beta-catenin/TCF4 sites in the Dkk-1 gene promoter have been reported to contribute to Dkk-1 activation, thus initiating a negative feedback loop<sup>[5]</sup>. In fact, Dkk-1 mRNA levels have been increased in a subset of colorectal cancer tissues compared to normal tissues<sup>[5]</sup>. In our study, Dkk-1, Dkk-3, Dkk-4 and Krm2 expression was correlated with beta-catenin over-expression in colorectal cancer tissues. Moreover, knockdown of Dkk-1 or Dkk-4 up-regulated levels of beta-catenin expression and down-regulated levels of phosphorylated beta-catenin. Therefore, Dkks and Krm2 could be directly and/or indirectly induced by Wnt signals, at least in part, as components of negative feedback loops; but, this mechanism may be lost or abolished in a certain subset of colorectal cancers by promoter hypermethylation. On the other hand, no correlation was found between expression levels of Dkks and Krm2 and beta-catenin over-expression in gastric or esophageal cancer tissues. Further analysis is required to clarify the mechanism of over-expression of Dkks and Krm2 in a subset of gastric and esophageal cancers.







**Figure 8** Enhancement of cancer cell growth and invasiveness by Dkk siRNA treatment. **A:** WST-8 assays after treatment with specific siRNA for the Dkk genes were performed to assess the role of the expression of Dkks in cancer cell proliferation; **B and C:** *In vitro* invasion assays after treatment with specific siRNA for the Dkk genes were performed to assess the role of the expression of Dkks in cancer cell invasiveness.

Next, we examined correlations of altered expression of Dkks and Krm2 with expression of the Wnt non-canonical pathway genes. It has been reported that the Wnt canonical pathway activation stabilizes beta-catenin and that the non-canonical pathway activates Rho, Rac, JNK, and PKC or activates CaMK II<sup>[26]</sup>. The TAK1-NLK MAPK cascade is activated by the non-canonical Wnt-5A/Ca<sup>2+</sup> pathway and antagonizes canonical Wnt/beta-catenin signaling<sup>[27,28]</sup>. In the present study, Rac activation was negatively correlated with Dkk-1 expression in colorectal cancer and with Dkk-2 and Dkk-3 in esophageal cancer. These results suggest that Dkk-1 down-regulation in colorectal cancer and Dkk-2 or Dkk-3 down-regulation in esophageal cancer play a role in Rac activation. Moreover, knockdown of Dkk-1 using Dkk-1-specific siRNA induced Rac phosphorylation. On the other hand, CaMKII activation showed a weak positive correlation with Dkk-2 and Krm2 in esophageal cancer. Although further investigation is required, these results suggest additional effects of Dkks and Krm2 on the Wnt non-canonical pathway.

In colorectal cancers with beta-catenin over-expression, Dkk-1 expression levels were significantly lower in those with lymph node metastasis than in those without lymph node metastasis. Dkk-1 promoter has been reported to be hypermethylated in advanced Dukes' C and Dukes' D colorectal cancers<sup>[7]</sup>. Transfection of Dkk-3 has been reported to reduce invasion of osteosarcoma and malignant melanoma<sup>[9]</sup>. We further revealed that down-regulation of Dkks expression by siRNA resulted in a significant increase in esophageal and colon cancer cell growth and invasion *in vitro*. Thus, down-regulation of Dkks may contribute to the more aggressive phenotype of esophageal and colorectal cancer cells.

Our results indicate that promoter hypermethylation is an important mechanism of silencing of Dkk family in gastrointestinal cancer. Suzuki *et al.*<sup>[29,30]</sup> reported epigenetic inactivation of secreted frizzled-related proteins (sFRPs) in colorectal and gastric cancer. We reported silencing of the Wnt inhibitory factor-1 gene due to promoter hypermethylation in gastrointestinal tumors<sup>[18]</sup>. Thus, CpG island promoter hypermethylation is a common mechanism of the inactivation of extracellular Wnt

antagonists in gastrointestinal cancer. Activated Wnt signal pathway, characterized by the stabilization of beta-catenin, plays an important role in most gastrointestinal cancers. Modulation of the Wnt pathway, through reversal of extracellular Wnt antagonists silencing by demethylating agents, may be a potential target for treatment and/or prevention of gastrointestinal cancer.

## COMMENTS

### Background

Dickkopfs (Dkks) are secreted antagonists of Wnt signaling pathway. Activated Wnt signal pathway, characterized by the stabilization of beta-catenin, plays an important role in most gastrointestinal cancers. Extracellular Wnt antagonists such as secreted frizzled-related proteins (SFRPs) and WIF-1 is frequently inactivated in gastrointestinal cancer. Thus, Dkks may be also inactivated in gastrointestinal cancer.

### Research frontiers

Epigenetic transcriptional silencing of tumor suppressor genes plays a key role in gastrointestinal cancer and becomes one of the most important research areas. CpG island promoter hypermethylation is a common mechanism for the inactivation of extracellular Wnt antagonists such as SFRPs and WIF-1 in gastrointestinal cancer.

### Innovations and breakthroughs

The expression profiles and epigenetic alterations of Dkks (Dkk1, Dkk2, Dkk3 and Dkk4) and Kremen2 (Krm2) genes were systematically analyzed in many gastrointestinal cancer cell lines and tissues by using RT-PCR, tissue microarray analysis, and methylation specific PCR (MSP).

### Applications

Our study demonstrated that down-regulation of the Dkk gene family and Krm2 gene associated to promoter hypermethylation is frequently involved in gastrointestinal tumorigenesis. Hypermethylated Dkks could be a marker for screening gastrointestinal cancer. Modulation of the Wnt pathway, through reversal of Dkks and/or Krm2 gene silencing by demethylating agents, may be a potential target for treatment and/or prevention of gastrointestinal cancer.

### Peer review

This paper studied alterations of Dkks and Krm2 in gastrointestinal cancer. The authors showed that down-regulation of the Dkks and Krm2 associated to promoter hypermethylation is frequently involved in gastrointestinal tumorigenesis. Hypermethylated Dkks could be a marker for screening gastrointestinal cancer and reactivation of Dkks and/or Krm2 gene could be a promising strategy for treatment and/or prevention of gastrointestinal cancer.

## REFERENCES

- 1 **Kawano Y**, Kypta R. Secreted antagonists of the Wnt signalling pathway. *J Cell Sci* 2003; **116**: 2627-2634
- 2 **Katoh M**, Katoh M. WNT signaling pathway and stem cell signaling network. *Clin Cancer Res* 2007; **13**: 4042-4045
- 3 **Glinka A**, Wu W, Delius H, Monaghan AP, Blumenstock C, Niehrs C. Dickkopf-1 is a member of a new family of secreted proteins and functions in head induction. *Nature* 1998; **391**: 357-362
- 4 **Krupnik VE**, Sharp JD, Jiang C, Robison K, Chickering TW, Amaravadi L, Brown DE, Guyot D, Mays G, Leiby K, Chang B, Duong T, Goodearl AD, Gearing DP, Sokol SY, McCarthy SA. Functional and structural diversity of the human Dickkopf gene family. *Gene* 1999; **238**: 301-313
- 5 **Gonzalez-Sancho JM**, Aguilera O, Garcia JM, Pendas-Franco N, Pena C, Cal S, Garcia de Herreros A, Bonilla F, Munoz A. The Wnt antagonist DICKKOPF-1 gene is a downstream target of beta-catenin/TCF and is downregulated in human colon cancer. *Oncogene* 2005; **24**: 1098-1103
- 6 **Wang J**, Shou J, Chen X. Dickkopf-1, an inhibitor of the Wnt signaling pathway, is induced by p53. *Oncogene* 2000; **19**: 1843-1848
- 7 **Aguilera O**, Fraga MF, Ballestar E, Paz MF, Herranz M, Espada J, Garcia JM, Munoz A, Esteller M, Gonzalez-Sancho JM. Epigenetic inactivation of the Wnt antagonist DICKKOPF-1 (DKK-1) gene in human colorectal cancer. *Oncogene* 2006; **25**: 4116-4121
- 8 **Lee AY**, He B, You L, Xu Z, Mazieres J, Reguart N, Mikami I, Batra S, Jablons DM. Dickkopf-1 antagonizes Wnt signaling independent of beta-catenin in human mesothelioma. *Biochem Biophys Res Commun* 2004; **323**: 1246-1250
- 9 **Kuphal S**, Lodermeier S, Bataille F, Schuierer M, Hoang BH, Bosserhoff AK. Expression of Dickkopf genes is strongly reduced in malignant melanoma. *Oncogene* 2006; **25**: 5027-5036
- 10 **Mao B**, Wu W, Davidson G, Marhold J, Li M, Mechler BM, Delius H, Hoppe D, Stannek P, Walter C, Glinka A, Niehrs C. Kremen proteins are Dickkopf receptors that regulate Wnt/beta-catenin signalling. *Nature* 2002; **417**: 664-667
- 11 **Mao B**, Niehrs C. Kremen2 modulates Dickkopf2 activity during Wnt/LRP6 signaling. *Gene* 2003; **302**: 179-183
- 12 **Nozaki I**, Tsuji T, Iijima O, Ohmura Y, Andou A, Miyazaki M, Shimizu N, Namba M. Reduced expression of REIC/Dkk-3 gene in non-small cell lung cancer. *Int J Oncol* 2001; **19**: 117-121
- 13 **Tsuji T**, Nozaki I, Miyazaki M, Sakaguchi M, Pu H, Hamazaki Y, Iijima O, Namba M. Antiproliferative activity of REIC/Dkk-3 and its significant down-regulation in non-small-cell lung carcinomas. *Biochem Biophys Res Commun* 2001; **289**: 257-263
- 14 **Kobayashi K**, Ouchida M, Tsuji T, Hanafusa H, Miyazaki M, Namba M, Shimizu N, Shimizu K. Reduced expression of the REIC/Dkk-3 gene by promoter-hypermethylation in human tumor cells. *Gene* 2002; **282**: 151-158
- 15 **Roman-Gomez J**, Jimenez-Velasco A, Agirre X, Castillejo JA, Navarro G, Barrios M, Andreu EJ, Prosper F, Heiniger A, Torres A. Transcriptional silencing of the Dickkopfs-3 (Dkk-3) gene by CpG hypermethylation in acute lymphoblastic leukaemia. *Br J Cancer* 2004; **91**: 707-713
- 16 **Hsieh SY**, Hsieh PS, Chiu CT, Chen WY. Dickkopf-3/REIC functions as a suppressor gene of tumor growth. *Oncogene* 2004; **23**: 9183-9189
- 17 **Katoh Y**, Katoh M. Comparative genomics on DKK2 and DKK4 orthologs. *Int J Mol Med* 2005; **16**: 477-481
- 18 **Taniguchi H**, Yamamoto H, Hirata T, Miyamoto N, Oki M, Noshio K, Adachi Y, Endo T, Imai K, Shinomura Y. Frequent epigenetic inactivation of Wnt inhibitory factor-1 in human gastrointestinal cancers. *Oncogene* 2005; **24**: 7946-7952
- 19 **Hirata T**, Yamamoto H, Taniguchi H, Horiuchi S, Oki M, Adachi Y, Imai K, Shinomura Y. Characterization of the immune escape phenotype of human gastric cancers with and without high-frequency microsatellite instability. *J Pathol* 2007; **211**: 516-523
- 20 **Herman JG**, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996; **93**: 9821-9826
- 21 **Cameron EE**, Bachman KE, Myohanen S, Herman JG, Baylin SB. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* 1999; **21**: 103-107
- 22 **Taniguchi H**, Yamamoto H, Akutsu N, Noshio K, Adachi Y, Imai K, Shinomura Y. Transcriptional silencing of hedgehog-interacting protein by CpG hypermethylation and chromatin structure in human gastrointestinal cancer. *J Pathol* 2007; **213**: 131-139
- 23 **Miyamoto N**, Yamamoto H, Taniguchi H, Miyamoto C, Oki M, Adachi Y, Imai K, Shinomura Y. Differential expression of angiogenesis-related genes in human gastric cancers with and those without high-frequency microsatellite instability. *Cancer Lett* 2007; **254**: 42-53
- 24 **Yamamoto H**, Vinitketkumnuen A, Adachi Y, Taniguchi H, Hirata T, Miyamoto N, Noshio K, Imsumran A, Fujita M, Hosokawa M, Hinoda Y, Imai K. Association of matrilysin-2 (MMP-26) expression with tumor progression and activation of MMP-9 in esophageal squamous cell carcinoma. *Carcinogenesis* 2004; **25**: 2353-2360
- 25 **Cho JH**, Kimura H, Minami T, Ohgane J, Hattori N, Tanaka S, Shiota K. DNA methylation regulates placental lactogen I gene expression. *Endocrinology* 2001; **142**: 3389-3396
- 26 **Katoh M**. WNT/PCP signaling pathway and human cancer (review). *Oncol Rep* 2005; **14**: 1583-1588
- 27 **Ishitani T**, Ninomiya-Tsuji J, Nagai S, Nishita M, Meneghini M, Barker N, Waterman M, Bowerman B, Clevers H, Shibuya H, Matsumoto K. The TAK1-NLK-MAPK-related pathway antagonizes signalling between beta-catenin and transcription factor TCF. *Nature* 1999; **399**: 798-802
- 28 **Ishitani T**, Kishida S, Hyodo-Miura J, Ueno N, Yasuda J, Waterman M, Shibuya H, Moon RT, Ninomiya-Tsuji J, Matsumoto K. The TAK1-NLK mitogen-activated protein kinase cascade functions in the Wnt-5a/Ca(2+) pathway to antagonize Wnt/beta-catenin signaling. *Mol Cell Biol* 2003; **23**: 131-139
- 29 **Suzuki H**, Watkins DN, Jair KW, Schuebel KE, Markowitz SD, Chen WD, Pretlow TP, Yang B, Akiyama Y, Van Engeland M, Toyota M, Tokino T, Hinoda Y, Imai K, Herman JG, Baylin SB. Epigenetic inactivation of SFRP genes allows constitutive WNT signaling in colorectal cancer. *Nat Genet* 2004; **36**: 417-422
- 30 **Nojima M**, Suzuki H, Toyota M, Watanabe Y, Maruyama R, Sasaki S, Sasaki Y, Mita H, Nishikawa N, Yamaguchi K, Hirata K, Itoh F, Tokino T, Mori M, Imai K, Shinomura Y. Frequent epigenetic inactivation of SFRP genes and constitutive activation of Wnt signaling in gastric cancer. *Oncogene* 2007; **26**: 4699-4713

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# Cost-effectiveness analysis of chemotherapy for advanced gastric cancer in China

Xin-Zu Chen, Kun Jiang, Jian-Kun Hu, Bo Zhang, Hong-Feng Gou, Kun Yang, Zhi-Xin Chen, Jia-Ping Chen

Xin-Zu Chen, Jian-Kun Hu, Bo Zhang, Kun Yang, Zhi-Xin Chen, Jia-Ping Chen, Department of Gastrointestinal Surgery, and Multidisciplinary Treatment Team of Gastrointestinal Tumors, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Kun Jiang, Department of Integrated Western and Traditional Chinese Medicine, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

Hong-Feng Gou, Department of Oncology, West China Hospital, Sichuan University, Chengdu 610041, Sichuan Province, China

**Author contributions:** Chen XZ was responsible for the research design, data extraction and statistics; Jiang K for the literature search, full-text retrieval and data extraction; medical student Yang K for the literature search and full-text retrieval; Gou HF for the result interpretation; Hu JK and Zhang B for the proof of results and manuscript; Chen ZX and Chen JP for the academic instructions.

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**Correspondence to:** Professor Jian-Kun Hu, Department of Gastrointestinal Surgery, West China Hospital, Sichuan University, Guoxuexiang No. 37, Chengdu 610041, Sichuan Province, China. [hujkwch@126.com](mailto:hujkwch@126.com)

Telephone: +86-28-85422879 Fax: +86-28-85164035

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## Abstract

**AIM:** To assess the economics of various chemotherapeutic regimens for advanced gastric cancer (AGC), and to select the best cost-effective regimen for the common Chinese patients.

**METHODS:** Data source used in this study was the Chinese Biomedical Disk Database. Patients were diagnosed as AGC and any regimen was eligible. Outcome measures included median survival time (MST) and percentage of complete and partial response (CR+PR). Economic statistics was per capita direct medical cost (DMC) of a single cycle. TreeAge Pro Healthcare 2007 software was used to carry out cost-effectiveness and incremental cost-effectiveness analysis. Sensitivity analyses were applied by altering willingness-to-pay and annual discount rate, and also re-analyzed by excluding the studies with apparent heterogeneity.

**RESULTS:** Seven retrospective economics studies on 760 patients were included. 5-fluorouracil-based regimens were universal, and also some new agents were involved, such as docetaxel, paclitaxel, and

oxaliplatin. By processing analysis, we could recommend etoposide, leucovorin and 5-fluorouracil (ELF) regimen as preference, with a DMC/MST ratio of 2543 RMB/11.7 mo and a DMC/CR+PR ratio of 2543 RMB/53.3%. Uracil-tegafur, etoposide and cisplatin (FEP) or 5-fluorouracil, adrimycin/epirubin and mitomycin (FAM) regimens could be regarded as optional first-line chemotherapy for AGC in common Chinese patients. With no regard for willingness-to-pay, the docetaxel, cisplatin and 5-fluorouracil (DCF) regimen could be chosen as either a first- or a second-line chemotherapy, with a DMC/CR+PR ratio of 9979 RMB/56.3%.

**CONCLUSION:** 5-fluorouracil regimens are still considered the mainstream for AGC, while new agents such as taxanes are optional. More randomized clinical trials are required before any mandatory recommendation of certain regimens for patients with AGC in China is made.

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**Key words:** Advanced gastric cancer; Chemotherapy; 5-fluorouracil; Taxanes; Cost-effectiveness

**Peer reviewer:** Takafumi Ando, MD, PhD, Department of Gastroenterology, Nagoya University Graduate School of Medicine, Therapeutic Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

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## INTRODUCTION

Gastric cancer is the fourth most common malignancy worldwide, and China is one of the countries with a high incidence of the disease<sup>[1]</sup>. It is one of the most common causes for cancer mortality and leads to approximately 160 000 deaths annually in China<sup>[2]</sup>. Surgery remains the only established curative treatment for this disease in resectable stages<sup>[3]</sup>. However, about 84% of patients with gastric cancer will have advanced disease, and their median survival time (MST) is only 3-4 mo if they are not treated with chemotherapy<sup>[4]</sup>. Chemotherapy for advanced gastric



cancer (AGC) can improve either time-to-progression or MST, and are well tolerated<sup>[4]</sup>. However, recommended regimens are still controversial since their survival benefit appears marginal in some studies<sup>[3]</sup>. Additionally, in China, a developing country with a huge population, oncologists need to know which regimen is the best cost-effective for the common Chinese patients. The present study was to review the Chinese literature and to make an economic assessment of various regimens for AGC.

## MATERIALS AND METHODS

### Search strategy

We searched the Chinese Biomedical Disk Database (CBMdisc) as the data source. The search strategy was ("gastric cancer" OR "gastric carcinoma" OR "gastric adenocarcinoma") AND ("economics" OR "cost-effectiveness" OR "CEA"). There was no limitation to publication year. Two reviewers (Chen XZ and Jiang K) carried out selection and assessment independently.

### Inclusion criteria

Either prospective or retrospective controlled studies were eligible; but, case serial reports were excluded. Patients were diagnosed having gastric cancer by gastric endoscopy and biopsy or postoperative pathological test. The staging system used was either UICC or Japanese TNM classification. Patients with early gastric cancer (stage I) were excluded. Any regimen of chemotherapy was acceptable for the present analysis. All patients should receive at least two cycles of chemotherapy. Surgery was either resection or explorative laparotomy. Any other therapies were ineligible, such as radiotherapy and biotherapy. The MST (month) was used as the primary outcome measure, or/and percentage of complete and partial response (CR+PR, %) as the secondary outcome measure. The economic parameter and direct medical cost (DMC, RMB) of a single cycle should be available from literature. The criteria for CR/PR were the standard World Health Organization (WHO) tumor response criteria. DMC contained the expenditure of drugs, tests, treatment, nursing and hospitalization.

### Data extraction and synthesis

Basic information about studies was extracted by two reviewers (Chen XZ and Jiang K) independently, including publication year, city of the hospital, sample and demography, and details of regimens. The effectiveness data contained percentage of CR+PR and length of MST. The percentage of CR+PR was numerical data, and was accumulated as  $100 \times (\sum n_i) / (\sum N_i)$ . The length of MST was parameter data, and was synthesized as  $[\sum (x_i N_i)] / (\sum N_i)$ . The abbreviations were respectively shown as ( $n$ ) = number of patients reaching CR or PR criteria in any regimen of individual study, ( $N$ ) = number of patients undertaking any regimen of individual study, ( $x$ ) = months of MST in any regimen of individual study, and ( $i$ ) = number of included studies for any regimen. The economic data were DMC, and synthesized as  $[\sum (y_i N_i)] / (\sum N_i)$ . The abbreviations were shown as ( $y$ ) = per capita DMC (¥, RMB) in a single cycle of any regimen of individual study, all of which were

converted to the 2007 price by discount rate. The annual discount rate was assumed as 1% initially, and ( $n$ ), ( $N$ ) and ( $i$ ) were the same as in the above formulas.

### Statistical analysis

TreeAge Pro Healthcare 2007 software was used in modeling and analyses. Both cost-effectiveness analysis and incremental cost-effectiveness analysis were considered, with C/E ratio and incremental C/E ( $\Delta C / \Delta E$ ) ratio calculated, respectively. The regimen of the lowest cost was selected as the common baseline for other regimens to refer to. For the thresholds, we enacted the expected MST as  $\geq 9$  mo, CR+PR percentage as  $\geq 50\%$ , and willingness-to-pay (WTP) for a single cycle as  $\leq 3000$  RMB (DMC/MST ratio  $\leq 333.3$  RMB, and DMC/CR+PR ratio  $\leq 60$  RMB). Once uncertainty was met in decision making, sensitivity analysis was carried out by alternating the willing-to-pay (WTP) from 1000 RMB to 10000 RMB, or the discount rate from 1% to 10%, or even excluding the studies with a potential heterogeneity.

## RESULTS

### Literature number

Seven studies on 760 patients were included in the present analysis<sup>[5-11]</sup>. All the included studies were retrospective case-control cost-effectiveness analyses. The detailed information is listed in Table 1. Eleven regimens were involved, eight of which were 5-fluorouracil-based and two were uracil-tegafur-based. One study included stages II and III mainly<sup>[5]</sup>. The components of reported DMC were different to some extent. One study, on capecitabine and cisplatin regimen versus oxaplatin and 5-fluorouracil regimen, was excluded for only overall cost of all cycles retrieved, but per capita DMC of a single cycle<sup>[12]</sup>.

### Data retrieval and synthesis

The primary data about the clinical and economic outcomes were retrieved (Table 2), and the data were synthesized according to the method specified before (Table 3). The data about the overall survival were not enough to be analyzed. The MST of each regimen ranged from 6.1 to 11.7 mo, and CR+PR percentage from 21.0% to 56.3%. However, the MST data about the TCF, DCF and FOLFOX regimens with new chemotherapeutic agents were not reported. The per capita DMC of a single cycle ranged from 1756.95 RMB to 9979.00 RMB.

### Cost-effectiveness analysis

The C/E ratios of DMC/MST and DMC/CR+PR were calculated, respectively (Table 4). The ELF regimen had the longest MST (11.7 mo), and the DCF regimen had the highest percentage of CR+PR (56.3%). The FAM regimen was of the lowest DMC/MST ratio (183.17 RMB for per 1-mo survival), and the FEP regimen was of the lowest DMC/CR+PR ratio (42.46 RMB for per 0.01 probability of CR+PR). The FAMTX regimen with the lowest per capita DMC in a single cycle was selected as the common baseline for other regimens to refer to. Incremental cost-effectiveness analysis showed that FAM and ELF regimens

Table 1 General information obtained from the included studies

Study and publication (yr)	Area of China	Design	Sample	Weight (%)	Regimens	Staging				Operation	Outcome measures		Median follow-up period	
						I	II	III	IV		Effectiveness	Economics		
Dong <sup>[5]</sup>	Western-northern	Case-control study	44	5.8	Two cycles at least, and 3-4 wk for one cycle.	FAM EAP ELF	0 0 0	7 5 10	9 6 4	0 2 1	UR	(1) CR+PR (WHO tumor response criteria) (2) Median survival time	DMC contained: (1) Drugs (2) Tests (3) Treatment for ADR	UR
Yu <sup>[6]</sup>	Eastern-southern	Case-control study	41	5.4	Four cycles totally, and 28 d for one cycle.	FAM EAP ELF	0 0 0	0 0 0	5 3 5	10 8 9	None	(1) CR+PR (WHO tumor response criteria) (2) Median survival time	DMC contained: (1) Drugs (2) Treatment for ADR	UR
Ding <sup>[7]</sup>	Eastern-southern	Case-control study	49	6.4	Two cycles at least, and 3-4 wk for one cycle.	FAM EAP ELF	0 0 0	1 1 2	8 5 8	9 9 6	None	(1) CR+PR (WHO tumor response criteria) (2) Median survival time	DMC contained: (1) Drugs (2) Treatment for ADR	UR
Qian <sup>[8]</sup>	Eastern-southern	Case-control study	226	29.7	Two cycles at least, and 3 wk for one cycle.	FAM UFTM FEP LFP/M	Almost composed of stage IV as reported.				Unresectable disease, or palliative operation, or recurrence/metastases after curative operation.	(1) CR+PR (WHO tumor response criteria) (2) Median survival time	DMC contained: (1) Drugs (2) Treatment (3) Fee for berth	UR
Yang <sup>[9]</sup>	Eastern-northern	Case-control study	256	33.7	Three cycles at least, and 3 wk for one cycle.	FAMTX ECF	All unresectable newly diagnosed patients.				None	(1) CR+PR (2) Median survival time	DMC contained: (1) Drugs (2) Treatment for ADR	26.9 mo
Zhou <sup>[10]</sup>	Eastern-southern	Case-control study	48	6.3	Two cycles at least, and 4 wk for one cycle.	TCF FOLFOX	UR				UR	(1) CR+PR (WHO tumor response criteria) (2) Time-to-progression	DMC contained: (1) Drugs (2) Tests (3) Treatment	UR
Liu <sup>[11]</sup>	Eastern-southern	Case-control study	96	12.6	All six cycles.	DCF ECF FOLFOX	0 0 0	0 0 0	30 29 31	2 1 3	UR	CR+PR (Chinese association of gastric cancer response criteria)	DMC contained: (1) Drugs (2) Treatment (3) Fee for berth	UR

CR: Complete response; PR: Partial response; DMC: Per capita direct medical cost of single cycle; 5FU: 5-fluorouracil; ADM: Adriamycin; EPI: Epirubicin; MMC: Mitomycin; DDP: Cisplatin; VP-16: Etoposide; CF: Calcium Leucovorin; UFT: Uracil-tegafur; MTX: Methotrexate; PTX: Paclitaxel; DXL: Docetaxel; L-OHP: Oxaliplatin; UR: Unretrieved data; FAM: 5FU+ADM/EPI+MMC; EAP: DDP+VP-16+ADM/EPI; ELF: 5FU+VP-16+CF; UFTM: UFT+MMC; LFP/M: 5FU+CF+DDP+MMC; FAMTX: 5FU+CF+ADM+MTX; ECF: 5FU+EPI+DDP; TCF: 5FU+DDP+PTX; FOLFOX: 5FU+CF+L-OHP; DCF: 5FU+CF+DXL+DDP.

were not dominated in the DMC/MST analysis. The FEP, ELF and DCF regimens were not dominated in the DMC/CR+PR analysis (Table 4 and Figure 1). According to our previous enacted thresholds, the considerable regimens included ELF, FAM, FEP, LFP/M, EAP, TCF and DCF, but only ELF regimen was conformed to all the thresholds.

### Sensitivity analysis

When altering the willingness-to-pay (WTP), if we want to get the longest survival time, the ELF regimen was

selected without doubt, while if we want to get the best tumor response probability, the ELF regimen was selected on WTP below 2579.00 RMB, while the DCF regimen was selected beyond 2579.00 RMB (Figure 2). Additionally, the FAM, FEP and EAP regimens were conformed to the previous enacted thresholds, but inferior to the ELF and DCF regimens.

When altering the annual discount rate from 1% to 10%, the targeted EAP, FAM, ELF and FEP regimens were rejected once the annual discount rate was more than 1%, 3%, 4% and 8%, respectively, according to

Table 2 Primary data retrieved from literatures

Regimens studies	N	CR + PR		MST (mo)	DMC (¥, RMB)
		n	%		
5FU+ADM/EPI+MMC (FAM)					
Dong <sup>[5]</sup>	16	7	43.8	6.0	2439.78
Yu <sup>[6]</sup>	15	6	40.0	13.5	1965.00
Ding <sup>[7]</sup>	18	7	38.9	11.5	1888.50
Qian <sup>[8]</sup>	35	12	34.3	11.5	1509.78
DDP+VP-16+ADM/EPI (EAP)					
Dong <sup>[5]</sup>	13	7	53.8	8.0	2952.42
Yu <sup>[6]</sup>	12	6	50.0	9.0	2705.00
Ding <sup>[7]</sup>	15	8	53.3	8.5	2170.00
5FU+VP-16+CF (ELF)					
Dong <sup>[5]</sup>	15	7	46.7	9.0	3823.05
Yu <sup>[6]</sup>	14	8	57.1	13.0	1640.00
Ding <sup>[7]</sup>	16	9	56.3	13.0	1602.00
UFT+MMC (UFTM)					
Qian <sup>[8]</sup>	49	16	32.6	8.5	2322.80
UFT+DDP+VP-16 (FEP)					
Qian <sup>[8]</sup>	51	24	47.9	10.0	1902.90
5FU+CF+DDP+MMC (LFP/M)					
Qian <sup>[8]</sup>	91	40	44.0	9.0	2907.76
5FU+CF+ADM+MTX (FAMTX)					
Yang <sup>[9]</sup>	UR	UR	21.0	6.1	1705.28
5FU+EPI+DDP (ECF)					
Yang <sup>[9]</sup>	UR	UR	46.0	8.7	1526.67
Liu <sup>[11]</sup>	30	12	40.0	UR	4158.00
5FU+DDP+PTX (TCF)					
Zhou <sup>[10]</sup>	22	11	50.0	UR	2640.60
5FU+CF+DXL+DDP (DCF)					
Liu <sup>[11]</sup>	32	18	56.3	UR	9979.00
5FU+CF+L-OHP (FOLFOX)					
Zhou <sup>[10]</sup>	26	12	46.1	UR	1588.99
Liu <sup>[11]</sup>	34	18	52.9	UR	4498.00

CR: Complete response; PR: Partial response; MST: Median survival time; DMC: Per capita direct medical cost of single cycle; 5FU: 5-fluorouracil; ADM: Adriamycin; EPI: Epirubicin; MMC: Mitomycin; DDP: Cisplatin; VP-16: Etoposide; CF: Calcium Leucovorin; UFT: Uracil-tegafur; MTX: Methotrexate; PTX: Paclitaxel; DXL: Docetaxel; L-OHP: Oxaliplatin; UR: Unretrieved data.

the previous enacted thresholds (Figure 3). The ECF and TCF regimens were not rejected based on these sensitivity analyses. If considering the discount rate as an annual increase in Chinese consumption price index, i.e. 1.0%-3.9% from 1999 to 2005<sup>[13]</sup>, we assumed that the discount rate increased from 1.0% to 4.0%. Thus, only ELF, FEP, ECF and TCF regimens were not rejected in this aspect.

Additionally, further sensitivity analyses were carried out by excluding those studies with a potential heterogeneity (Table 5). After excluding the two studies contaminated with stage II disease<sup>[5,7]</sup> on the FAM, EAP and ELF regimens, re-analysis was carried out, showing that the FAM and ELF regimens were not dominated.

Considering the economic gap between northern and southern areas, or western and eastern areas of China, we excluded the two studies on FAM, EAP, ELF, FAMTX and ECF regimens conducted in relatively poor developing areas<sup>[5,9]</sup>, and the cost-effectiveness was re-analyzed, showing that only ELF regimen was not dominated.

If only the recent studies published within 5 years (2003-2007)<sup>[9-11]</sup> were included, the re-analysis of FAMTX, ECF, TCF, DCF and FOLFOX regimens showed that ECF, TCF and DCF regimens were not dominated.

Table 3 Accumulated and synthesized data

Regimens	Accumulated n	Synthesized MST (mo)	Accumulated CR + PR (%)	Synthesized DMC <sup>1</sup> (¥, RMB)
5FU+ADM/EPI+MMC (FAM)	84	10.8	38.1	1978.23
5FU+VP-16+CF (ELF)	45	11.7	53.3	2543.12
5FU+CF+DDP+MMC (LFP/M)	91	9.0	44.0	3056.08
5FU+CF+ADM+MTX (FAMTX)	UR	6.1	21.0	1756.95
5FU+EPI+DDP (ECF)	UR	8.7	44.4 <sup>2</sup>	2276.43 <sup>2</sup>
5FU+DDP+PTX (TCF)	22	UR	50.0	2667.01
5FU+CF+DXL+DDP (DCF)	32	UR	56.3	9979.00
5FU+CF+L-OHP (FOLFOX)	60	UR	50.0	3244.31
UFT+MMC (UFTM)	49	8.5	32.7	2441.29
UFT+DDP+VP-16 (FEP)	51	10.0	47.1	1999.97
DDP+VP-16+ADM/EPI (EAP)	40	8.5	52.5	2790.23

<sup>1</sup>All the cost data were converted to 2007 price by preset discount rate (1%);

<sup>2</sup>The data were synthesized by giving the weights equal to those of the studies (Table 1). CR: Complete response; PR: Partial response; MST: Median survival time; DMC: Per capita direct medical cost of single cycle; 5FU: 5-fluorouracil; ADM: Adriamycin; EPI: Epirubicin; MMC: Mitomycin; DDP: Cisplatin; VP-16: Etoposide; CF: Calcium Leucovorin; UFT: Uracil-tegafur; MTX: Methotrexate; PTX: Paclitaxel; DXL: Docetaxel; L-OHP: Oxaliplatin; UR: Unretrieved data.

However, the incremental cost-effectiveness analysis found that paclitaxel regimen was paid more than 69.75 RMB per cycle to gain a higher 0.01 probability of CR+PR more than ECF regimen, and docetaxel regimen was paid 1160.63 RMB more than TCF regimen. However, the outcome data about MST were insufficient in recent studies.

If the studies weighted more than 25% of the overall included participants influencing the results most were excluded<sup>[8,9]</sup>, the FAM, ELF and DCF regimens were not dominated. The docetaxel regimen was more expensive to gain a higher CR+PR rate than FAM and ELF regimens.

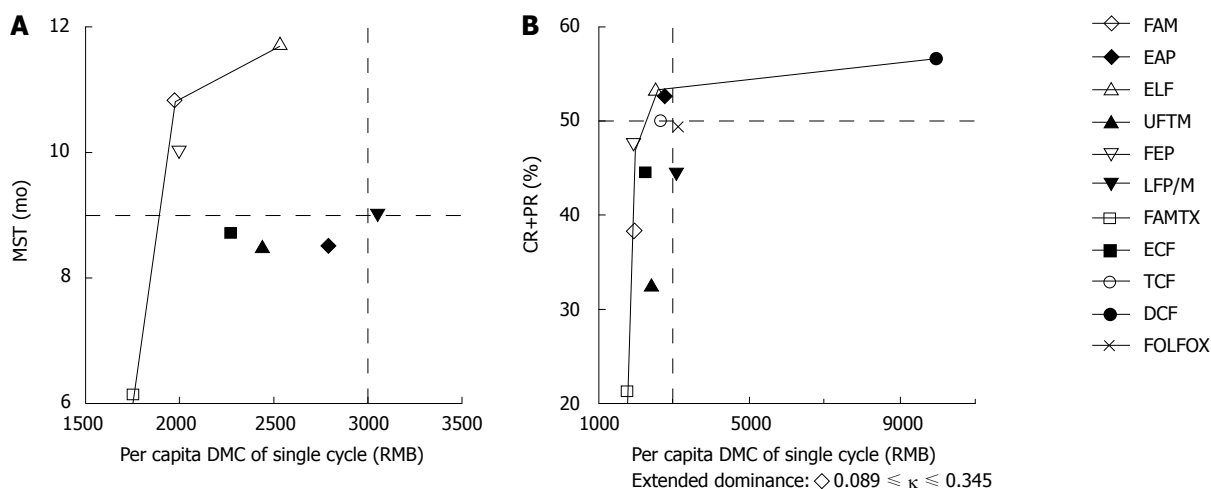
Finally, if our previous enacted thresholds were used to assess each regimen in excluding sensitivity analysis (Table 5), the results in CR+PR aspect indicated that only TCF regimen conformed to the threshold limitation, and with no regard for publication date, EAP or ELF regimen was acceptable. Besides, in MST aspect, regimens with new agents were lack of outcome data. With no regard for publication date, either FAM or ELF regimen was better in improving the median survival according to the threshold limitation.

In conclusion, although FAM or FEP regimen was the best cost-effective choice, ELF regimen trended to be paid more attention to gaining a better response and survival outcome. The taxanes regimen was also attractive in the case of a high level of willingness-to-pay.

Table 4 Direct medical cost-effectiveness analyses

Regimens	Cost (¥, RMB)	Incr cost (¥, RMB)	Eff	Incr Eff	C/E (¥, RMB)	Incr C/E (ICER) (¥, RMB)
DMC/MST analyses						
5Fu+CF+ADM+MTX (FAMTX) <sup>1</sup>	1756.95	-	6.1	-	288.02	Baseline
5Fu+ADM/EPI+MMC (FAM)	1978.23	221.28	10.8	4.7	183.17	47.08
UFT+DDP+VP-16 (FEP)	1999.97	21.74	10.0	-0.8	200.00	Dominated
5Fu+EPI+DDP (ECF)	2276.43	298.20	8.7	-2.1	261.66	Dominated
UFT+MMC (UFTM)	2441.29	463.06	8.5	-2.3	287.21	Dominated
5Fu+VP-16+CF (ELF)	2543.12	564.89	11.7	0.9	217.36	627.66
DDP+VP-16+ADM/EPI (EAP)	2790.23	247.11	8.5	-3.2	328.26	Dominated
5Fu+CF+DDP+MMC (LFP/M)	3056.08	512.96	9.0	-2.7	339.56	Dominated
DMC/CR+PR analyses						
5Fu+CF+ADM+MTX (FAMTX) <sup>1</sup>	1756.95	-	21.0	-	83.66	Baseline
5Fu+ADM/EPI+MMC (FAM)	1978.23	221.28	38.1	17.1	51.92	Extended dominance
UFT+DDP+VP-16 (FEP)	1999.97	21.74	47.1	9	42.46	2.42
5Fu+EPI+DDP (ECF)	2276.43	276.46	44.4	-2.7	51.27	Dominated
UFT+MMC (UFTM)	2441.29	441.32	32.7	-14.4	74.66	Dominated
5Fu+VP-16+CF (ELF)	2543.12	543.15	53.3	6.2	47.71	87.6
5Fu+DDP+PTX (TCF)	2667.01	123.89	50.0	-3.3	53.34	Dominated
DDP+VP-16+ADM/EPI (EAP)	2790.23	247.11	52.5	-0.8	53.15	Dominated
5Fu+CF+DDP+MMC (LFP/M)	3056.08	512.96	44.0	-9.3	69.46	Dominated
5Fu+CF+L-OHP (FOLFOX)	3244.31	701.19	50.0	-3.3	64.89	Dominated
5Fu+CF+DXL+DDP (DCF)	9979.00	7435.88	56.3	3.0	177.25	2478.63

<sup>1</sup>FAMTX regimen was selected as the common baseline for other regimens to refer to. Incr cost: Incremental cost; Eff: Effectiveness; Incr Eff: Incremental effectiveness; CR: Complete response; PR: Partial response; MST: Median survival time; DMC: Per capita direct medical cost of single cycle; 5Fu: 5-fluorouracil; ADM: Adriamycin; EPI: Epirubicin; MMC: Mitomycin; DDP: Cisplatin; VP-16: Etoposide; CF: Calcium Leucovorin; UFT: Uracil-tegafur; MTX: Methotrexate; PTX: Paclitaxel; DXL: Docetaxel; L-OHP: Oxaliplatin.



**Figure 1** Plots of cost-effectiveness analyses for DMC/MST data (the MST data of TCF, DCF and FOLFOX regimens were not reported) (A), and DMC/CR+PR (B). The regimens under the line are dominated in incremental cost-effectiveness analyses.

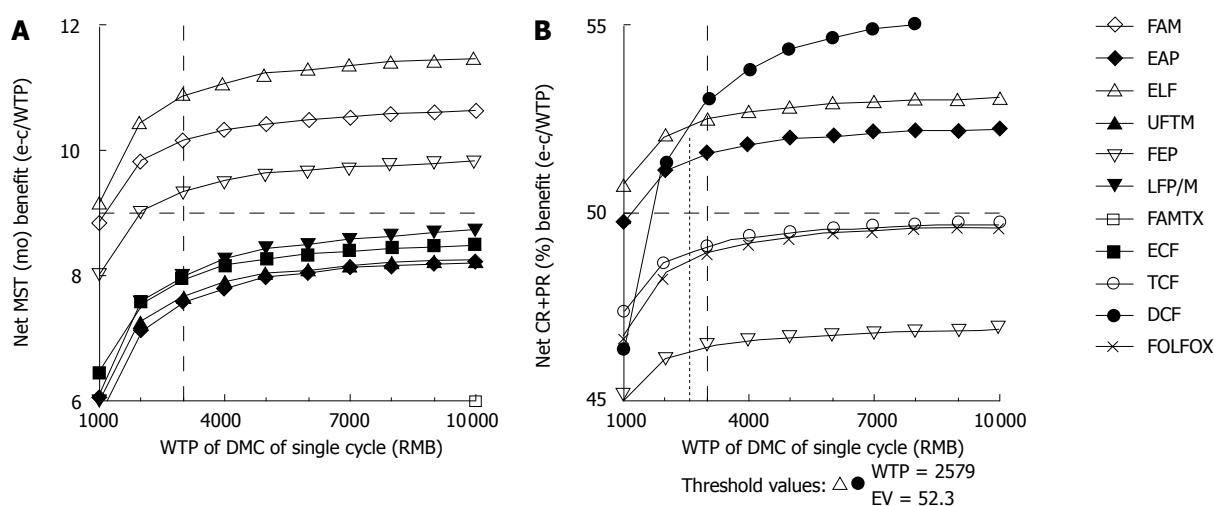
## DISCUSSION

The present cost-effectiveness analysis was based on 7 Chinese retrospective economic case-control studies with a limited sample, which might contain apparent observation bias. The level of evidence source was low (level 3b according to Oxford standard)<sup>[14]</sup>, and there was a potential heterogeneity in some aspects. One study<sup>[5]</sup> half patients had stage II disease, and another study<sup>[7]</sup> was also contaminated with patients of the disease at stage II (Table 1). Moreover, although all the studies reported the information about DMC, the detailed items, such as fee counted in some studies, were slightly different, but not in the others (Table 1). The homogeneity in

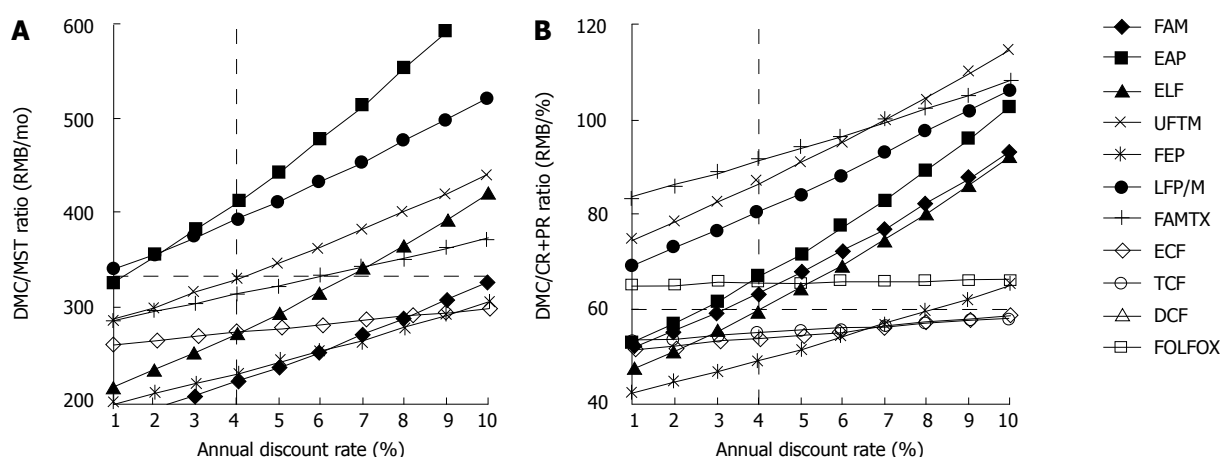
participants should also be questioned, because some studies included patients with recurrence/metastases after curative operation for the disease, while others were newly diagnosed as AGC. Therefore, these factors might remarkably influence the validity of the present analysis.

To our knowledge, there is no standard chemotherapy protocol for AGC worldwide. However, 5-fluorouracil-based regimens have been recommended as a standard modality of chemotherapy for gastric cancer<sup>[1,3]</sup>. Cisplatin plus 5Fu (FP) or etoposide (EP) in the treatment of several types of cancer, including AGC, has been widely used in the treatment of cancer due to their synergistic activity *in vitro*<sup>[15]</sup>. Based on the current available evidence, 5FU-based regimens are universally used in the treatment





**Figure 2** Sensitivity analyses by altering the willingness-to-pay from 1000 RMB to 10000 RMB for DMC, DMC/MST data (the MST data of TCF, DCF and FOLFOX regimens were not reported) (A), and DMC/CR+PR (the curves for FAM, UFTM, LFP/M, FAMTX and ECF regimens are below the bottom of the chart) (B).



**Figure 3** Sensitivity analysis by altering the annual discount rate from 1% to 10% for DMC, DMC/MST data (the MST data of TCF, DCF and FOLFOX regimens were not reported) (A), and DMC/CR+PR (the curve for DCF regimen is beyond the top of the chart) (B).

of Chinese patients, and uracil-tegafur-based regimens are also optional. In the present study, the ELF and FAM regimens had a longer MST of over 10 mo. The ECF regimen was recommended for AGC in NCCN guideline, which appears to improve MST and quality of life<sup>[1]</sup>. However, the results of studies in China are compromising and less cost-effective. Thus, we cannot simply transplant the Western experience to the common Chinese patients, and the ECF regimen requires more prospective randomized controlled trials to confirm its efficacy for the common Chinese patients.

Moreover, some new agents have been applied to the treatment of AGC, such as paclitaxel, docetaxel, oxaliplatin and capecitabine, usually used as second-line regimens. The present literature review found that regimens with the new agents would get a relatively higher CR+PR percentage, but increased the DMC. A retrospective study showed that capecitabine (Xeloda) plus cisplatin regimen could reach a CR+PR percentage of 64.3%<sup>[12]</sup>. Capecitabine is considered a promising agent for the treatment of gastric cancer. Phase III trials of capecitabine-based regimens, comparing its efficacy and safety with those of parenteral

5FU-based regimens in the first-line metastatic setting, are important<sup>[16]</sup>. It was reported that oxaliplatin regimens also have a good efficacy and acceptable safety profile in AGC<sup>[17]</sup>. It has been shown that oxaliplatin, folinic acid, 5-fluorouracil and irinotecan (COFFI) regimen could reach a 67% CR+PR rate in AGC<sup>[17]</sup>, while FOLFOX-4 regimen as a first-line therapy for elderly patients could reach 31%<sup>[18]</sup>, suggesting that irinotecan might play a positive role in the treatment of AGC, and other studies reported that irinotecan, 5-fluorouracil and folinic acid (FOLFIRI) regimen is a very promising and useful treatment of AGC<sup>[19,20]</sup>.

Based on the available data, we could recommend ELF regimen as the preference, and FAM or FEP regimen as a first- or a second-line therapy for AGC in common Chinese patients. With no regard for willingness-to-pay, the DCF regimen can be chosen as either the first-line chemotherapy or the second-line chemotherapy. A recent study showed that combined docetaxel, cisplatin and fluorouracil (DCF) regimen not only significantly improves clinical benefit, quality of life, disease progression and overall survival compared with CF regimen<sup>[21]</sup>. Besides,

Table 5 Sensitivity analyses by excluding the studies with a potential heterogeneity

Regimens	Excluding studies contaminated with stage II diseases			Excluding studies from poorly developing areas of China			Excluding studies published before the last 5 years			Excluding the studies weighted more than 25% of overall sample		
	Cost	Eff	ICER	Cost	Eff	ICER	Cost	Eff	ICER	Cost	Eff	ICER
Sensitivity analyses of DMC/MST ratio (RMB/mo)												
5FU+ADM/EPI+MMC (FAM)	1749.10 <sup>1</sup>	12.1	Baseline	1822.06	11.9	DT	-	-	-	2257.82 <sup>1</sup>	10.3	Baseline
5FU+VP-16+CF (ELF)	1775.88	13.0	29.76	1744.78 <sup>1</sup>	13.0	Baseline	-	-	-	2543.12	11.7	203.79
5FU+CF+DDP+MMC (LFP/M)	3056.08	9.0	DT	3056.08	9.0	DT	-	-	-	-	-	-
5FU+CF+ADM+MTX (FAMTX)	1756.95	6.1	DT	-	-	-	1756.95 <sup>1</sup>	6.1	Baseline	-	-	-
5FU+EPI+DDP (ECF)	2276.43	8.7	DT	-	-	-	2276.43	8.7	199.8	-	-	-
5FU+DDP+PTX (TCF)	-	-	-	-	-	-	-	-	-	-	-	-
5FU+CF+DXL+DDP (DCF)	-	-	-	-	-	-	-	-	-	-	-	-
5FU+CF+L-OHP (FOLFOX)	-	-	-	-	-	-	-	-	-	-	-	-
UFT+MMC (UFTM)	2441.29	8.5	DT	2441.29	8.5	DT	-	-	-	-	-	-
UFT+DDP+VP-16 (FEP)	1999.97	10.0	DT	1999.97	10.0	DT	-	-	-	-	-	-
DDP+VP-16+ADM/EPI (EAP)	2929.13	9.0	DT	2594.35	8.7	DT	-	-	-	2790.23	8.5	DT
Sensitivity analyses of DMC/CR+PRratio (RMB/%)												
5FU+ADM/EPI+MMC (FAM)	1749.10 <sup>1</sup>	36.0	Baseline	1822.06	36.8	DT	-	-	-	2257.82 <sup>1</sup>	40.8	Baseline
5FU+VP-16+CF (ELF)	1775.88	57.1	1.27	1744.78 <sup>1</sup>	56.7	Baseline	-	-	-	2543.12	53.3	22.82
5FU+CF+DDP+MMC (LFP/M)	3056.08	44.0	DT	3056.08	44.0	DT	-	-	-	-	-	-
5FU+CF+ADM+MTX (FAMTX)	1756.95	21.0	DT	-	-	-	1756.95 <sup>1</sup>	21.0	Baseline	-	-	-
5FU+EPI+DDP (ECF)	2276.43	44.4	DT	4158.00	40.0	DT	2276.43	44.4	22.2	4158.00	40	DT
5FU+DDP+PTX (TCF)	2667.01	50.0	DT	2667.01	50.0	DT	2667.01	50.0	69.75	2667.01	50	DT
5FU+CF+DXL+DDP (DCF)	9979.00	56.3	DT	9979.00	56.3	DT	9979.00	56.3	1160.63	9979.00	56.3	2478.63
5FU+CF+L-OHP (FOLFOX)	3244.31	50.0	DT	3244.31	50.0	DT	3244.31	50.0	DT	3244.31	50	DT
UFT+MMC (UFTM)	2441.29	32.7	DT	2441.29	32.7	DT	-	-	-	-	-	-
UFT+DDP+VP-16 (FEP)	1999.97	47.1	DT	1999.97	47.1	DT	-	-	-	-	-	-
DDP+VP-16+ADM/EPI (EAP)	2929.13	50.0	DT	2594.35	51.9	DT	-	-	-	2790.23	52.5	DT

<sup>1</sup>The regimen with the lowest direct medical cost in a single cycle was selected as the common baseline for other regimens to refer to. Eff: Effectiveness; ICER: Incremental cost/effectiveness ratio; CR: Complete response; PR: Partial response; MST: Median survival time; DMC: Per capita direct medical cost of single cycle; DT: Dominated by just the less costly one; 5FU: 5-fluorouracil; ADM: Adriamycin; EPI: Epirubicin; MMC: Mitomycin; DDP: Cisplatin; VP-16: Etoposide; CF: Calcium Leucovorin; UFT: Uracil-tegafur; MTX: Methotrexate; PTX: Paclitaxel; DXL: Docetaxel; L-OHP: Oxaliplatin.

paclitaxel regimen is also considered an active and well tolerated therapy for AGC<sup>[22]</sup>. Generally, the TCF regimen is recommended as a second- or a third-line therapy for AGC, with a tolerable and acceptable toxicity profile<sup>[23,24]</sup>. It was reported that PTX plus 5FU as a first-line chemotherapy can prolong the MST and 1-year survival rate<sup>[25]</sup>. However, another study reported that only 27% of patients with measurable disease have achieved a MST of 26 wk<sup>[26]</sup>. Thus, chemotherapy with docetaxel appears to be promising in the treatment of AGC, while chemotherapy with paclitaxel needs further confirmation<sup>[27]</sup>. Based on the availability of agents, physician/patient expectations and the “standard” regimens will disappear in the future oncology fields of individualized therapy<sup>[28]</sup>.

In conclusion, 5-fluorouracil regimens, especially ELF regimens, are still recommended as the mainstream for AGC in China, while some new agents are optional, such as taxanes. More randomized clinical trials are required before mandatory recommendation for certain regimens for patients with AGC in China is made.

## COMMENTS

### Background

Gastric cancer is the forth common malignancy worldwide, and China is one of the countries with a high incidence of the disease. About 84% of patients with Gastric cancer will progress to its advanced stage in about 84% of patients. If they do not receive chemotherapy, their median survival time (MST) is only 3-4 mo. Studies

have found that chemotherapy for Advanced gastric cancer (AGC) might improve the MST and can be well tolerated. However, recommendation for regimens is still controversial, since the survival appears marginal in some studies.

### Research frontiers

In China, a developing country with a huge population, oncologists need to know which regimen is the best cost-effective for the common Chinese patients. The present paper reviews the Chinese literatures and makes an economics assessment of various regimens for AGC.

### Innovations and breakthroughs

Some new agents have been applied to the treatment of AGC, such as paclitaxel, docetaxel, oxaliplatin and capecitabine, usually used as the second-line regimen drugs. The present literature review found that regimens with new agents could get a relatively higher CR+PR percentage while increasing the direct medical cost (DMC).

### Applications

In any case, regimens with 5-fluorouracil are still considered the mainstream for the treatment of AGC, while new agents are optional, such as taxane. More randomized clinical trials are required before mandatory regimen recommendations are made for patients with AGC in China.

### Terminology

AGC is an adenocarcinoma of the stomach at a more advanced stage than T1 stage, i.e. the primary lesion penetrates into tissues beyond the mucosal layer, which can be divided as a locally advanced or metastatic disease. 5-fluorouracil (5FU), a chemotherapeutic agent used to treat several types of cancer, including digestive, head and neck cancers, can prevent cells from making DNA and RNA to disrupt the growth of cancer cells. Taxanes are a group of chemotherapeutic agents including paclitaxel (PTX) and docetaxel (DXL), which are also able to prevent the growth of cancer cells.

## Peer review

This paper reports a health economics assessment of various chemotherapeutic regimens for AGC, and the authors selected the best cost-effective regimen for common Chinese patients. Although they concluded that more randomized clinical trials are required before any mandatory recommendation of certain regimens is made for patients with AGC in China, this paper offers a lot of important information.

## REFERENCES

- 1 **National Comprehensive Cancer Network.** Clinical practice guidelines in oncology: gastric cancer, 2007
- 2 **Chen XM,** Chen GY, Wang ZR, Zhu FS, Wang XL, Zhang X. Detection of micrometastasis of gastric carcinoma in peripheral blood circulation. *World J Gastroenterol* 2004; **10**: 804-808
- 3 **Hu JK,** Li CM, Chen XZ, Chen ZX, Zhou ZG, Zhang B, Chen JP. The effectiveness of intravenous 5-fluorouracil-containing chemotherapy after curative resection for gastric carcinoma: A systematic review of published randomized controlled trials. *J Chemother* 2007; **19**: 359-375
- 4 **Rivera F,** Vega-Villegas ME, Lopez-Brea MF. Chemotherapy of advanced gastric cancer. *Cancer Treat Rev* 2007; **33**: 315-324
- 5 **Dong YL,** Li R. Cost-effectiveness analysis of three chemotherapy regimens for gastric cancer. *Zhongguo Yaoxue Zazhi* 1999; **34**: 627-628
- 6 **Yu LX,** Lv ZC. Cost-effectiveness analysis of three chemotherapy regimens for gastric cancer. *Zhongguo Yiyuan Yaoxue Zazhi* 1999; **19**: 552-553
- 7 **Ding WX,** Zhai ZY. Cost-effectiveness analysis of three chemotherapy regimens for gastric cancer. *Zhenjiang Yixueyuan Xuebao* 2000; **10**: 89-90
- 8 **Qian SS,** Qin LR. Cost-effectiveness analysis of four chemotherapy regimens for advanced gastric cancer. *Yaowu Liuxingxue Zazhi* 2002; **11**: 250-251
- 9 **Yang L,** Cui CX, Wang JW. Health economics assessment of chemotherapy for gastric cancer. *Zhongguo Quanke Yixue* 2004; **7**: 1640-1641
- 10 **Zhou T,** Yang QL, Ling Y. Cost-effectiveness analysis of two new chemotherapeutic agent containing regimens for advanced gastric cancer. *Shiyong Linchuang Yiyao Zazhi* 2006; **10**: 28-29
- 11 **Liu YX,** Zhang J, Chen SX, Li JW. Cost-effectiveness analysis of three chemotherapeutic schemes for advanced gastric cancer. *Yaoyue Shijian Zazhi* 2007; **25**: 117-120
- 12 **Yang Q,** Chen DS. Cost-effectiveness analysis of three chemotherapy regimens for gastric cancer. *Zhongguo Yiyuan Yaoyue Zazhi* 2007; **27**: 230-232
- 13 **Liu SH,** Chen QS. Predictive goal of regulation of the residents' consumer price index in China. *Jing Ji Shi* 2005; **10**: 50-52
- 14 **Phillips B,** Ball C, Sackett D, Badenoch D, Straus S, Haynes B, Dawes M. Oxford Centre for Evidence-based Medicine Levels of Evidence (May 2001). Oxford: Centre for Evidence-Based Medicine
- 15 **Icli F,** Celik I, Aykan F, Uner A, Demirkazik A, Ozet A, Ozguroglu M, Tas F, Akbulut H, Firat D. A randomized Phase III trial of etoposide, epirubicin, and cisplatin versus 5-fluorouracil, epirubicin, and cisplatin in the treatment of patients with advanced gastric carcinoma. Turkish Oncology Group. *Cancer* 1998; **83**: 2475-2480
- 16 **Ajani J.** Review of capecitabine as oral treatment of gastric, gastroesophageal, and esophageal cancers. *Cancer* 2006; **107**: 221-231
- 17 **Chiesa MD,** Buti S, Tomasello G, Negri F, Buononato M, Brunelli A, Lazzarelli S, Brighenti M, Donati G, Passalacqua R. A pilot phase II study of chemotherapy with oxaliplatin, folinic acid, 5-fluorouracil and irinotecan in metastatic gastric cancer. *Tumori* 2007; **93**: 244-247
- 18 **Nardi M,** Azzarello D, Maisano R, Del Medico P, Giannicola R, Raffaele M, Zavettieri M, Costarella S, Falzea A. FOLFOX-4 regimen as first-line chemotherapy in elderly patients with advanced gastric cancer: a safety study. *J Chemother* 2007; **19**: 85-89
- 19 **Beretta E,** Di Bartolomeo M, Buzzoni R, Ferrario E, Mariani L, Gevorgyan A, Bajetta E. Irinotecan, fluorouracil and folinic acid (FOLFIRI) as effective treatment combination for patients with advanced gastric cancer in poor clinical condition. *Tumori* 2006; **92**: 379-383
- 20 **Kim SG,** Oh SY, Kwon HC, Lee S, Kim JH, Kim SH, Kim HJ. A phase II study of irinotecan with bi-weekly, low-dose leucovorin and bolus and continuous infusion 5-fluorouracil (modified FOLFIRI) as salvage therapy for patients with advanced or metastatic gastric cancer. *Jpn J Clin Oncol* 2007; **37**: 744-749
- 21 **Ajani JA,** Moiseyenko VM, Tjulandin S, Majlis A, Constenla M, Boni C, Rodrigues A, Fodor M, Chao Y, Voznyi E, Marabotti C, Van Cutsem E. Clinical benefit with docetaxel plus fluorouracil and cisplatin compared with cisplatin and fluorouracil in a phase III trial of advanced gastric or gastroesophageal cancer adenocarcinoma: the V-325 Study Group. *J Clin Oncol* 2007; **25**: 3205-3209
- 22 **Lokich JJ,** Sonneborn H, Anderson NR, Bern MM, Coco FV, Dow E, Oliynyk P. Combined paclitaxel, cisplatin, and etoposide for patients with previously untreated esophageal and gastroesophageal carcinomas. *Cancer* 1999; **85**: 2347-2351
- 23 **Kim YH,** Shin SW, Kim BS, Kim JH, Kim JG, Mok YJ, Kim CS, Rhyu HS, Hyun JH, Kim JS. Paclitaxel, 5-fluorouracil, and cisplatin combination chemotherapy for the treatment of advanced gastric carcinoma. *Cancer* 1999; **85**: 295-301
- 24 **Yamaguchi K,** Nakagawa S, Yabusaki H, Nashimoto A. Combination chemotherapy with 5-fluorouracil, cisplatin and paclitaxel for pretreated patients with advanced gastric cancer. *Anticancer Res* 2007; **27**: 3535-3539
- 25 **Ninomiya M,** Kondo K, Matsuo K, Hirabayashi N, Kojima H, Kobayashi M, Kawamura S, Ando T, Musha N, Konno H, Nagata N, Usuki H, Miyashita Y, Oba K, Morita S, Sakamoto J. Multicenter phase II trial of combination chemotherapy with weekly paclitaxel and 5-fluorouracil for the treatment of advanced or recurrent gastric carcinoma. *J Chemother* 2007; **19**: 444-450
- 26 **Kim YH,** Shin SW, Kim BS, Kim JH, Kim JG, Mok YJ, Kim CS, Rhyu HS, Hyun JH, Kim JS. Paclitaxel, 5-fluorouracil, and cisplatin combination chemotherapy for the treatment of advanced gastric carcinoma. *Cancer* 1999; **85**: 295-301
- 27 **Roth AD,** Ajani J. Docetaxel-based chemotherapy in the treatment of gastric cancer. *Ann Oncol* 2003; **14** Suppl 2: ii41-ii44
- 28 **Ajani JA.** Standard of care for gastric cancer based on meta-analysis? Treading on thin ice or it is very nice! *J Clin Oncol* 2006; **24**: 5473-5474; author reply 5474-5476

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## Correlation between *rpoB* gene mutation in *Mycobacterium avium* subspecies *paratuberculosis* and clinical rifabutin and rifampicin resistance for treatment of Crohn's disease

Daniel R Beckler, Sammer Elwasila, George Ghobrial, John F Valentine, Saleh A Naser

Daniel R Beckler, Sammer Elwasila, George Ghobrial, Saleh A Naser, Department of Molecular Biology and Microbiology, Burnett School of Biomedical Sciences, Center for Biomolecular Sciences, College of Medicine, University of Central Florida, Florida, FL 32816, United States

John F Valentine, Department of Medicine, University of Florida, Gainesville, Florida, FL 32810, United States

**Author contributions:** Beckler DR, Elwasila S, Ghobrial G, Valentine JF, Naser SA contributed equally to the work; Beckler DR, Elwasila S, and Ghobrial G participated in the experiments design and the data collection; Valentine JF provided the clinical samples and assisted in data interpretation; Naser SA participated in research design, supervising the daily experiments, interpretation of the data and editing the manuscript.

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**Correspondence to:** Saleh A Naser, Professor, Department of Molecular Biology and Microbiology, Burnett School of Biomedical Sciences, College of Medicine, University of Central Florida, 4000 Central Florida Blvd, Florida, FL 32816, United States. [nasers@mail.ucf.edu](mailto:nasers@mail.ucf.edu)

Telephone: +1-407-823-0955 Fax: +1-407-823-0956

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### Abstract

**AIM:** To investigate overlapping regions of the *rpoB* gene previously involved with rifamycin resistance in *M. tuberculosis* and seek correlation between *rpoB* mutations in clinical MAP strains with susceptibility to RIF and RFB.

**METHODS:** We designed a molecular-based PCR method for the evaluation of rifabutin (RFB) and rifampicin (RIF) resistance based on probable determinant regions within the *rpoB* gene of MAP, including the 81 bp variable site located between nucleotides 1363 and 1443. The minimum inhibitory concentration (MIC) for RIF was also determined against 11 MAP isolates in attempt to seek correlation with *rpoB* sequences.

**RESULTS:** We determined that MAP strain 18 had an MIC of  $> 30$  mg/L and  $\leq 5$  mg/L for RIF and RFB respectively, and a significant and novel *rpoB* mutation C1367T, compared to an MIC of  $\leq 1.0$  mg/L for both drugs in the wild type MAP. The 30-fold increase in the MIC was a direct result of the *rpoB* mutation C1367T, which caused an amino acid change Thr456 to Ile456 in the drug's binding site. In addition, MAP strain 185 contained five silent *rpoB* mutations and exhibited an MIC comparable to the wild-type. Moreover, our *in vitro*

selected mutation in MAP strain UCF5 resulted in the generation of a new resistant strain (UCF5-RIF16r) that possessed T1442C *rpoB* mutation and an MIC  $> 30$  mg/L and  $> 10$  mg/L for RIF and RFB respectively. Sequencing of the entire *rpoB* gene in MAP strains UCF4, 18, and UCF5-RIF16r revealed an *rpoB* mutation A2284C further downstream of the 81 bp variable region in UCF4, accounting for observed slight increase in MIC. In addition, no other significant mutations were found in strains 18 and UCF-RIF16r.

**CONCLUSION:** The data clearly illustrates that clinical and *in vitro*-selected MAP mutants with *rpoB* mutations result in resistance to RIF and RFB, and that a single amino acid change in the beta subunit may have a significant impact on RIF resistance. Unconventional drug susceptibility testing such as our molecular approach will be beneficial for evaluation of antibiotic effectiveness. This molecular approach may also serve as a model for other drugs used for treatment of MAP infections.

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**Key words:** *Mycobacterium paratuberculosis*; Crohn's disease; Rifabutin; Rifampicin; *rpoB*; Minimum inhibitory concentration

**Peer reviewer:** Francesco Feo, Professor, Dipartimento di Scienze Biomediche, Sezione di Patologia Sperimentale e Oncologia, Università di Sassari, Via P. Manzella 4, Sassari 07100, Italy

Beckler DR, Elwasila S, Ghobrial G, Valentine JF, Naser SA. Correlation between *rpoB* gene mutation in *Mycobacterium avium* subspecies *paratuberculosis* and clinical rifabutin and rifampicin resistance for treatment of Crohn's disease. *World J Gastroenterol* 2008; 14(17): 2723-2730 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2723.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2723>

### INTRODUCTION

Crohn's disease (CD) is an inflammatory bowel disease that detrimentally affects the epithelial lining of the digestive tract and presents with symptoms such as diarrhea, weight loss, abdominal pain, and constipation<sup>[1]</sup>. Despite the gross similarity of histological and pathological characteristics between CD and the inflammatory intestinal disease found



in cattle with Johne's disease (JD), both diseases remain distinct<sup>[1,2]</sup>. There is a strong debate currently between CD's potential autoimmune cause and its relation to bacteria, and either concept has yet to be proven. *Mycobacterium avium* subspecies *paratuberculosis* (MAP) is known to be the causative agent of JD and has previously been implicated in etiological studies of CD<sup>[2-8]</sup>. Available information regarding MAP's role in CD pathogenesis has promoted the ongoing studies to continue investigating the relationship between the MAP bacterium and the human bowel disease<sup>[3]</sup>. A most recent report has shown that antibiotics including rifabutin (RFB) is beneficial to CD patients despite the fact that the clinical trial study met with a few shortcomings regarding drug dosage, duration, and lack of MAP testing for the participating subjects<sup>[9]</sup>. The latter is best detected using PCR-based assays that amplify regions of the insertion sequence IS900 as shown by many investigators<sup>[10-12]</sup>.

RFB and rifampicin (RIF) are antibiotics that belong to the rifamycin drug family and are very similar in chemical structure. The function of these antibiotics are to inhibit the growth of bacteria, specifically by binding to the beta subunit of RNA polymerase through direct and indirect amino acid interactions, and preventing the production of nascent RNA transcripts<sup>[13]</sup>. The *rpoB* gene in prokaryotes encodes the beta subunit, and mutations within this gene result in a higher level of resistance to the rifamycin antibiotics in several bacteria<sup>[14-19]</sup>. Moreover, mutations within an 81 base pair region in *rpoB* spanning nucleotides 1276-1356 in *M. tuberculosis* have been shown to contain the majority of alterations relating to rifamycin resistance<sup>[20]</sup>. In addition, mutations at the beginning of *rpoB* are associated with rifamycin resistance in some strains of *M. tuberculosis*. However, these occurrences are not as prevalent as the former<sup>[15,21]</sup>.

Previous reports have shown that RFB may potentially serve as a therapeutic agent for the treatment of CD<sup>[22-27]</sup>, and as an effective drug against MAP<sup>[28]</sup>. In addition, other treatments such as anti-inflammatory agents have been proved to relieve symptoms of CD<sup>[29]</sup>; however these treatments are not as beneficial as antibiotic therapy<sup>[22]</sup>. As a result, more attention has been focused on the possible use of antibiotics as an alternative remedy. However, a screening method for RIF and RFB resistant strains of MAP has yet to be established. Therefore, as CD therapy becomes more focused on antibiotics, it is essential to develop a method for susceptibility testing in order to detect and monitor MAP strains for RIF and RFB resistance.

Unlike other prokaryotes, MAP is very fastidious and requires unusual *in vitro* growth conditions, including the addition of *Mycobacterin J*. Additionally, MAP cultured from CD samples has shown to lack a cell wall, and this characteristic is a major contributing factor to the complexity of the bacteria's primary isolation process<sup>[11,30,31]</sup>. Consequently, conventional drug susceptibility tests involving MAP are not reliable and may result in inaccurate results. Therefore, the challenges faced when working with this bacterium must be counteracted *via* alternate approaches that could potentially result in the exposition of significant data in order to effectively treat MAP infections. In this study, we adopted a molecular approach based on PCR amplification, followed by nucleotide sequencing of regions within

the *rpoB* gene of MAP. We attempted to investigate the overlapping regions of the *rpoB* gene previously involved with rifamycin resistance in *M. tuberculosis*. The ultimate goal is to seek the correlation between *rpoB* mutations in clinical MAP strains with susceptibility to RIF and RFB.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

All clinical strains including UCF3, UCF4, UCF5, UCF7, UCF8, MAP18, MAP185, and 61a were isolated in our laboratory from clinical samples obtained from CD patients<sup>[32]</sup>. Each MAP isolate was taken from different patients; however the anatomical sources for each overlap. For instance, each MAP isolate was isolated from their corresponding anatomical sources of individual CD patients: UCF3, UCF4, UCF8, 18, and 61a from the ileal; UCF5 and UCF7 from ileocolonic tissue; and 185 from the mesenteric lymph node. These MAP strains originated from surgical tissue samples obtained from CD patients<sup>[32-34]</sup>. Cow2013 and Cow5 MAP strains were recently isolated from ground beef samples from two cattles with JD. Briefly, tissue samples were ground, homogenized, decontaminated and then inoculated into MGIT culture media with supplements including OADC, *Mycobacterin J* and PANTA as described previously<sup>[32]</sup>.

All American type culture collection (ATCC) strains were verified in our laboratory by biochemical and molecular testing, including MAP strain 43544, *M. avium* subspecies *avium* 25291, *M. tuberculosis* strain 25177, and *M. smegmatis* strain 607. All cultures were subcultured in 7H10 agar supplemented with oleic acid-albumin-dextrose-catalase (OADC) and *Mycobacterin J*. Plates were incubated at 37°C until visible colonies were observed. Colonies from pure culture verified by Ziehl-Neelsen acid fast stain and IS900-based PCR for MAP were used to inoculate BACTEC 7H9 broth culture supplemented with 500 µL OADC and 2.4 µmol/L *Mycobacterin J*. Growth index (GI) was read weekly until optimum growth index was observed. The fresh culture was then used for drug susceptibility testing and for molecular studies.

### Minimum inhibitory concentration (MIC) measurement

MIC for RIF and RFB were determined against all micro-organisms used for this study. Starting cultures with a GI range of 500-600 were found optimal for inoculation. The inoculum size for each Bactec bottle used in the drug susceptibility study was approximately  $1.0 \times 10^5$  CFUs. Serial dilutions of RIF concentration ranging from 0 to 4 mg/L were evaluated against all micro-organisms. MAP strains resistant to RIF > 1.0 mg/L were further tested against RIF concentrations of 10, 20 and 30 mg/L for MIC measurement. These MAP strains were also evaluated against RFB at concentrations ranging from 0.0 to 10 mg/L, excluding UCF4. MAP wild type was included as a control in each batch of analysis. Experiments were repeated for confirmations. Micro-organisms other than MAP were also evaluated for RIF concentrations ranging from 0.0 to 10.0 mg/L. The percentage of RIF or RFB inhibition was used to determine the level of susceptibility for each

Table 1 PCR primers used in this study

Primers	Sequence (5' to 3')	Amplified base pairs (bp) <sup>1</sup>	Amplicon length (bp)
IS900			
P90	GTTCGGGGCCGTCGCTTAGG	22-421	400
P91	GAGGTCGATCGCCACGTGA		
AV1	ATGTGTTGCTGTGTGGATGG	77-384	308
AV2	CCGCCGAATCAACTCCAG		
rpoB			
Efox1	TTGCCGGCCGAACCGACACA	1-721	721
Fox1r	TGTCGACGTCGAATCCAGC		
UCF1	TCGATGTCGCTGCTTTCTC	373-820	448
UCF2	GCTCGGTGATCTGCTCGTTG		
Fox1f	CGGTGTCATGGGTGACTTC	521-1509	989
DBR	GTAGTGGACGGGTGCACGTC		
Knight1	ACCACTTCGCAACCGCCGG	1191-1777	587
Knight2	ACTCGACCTCGCCGCCTTG		
Ex1a	AAGGTGGTCGACGGCGTGGT	1621-2340	720
Ex2a	GATCTCGTGCTCTCGATGT		
Ex3	ACGAGGACGCGATCCTCT	2261-2980	720
Ex4	TCGACACGATCTGGTTCGGC		
Ex5	GAACATCGACGGCAATCCCG	2901-3597	697
Hrox1	TCCGTCGAGGACCTGGCTTAA		

<sup>1</sup>Numbers represent nucleotide positions within each gene of MAP.

concentration of antibiotics as follows: % Inhibition = 1 minus (GI of Bactec culture without drug minus GI of Bactec culture with drug)/GI of Bactec culture without drug). The MIC was also determined for each bacterial sample, and was defined as the minimum concentration of antibiotics that induced inhibition of growth by 90%.

### Microbial genomic DNA extraction, nested PCR and nucleotide sequencing

Extraction of genomic DNA from mycobacterial isolates was performed for verification of the presence of the IS900 gene and for analysis of rpoB gene sequence. The DNA extraction, purification and quantification were performed as described previously<sup>[11]</sup>. Nested PCR for IS900 amplification was performed using the primers p90/91 and AV1/AV2 as listed in Table 1<sup>[11]</sup>. The size of amplified product was determined on 2% agarose gel. Appropriate negative controls for PCR consisting of sterile TE buffer or sterile water in place of the DNA template were used in parallel with each round of PCR preparation. Positive MAP DNA from strain ATCC 43015 was prepared independently and added to PCR tubes at a different facility using separate supplies.

Unlike the nested IS900-based PCR assay, only one round of PCR assays was developed for amplification of two different regions of the rpoB gene. Therefore, one PCR reaction contained the UCF1/UCF2 primers for amplification of 448 bp whereas the second PCR reaction contained the Knight1/Knight2 primers for amplification of 587 bp. The ingredients and protocol conditions of the rpoB-based PCR assays were as described previously<sup>[11]</sup>. Table 2 lists the nucleotide sequence for all primers used in this study. Each PCR product was purified from agarose using the Purelink Quick Gel Extraction Kit following the procedure described by the manufacturer (Invitrogen). Purified DNA was then quantitated and subjected to

nucleotide sequencing. The latter was performed using the GenomeLab DTCS-Quick Start Kit following the manufacturer's instructions (Beckman Coulter). Both DNA strands were sequenced for each PCR product using appropriate nucleotide primers at the Biomolecular Science Center DNA Sequencing Core Facility at the University of Central Florida. BLAST analysis was performed using the Pubmed.gov database, and the rpoB sequence from MAP strain K-10<sup>[35]</sup> was used as our reference wildtype control sequence for comparison purposes.

### In vitro selected RIF-MAP mutant

An RIF resistant MAP mutant (UCF5-RIF16r) was selected through the generation of rpoB mutation *via* adaptive resistance in the presence of antibiotics. This was performed by exposing our wild-type MAP strain UCF5 to increase the concentrations of RIF ranging from 1 to 16 mg/L. Initially, MAP strain UCF5 was inoculated into a BACTEC bottle containing 1 mg/L RIF. Following incubation, the surviving MAP cells were sub-cultured into a new BACTEC bottle with double RIF concentrations. The process was repeated several times until a new MAP strain (UCF5-RIF16r) was selected that was able to survive in the presence of 16 mg/L of RIF. This new resistant strain was then tested for MIC against RIF and RFB and investigated for possible rpoB mutations as described earlier.

## RESULTS

### Identification of MAP

Genomic DNA from all bacterial isolates was subjected to IS900-based PCR in order to confirm the identity of all MAP strains. This procedure involved two rounds of PCR, using p90/91 primers in the first round to amplify a 400 bp sequence. The use of AV1/AV2 in the second round of PCR amplified a 308 bp sequence and provided exceptional sensitivity and enhanced specificity for the confirmation of MAP. As expected, all 11 MAP isolates were confirmed for the presence of IS900. In addition, *M. avium* subspecies *avium*, *M. smegmatis* and *M. tuberculosis* displayed negative results for the presence of the IS900.

### rpoB amplification and sequence analysis

Following the IS900 PCR analysis, genomic DNA from each identified MAP strain was used as template for PCR employing rpoB primers (Table 1). Amplification of rpoB sequences enabled the possibility for amplicon purification, sequencing, and subsequently BLAST analysis. The use of primers UCF1/UCF2 and Knight1/Knight2 enabled the amplification of two regions of the rpoB gene of MAP. These regions overlapped similar sequences in the rpoB gene of *M. tuberculosis* previously associated with rifamycin resistance. Moreover, PCR with Knight1/Knight2 primers amplified a sequence that harbored the 81 bp variable site 1363-1443, a highly probable determinant region for rifamycin resistance in closely related bacteria. Amplicons from both regions of the rpoB gene were obtained by successful PCR.

Nucleotide sequencing of both rpoB regions for all MAP strains was performed for both forward and reverse primer reactions to exclude any possible errors in the data.

Table 2 rpoB and susceptibility data for all micro-organization

Microorganism	Strain	RIF MIC (mg/L)	RFB MIC (mg/L) <sup>1</sup>	Inhib. at 1 mg/L of RIF (%) <sup>2</sup>	rpoB BLAST result <sup>3</sup>	Amino acid change <sup>4</sup>
MAP	ATCC43544	≤ 1.0	≤ 1.0	91 ± 0.71	WT	NC
MAP	UCF3	≤ 1.0		94 ± 2.83	WT	NC
MAP	UCF4	≤ 2.5		79 ± 7.07	A2284C	N762H
MAP	UCF5	≤ 1.0		92.5 ± 0.71	WT	NC
MAP	UCF7	≤ 1.0		88 ± 5.66	WT	NC
MAP	UCF8	≤ 1.0		90 ± 1.41	WT	NC
MAP	18	≥ 30	≤ 5.0	42 ± 1.00	C1367T	T456I
MAP	185	≤ 4.0	≤ 1.0	61 ± 7.07	Silent	NC
MAP	Cow2013	≤ 1.0		95 ± 1.41	WT	NC
MAP	Cow5	≤ 1.0		96.5 ± 0.71	WT	NC
MAP	61a	≤ 1.0		90 ± 3.54	WT	NC
<i>M. avium</i> subspecies. <i>avium</i> <sup>5</sup>	ATCC25291	≤ 1.0		90 ± 1.41		
<i>M. tuberculosis</i> <sup>5</sup>	ATCC25177	≤ 1.0		92 ± 1.41		
<i>M. smegmatis</i> <sup>5</sup>	ATCC607	≥ 9.0		6.6 ± 1.7		
UCF5-RIF16r	Modified UCF5	≥ 30	≥ 10	32 ± 1.41	T1442C	L481P

<sup>1</sup>Limited investigation involving MAP18, MAP185, and UCF5-RIF16r; <sup>2</sup>Values are expressed as mean ± SD; <sup>3</sup>Wild type (WT) indicates identical investigated rpoB sequence compared to MAP K-10 strain, and differentially expressed rpoB mutations are indicated; <sup>4</sup>Amino acid positions correspond to MAP strain K-10 numbering system from Li *et al* 2005, and the alteration is indicated with single letter amino acid codes and the corresponding codon number. NC corresponds to no change in beta subunit sequence based on investigated rpoB regions; <sup>5</sup>rpoB sequence was not investigated.

Furthermore, sequence data was reported as completely accurate upon the confirmation of error-free reactions. In addition, rpoB sequences for all MAP strains were compared through BLAST analysis using MAP strain K-10 as a reference strain, and amino acid positions were numbered based on MAP strain K-10. The numbering system for *E. coli* provided by Ramaswamy *et al* in 1998 was also used in order to avoid discrimination of our observed results, and to enable a more general comparison of previously published data relevant to other mycobacterial species.

Sequence analysis found identical, or wild-type (WT), and non-identical sequences. MAP strains consisting of the latter were further characterized for silent mutations with no effect on amino acid expression, or mutations that differentially expressed the amino acid sequence of the beta subunit. Of the 11 MAP clinical isolates, 9 consisted of no rpoB mutation in the two regions initially investigated (Table 2). However, two MAP strains possessed rpoB mutations, and these consisted of MAP strain 18 and 185. The rpoB mutations for MAP strain 18 included C1367T and T1375C. The C1367T mutation had significant amino acid changes from Thr456 to Ile456 in the beta subunit of RNA polymerase, which corresponded to a Ser508 to Ile508 change according to the *E. coli* numbering system. To our knowledge, this amino acid change was considered novel as well as indicative of RIF and RFB resistance. The T1375C mutation had no effect on the beta subunit, and was, therefore, characterized as being silent (Table 2). Alternatively, MAP strain 185, which was also isolated in our laboratory from the surgical mesenteric lymph node tissues of a CD patient, showed a total of five rpoB mutations including T534C, T795C, C1335A, T1375C, and C1578T (Table 2). None of the five mutations altered the amino acid sequence in the beta subunit, and consequently were also termed silent. The effects of all rpoB mutations on RIF and RFB susceptibility were investigated following sequence analysis.

### Effect of rpoB mutations on MIC

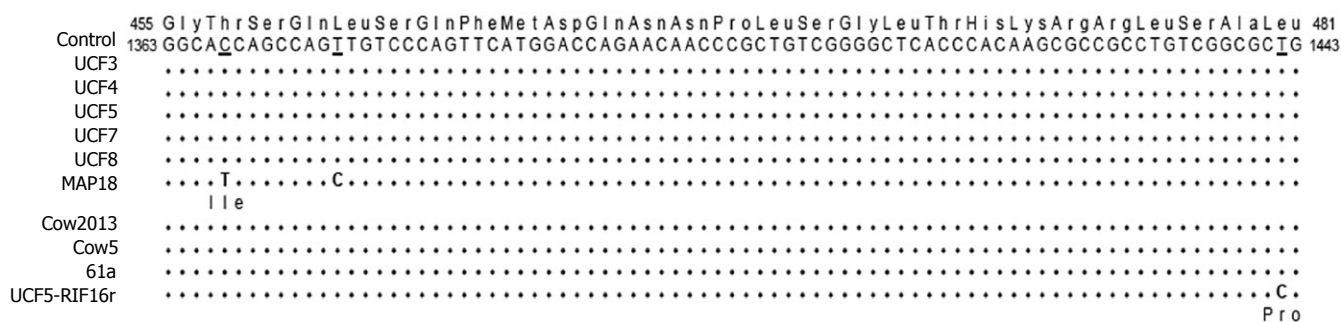
Correlation between rpoB sequence analysis and inhibitory

growth rates of RIF was assessed for all MAP strains. Initially, we tested all MAP strains in the presence of 1 mg/L RIF for comparison purposes (Table 2). This concentration was found to be the MIC of our wild-type control. Hence, our initial susceptibility test was to screen for suspicious growth characteristics against this concentration of RIF. Of the bacterial isolates studied, 8 MAP strains including ATCC43544 (control), UCF3, UCF5, UCF7-8, 61a, Cow2013, Cow5, and two non-MAP controls including *M. tuberculosis* and *M. avium* subspecies *avium* had an MIC ≤ 1.0 mg/L RIF (Table 2). In addition, there were no observed rpoB mutations in these 8 RIF susceptible MAP strains. The MIC for RIF of the remaining MAP strains 18, 185, UCF4, and *M. smegmatis* was determined as ≥ 30, ≤ 4.0, ≤ 2.5 and ≥ 9.0 mg/L, respectively (Table 2). Furthermore, MAP strains 18 and 185 had rpoB mutations in the 81 bp variable region as discussed earlier, and MAP strain UCF4 had no rpoB mutations within the two regions. Despite the lack of rpoB mutations, *M. smegmatis* had MIC of ≥ 9 mg/L RIF. RIF-resistance MAP strains were also evaluated for susceptibility against RFB, a more potent rifamycin antibiotic. Interestingly, the MIC for RFB against MAP strain 18 and 185 was ≤ 5.0 mg/L and ≤ 1.0 mg/L, respectively (Table 2). The MIC values for the drugs against all microorganisms are listed in Table 2.

### Correlation between selected rpoB resistant mutation and MIC in UCF5-RIF16r

An RIF resistant MAP strain, termed UCF5-RIF16r, was selected via culturing wild-type parent strain UCF5 in increasing concentrations of RIF over an extended time period. Specifically, this was accomplished by exposing parent strain UCF5 to a five-fold increase in RIF concentrations over approximately 2 mo. UCF5-RIF16r was then maintained in cultures with > 16.0 mg/L RIF. Genomic DNA was then extracted and followed by IS900-nested PCR and rpoB-based PCR analysis. Consequently, the newly selected resistant strain was confirmed for the





**Figure 1** Sequence alignment of 81 bp region in rpoB gene of MAP. The region overlaps the 81 bp rifamycin resistant determinant region within *M. tuberculosis*. For MAP, this sequence is harbored in the region amplified by primers Knight1/Knight2. Differentially expressed rpoB mutations are indicated for MAP strains 18 and UCF5-RIF16r. Base pairs and amino acids range from 1363-1443 and 456-481. Numbering of amino acids correspond to MAP strain K-10 beta subunit. Dots correspond to homologous bases and specific nucleotides are underlined in the control sequence. MAP strain 185 is excluded from diagram.

presence of IS900, and both regions of the rpoB gene were analyzed. We detected a single nucleotide rpoB mutation T1442C, which caused a differentially expressed amino acid from Leu481 to Pro481 in the beta subunit of RNA polymerase, which corresponded to a Leu533 to Pro533 change according to the *E. coli* numbering system (Table 2). Furthermore, the rpoB mutation was located within the 81 bp variable region, and the effect of the selected mutation on susceptibility to RIF was then investigated. The MIC for UCF5-RIF16r against RIF and RFB was  $\geq 30$  mg/L and  $\geq 10$  mg/L, respectively.

### Sequencing rpoB genes in MAP strains 18, UCF4 and UCF5-RIF16r

We attempted to sequence the entire 3.6 kb rpoB gene in order to determine the source of the variable increase in MIC for RIF against MAP strain UCF4, and to detect any other possible rpoB mutations leading to amino acid alterations in strains 18 and UCF5-RIF16r. We designed additional primers for PCR amplification of sequences outside that amplified by primers UCF1/UCF2 and Knight1/Knight2 (Table 1). After successful PCR amplification of these regions, nucleotide sequencing was performed. Through BLAST analysis, we found a significant rpoB mutation further downstream of the 81 bp variable region in MAP strain UCF4. Specifically, an A2284C mutation occurred within the region amplified by primers Ex1a/Ex2a. This mutation resulted in an Asn762 to His762 amino acid change, leading to a possible explanation for the higher MIC for RIF compared to our wild-type control strain 43544. No other rpoB mutations leading to amino acid changes were observed in MAP strains 18, UCF5-RIF16r, and UCF4. After obtaining collective sequence information for all MAP isolates, we aligned the 81 bp variable region to display the location of each mutation amino acid change that correlated with a high level of RIF resistance (Figure 1).

### Detection of rpoB in PBMNC infected with MAP

To show the potential for sequence analysis in the rpoB gene of MAP clinical isolates in correspondence with RIF and RFB resistance, we contaminated human PBMNC with  $1.0 \times 10^3$  CFU of MAP strain 18. Prokaryotic

genomic DNA was then extracted from the blood mixture, followed by IS900-PCR and rpoB-based PCR analysis. As expected, our protocol detected MAP in the blood sample and successfully amplified both regions of the rpoB gene of MAP (data not shown). The nucleotide sequence was then analyzed for the possible prediction of susceptibility to RIF or RFB.

## DISCUSSION

The main purpose of our study was (1) to characterize MAP's potential for developing RFB and RIF resistance, (2) associate RFB and RIF resistance with mutations in the rpoB gene of MAP, and (3) provide an effective protocol for detecting resistant mutations in MAP strains linked to CD. Despite the fact that *M. smegmatis* is known to be naturally resistant to rifamycins without rpoB mutations<sup>[36]</sup>, microorganisms such as *M. avium* spp. *avium*, *E. coli*, *H. pylori*, *S. aureus* and *M. kansasii* have laid the foundation for this association<sup>[14,16,18,19,21,37]</sup>. Moreover, various strains of closely related *M. tuberculosis* have set a fine trend for rifamycin resistance, as seen through rpoB mutations<sup>[6,17,21,38-46]</sup>. Our goal was to discover this trend in MAP, and develop an effective method for analyzing RFB and RIF resistance in the bacterium through rpoB-based PCR analysis.

Unlike *M. tuberculosis* and members of the *M. avium* complex (MAC), MAP is a fastidious micro-organism and requires prolonged incubation time. Consequently, conventional drug susceptibility testing against MAP strains is ineffective, thus leading us to develop an alternative method for determining drug resistance in MAP.

The UCF1/UCF2 and Knight1/Knight2 rpoB primers enabled the successful amplification and sequencing of two probable RFB and RIF resistant determinant regions (Table 1). These regions overlap similar sequences in the rpoB gene of *M. tuberculosis* that have displayed mutations upon rifamycin resistance, including the 81 bp region 1276-1356 within cluster I, and a region further located in the upstream of the beginning of rpoB<sup>[6,15,20]</sup>. Consequently, our determinant regions of interest in the rpoB gene of MAP were concluded based on the available information for closely related bacteria.

All MAP isolates were identified through an IS900 nested PCR reaction and each was investigated for rpoB



sequences and susceptibility tests against RIF. Those isolates suspected to be RIF resistant based on high MIC results were subjected to susceptibility tests against RFB. Consequently, the control, MAP strain 18 and UCF5-RIF16r were included in a RFB inhibition test. MAP strain 185 was also included in the RFB test due to the strain's suspicious susceptibility against RIF. Unlike RIF, RFB is not commercially available for laboratory use. Hence, information based on antibiotic resistance in our study was subjective mainly for RIF.

Our significant findings included the discovery of *rpoB* mutations within MAP strains 18, UCF5-RIF16r, and UCF4. Both strains 18 and UCF5-RIF16r showed a significant increase in the level of resistance due to amino acid alterations within the binding site of RIF. Moreover, upon our additional sequencing of the entire 3.6 kb *rpoB* gene for all three MAP strains along with a control, a significant *rpoB* mutation A2284C was uncovered leading to an Asn762 to His762 amino acid change in the beta subunit. These results justified the minor increase in MIC against RIF for strain UCF4. In addition, the complete sequencing of the *rpoB* in MAP strains 18 and UCF5-RIF16r proved that no additional *rpoB* mutations leading to an amino acid change were present, confirming the correlation between detected mutations and increased MIC in these micro-organisms. The finding illustrates that a single amino acid change in the *rpoB* gene of MAP may result in an increased level of resistance to RIF, which may have a significant clinical impact, specifically when the amino acid alterations occur within the drug's binding site.

Overall, our results display a novel *rpoB* mutation, C1367T, in MAP strain 18 not previously reported in the literature including *rpoB* studies in *E. coli* and *M. tuberculosis*. In addition, the A2284C *rpoB* mutation discovered in MAP strain UCF4 was also novel to our knowledge; however this mutation did not influence RIF resistance significantly compared to MAP strains 18 and UCF5-RIF16r. A possible explanation for this observation is that the mutation in strain UCF4 was located distant from the drug binding site. The selected *rpoB* mutation in strain UCF5-RIF16r has previously been reported to occur in *M. tuberculosis* as well as in *E. coli*<sup>[13]</sup>. More significantly, Leu481 in the beta subunit of MAP overlaps that of Leu413 in *Thermus aquaticus*, and this amino acid has been reported to make direct contacts with RIF in its bound state through Vanderwaals interactions<sup>[13]</sup>. Hence, the selected change from Leu481 to Pro481 in MAP strain UCF5-RIF16r may have affected the direct interaction between the beta subunit and the drug.

The rationale in selecting the RIF resistant mutant UCF5-RIF16r was to determine the potential for MAP to evolve adaptive resistance to RIF, and the location of the *rpoB* mutation that accounted for the resistance. This data, combined with our analysis of MAP strain 18, showed the potential for MAP to evolve resistance to RFB and RIF *in vivo* and *in vitro* through mutations in the 81 bp variable region. Collectively, after analyzing the resistance characteristics for MAP strain 18 and UCF5-RIF16r, we considered the *rpoB* 81 bp variable regions to be the most significant for determining resistant mutations for RFB and RIF.

MAP strain 185 consisting of five silent mutations within *rpoB* was not considered significant because they did not alter the beta subunit sequence. Moreover, we encountered unusual PCR results and difficulties in extracting genomic DNA from strain 185; therefore, we were unable to sequence its entire *rpoB* gene and account for the slight resistance to RIF. Hence, the mechanism of the partial resistance against RIF for this strain remained undetermined.

Our *rpoB*-based protocol on human blood mixed with MAP was accomplished through contaminating normal human blood with MAP strain 18, and extracting bacterial DNA directly from the blood mixture. As a result, a sufficient amount of bacterial genomic DNA was obtained for successful amplification of our regions of interest within *rpoB* (data not shown). The purpose of this approach was to assess the effectiveness of our protocol on CD patient blood.

Information regarding patient history may explain a rationale for the observed results. Ironically, the patients' medical history indicated that none of them was on anti-MAP treatment. Instead these patients had received a variety of anti-inflammatory and immunosuppressants. However, alarming new reports suggest that some of these drugs may contain antimicrobial activity, especially when tested against few strains of MAP<sup>[47]</sup>. Hence, it is inconclusive to rule out the possibility of antibiotic resistance development *via* alternative drugs.

In conclusion, through the application of our protocol on CD patient samples, we may assist in the determination of RFB and RIF susceptible MAP strains for the treatment of CD. The inconclusive results from the recent Australian clinical trial using RFB, calrithromycin and clofazamine for treatment of patients with CD<sup>[9]</sup> suggested either the absence of MAP or the presence of drug resistance MAP in non-responders. Therefore, detection of drug resistance in pathogens like MAP is now necessary. Our protocol may address the rationale for MAP resistance to RFB in these patients. Based upon the *in vitro* selection of a RFB and RIF resistant MAP strain (UCF5-RIF16r), it is likely that this trend occurs *in vivo*, as supported by the data from MAP strain 18. Furthermore, as RFB is applied more towards the treatment of CD, our protocol will be of utmost significance for the optimization of CD treatment with related antibiotics.

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## REFERENCES

- 1 Chiodini RJ. Crohn's disease and the mycobacterioses: a review and comparison of two disease entities. *Clin Microbiol Rev* 1989; 2: 90-117
- 2 Grant IR. Zoonotic potential of *Mycobacterium avium* ssp. paratuberculosis: the current position. *J Appl Microbiol* 2005; 98: 1282-1293
- 3 Chamberlin W, Graham DY, Hulten K, El-Zimaity HM, Schwartz MR, Naser S, Shafran I, El-Zaatari FA. Review article: *Mycobacterium avium* subsp. paratuberculosis as one cause of Crohn's disease. *Aliment Pharmacol Ther* 2001; 15:

- 337-346
- 4 **Chamberlin WM**, Naser SA. Integrating theories of the etiology of Crohn's disease. On the etiology of Crohn's disease: questioning the hypotheses. *Med Sci Monit* 2006; **12**: RA27-RA33
- 5 **Chiodini RJ**, Van Kruiningen HJ, Thayer WR, Merkal RS, Coutu JA. Possible role of mycobacteria in inflammatory bowel disease. I. An unclassified *Mycobacterium* species isolated from patients with Crohn's disease. *Dig Dis Sci* 1984; **29**: 1073-1079
- 6 **Hermon-Taylor J**, Bull TJ, Sheridan JM, Cheng J, Stellakis ML, Sumar N. Causation of Crohn's disease by *Mycobacterium avium* subspecies paratuberculosis. *Can J Gastroenterol* 2000; **14**: 521-539
- 7 **Hermon-Taylor J**. Protagonist. *Mycobacterium avium* subspecies paratuberculosis is a cause of Crohn's disease. *Gut* 2001; **49**: 755-756
- 8 **Thompson DE**. The role of mycobacteria in Crohn's disease. *J Med Microbiol* 1994; **41**: 74-94
- 9 **Selby W**, Pavli P, Crotty B, Florin T, Radford-Smith G, Gibson P, Mitchell B, Connell W, Read R, Merrett M, Ee H, Hetzel D. Two-year combination antibiotic therapy with clarithromycin, rifabutin, and clofazimine for Crohn's disease. *Gastroenterology* 2007; **132**: 2313-2319
- 10 **Gao A**, Mutharia L, Raymond M, Odumeru J. Improved template DNA preparation procedure for detection of *Mycobacterium avium* subsp. paratuberculosis in milk by PCR. *J Microbiol Methods* 2007; **69**: 417-420
- 11 **Naser SA**, Ghobrial G, Romero C, Valentine JF. Culture of *Mycobacterium avium* subspecies paratuberculosis from the blood of patients with Crohn's disease. *Lancet* 2004; **364**: 1039-1044
- 12 **Romero C**, Hamdi A, Valentine JF, Naser SA. Evaluation of surgical tissue from patients with Crohn's disease for the presence of *Mycobacterium avium* subspecies paratuberculosis DNA by in situ hybridization and nested polymerase chain reaction. *Inflamm Bowel Dis* 2005; **11**: 116-125
- 13 **Campbell EA**, Korzheva N, Mustaev A, Murakami K, Nair S, Goldfarb A, Darst SA. Structural mechanism for rifampicin inhibition of bacterial rna polymerase. *Cell* 2001; **104**: 901-912
- 14 **Glocker E**, Bogdan C, Kist M. Characterization of rifampicin-resistant clinical *Helicobacter pylori* isolates from Germany. *J Antimicrob Chemother* 2007; **59**: 874-879
- 15 **Heep M**, Brandstatter B, Rieger U, Lehn N, Richter E, Rusch-Gerdes S, Niemann S. Frequency of rpoB mutations inside and outside the cluster I region in rifampin-resistant clinical *Mycobacterium tuberculosis* isolates. *J Clin Microbiol* 2001; **39**: 107-110
- 16 **Jin DJ**, Gross CA. Characterization of the pleiotropic phenotypes of rifampin-resistant rpoB mutants of *Escherichia coli*. *J Bacteriol* 1989; **171**: 5229-5231
- 17 **Klein JL**, Brown TJ, French GL. Rifampin resistance in *Mycobacterium kansasii* is associated with rpoB mutations. *Antimicrob Agents Chemother* 2001; **45**: 3056-3058
- 18 **Murphy CK**, Mullin S, Osburne MS, van Duzer J, Siedlecki J, Yu X, Kerstein K, Cynamon M, Rothstein DM. In vitro activity of novel rifamycins against rifampicin-resistant *Staphylococcus aureus*. *Antimicrob Agents Chemother* 2006; **50**: 827-834
- 19 **Obata S**, Zwolska Z, Toyota E, Kudo K, Nakamura A, Sawai T, Kuratsugi T, Kirikae T. Association of rpoB mutations with rifampicin resistance in *Mycobacterium avium*. *Int J Antimicrob Agents* 2006; **27**: 32-39
- 20 **Ramaswamy S**, Musser JM. Molecular genetic basis of antimicrobial agent resistance in *Mycobacterium tuberculosis*: 1998 update. *Tuber Lung Dis* 1998; **79**: 3-29
- 21 **Heep M**, Rieger U, Beck D, Lehn N. Mutations in the beginning of the rpoB gene can induce resistance to rifamycins in both *Helicobacter pylori* and *Mycobacterium tuberculosis*. *Antimicrob Agents Chemother* 2000; **44**: 1075-1077
- 22 **Borody TJ**, Bilkey S, Wettstein AR, Leis S, Pang G, Tye S. Antimycobacterial therapy in Crohn's disease heals mucosa with longitudinal scars. *Dig Liver Dis* 2007; **39**: 438-444
- 23 **Borody TJ**, Leis S, Warren EF, Surace R. Treatment of severe Crohn's disease using antimycobacterial triple therapy--approaching a cure? *Dig Liver Dis* 2002; **34**: 29-38
- 24 **Chamberlin W**, Ghobrial G, Chehtane M, Naser SA. Successful treatment of a Crohn's disease patient infected with bacteremic *Mycobacterium paratuberculosis*. *Am J Gastroenterol* 2007; **102**: 689-691
- 25 **Gui GP**, Thomas PR, Tizard ML, Lake J, Sanderson JD, Hermon-Taylor J. Two-year-outcomes analysis of Crohn's disease treated with rifabutin and macrolide antibiotics. *J Antimicrob Chemother* 1997; **39**: 393-400
- 26 **Hermon-Taylor J**. Treatment with drugs active against *Mycobacterium avium* subspecies paratuberculosis can heal Crohn's disease: more evidence for a neglected public health tragedy. *Dig Liver Dis* 2002; **34**: 9-12
- 27 **Shafraan I**, Kugler L, El-Zaatari FA, Naser SA, Sandoval J. Open clinical trial of rifabutin and clarithromycin therapy in Crohn's disease. *Dig Liver Dis* 2002; **34**: 22-28
- 28 **Williams SL**, Harris NB, Barletta RG. Development of a firefly luciferase-based assay for determining antimicrobial susceptibility of *Mycobacterium avium* subsp. paratuberculosis. *J Clin Microbiol* 1999; **37**: 304-309
- 29 **Pizarro TT**, Cominelli F. Cytokine therapy for Crohn's disease: advances in translational research. *Annu Rev Med* 2007; **58**: 433-444
- 30 **Hermon-Taylor J**, Barnes N, Clarke C, Finlayson C. *Mycobacterium paratuberculosis* cervical lymphadenitis, followed five years later by terminal ileitis similar to Crohn's disease. *BMJ* 1998; **316**: 449-453
- 31 **Naser SA**, Shafraan I, Schwartz D, El-Zaatari F, Biggerstaff J. In situ identification of mycobacteria in Crohn's disease patient tissue using confocal scanning laser microscopy. *Mol Cell Probes* 2002; **16**: 41-48
- 32 **Schwartz D**, Shafraan I, Romero C, Piromalli C, Biggerstaff J, Naser N, Chamberlin W, Naser SA. Use of short-term culture for identification of *Mycobacterium avium* subsp. paratuberculosis in tissue from Crohn's disease patients. *Clin Microbiol Infect* 2000; **6**: 303-307
- 33 **Motiwala AS**, Strother M, Amonsin A, Byrum B, Naser SA, Stabel JR, Shulaw WP, Bannantine JP, Kapur V, Sreevatsan S. Molecular epidemiology of *Mycobacterium avium* subsp. paratuberculosis: evidence for limited strain diversity, strain sharing, and identification of unique targets for diagnosis. *J Clin Microbiol* 2003; **41**: 2015-2026
- 34 **Wu CW**, Glasner J, Collins M, Naser S, Talaat AM. Whole-genome plasticity among *Mycobacterium avium* subspecies: insights from comparative genomic hybridizations. *J Bacteriol* 2006; **188**: 711-723
- 35 **Li L**, Bannantine JP, Zhang Q, Amonsin A, May BJ, Alt D, Banerji N, Kanjilal S, Kapur V. The complete genome sequence of *Mycobacterium avium* subspecies paratuberculosis. *Proc Natl Acad Sci USA* 2005; **102**: 12344-12349
- 36 **Hetherington SV**, Watson AS, Patrick CC. Sequence and analysis of the rpoB gene of *Mycobacterium smegmatis*. *Antimicrob Agents Chemother* 1995; **39**: 2164-2166
- 37 **Xu M**, Zhou YN, Goldstein BP, Jin DJ. Cross-resistance of *Escherichia coli* RNA polymerases conferring rifampin resistance to different antibiotics. *J Bacteriol* 2005; **187**: 2783-2792
- 38 **Ahmad S**, Mokaddas E. The occurrence of rare rpoB mutations in rifampicin-resistant clinical *Mycobacterium tuberculosis* isolates from Kuwait. *Int J Antimicrob Agents* 2005; **26**: 205-212
- 39 **Aktas E**, Durmaz R, Yang D, Yang Z. Molecular characterization of isoniazid and rifampin resistance of *Mycobacterium tuberculosis* clinical isolates from Malatya, Turkey. *Microb Drug Resist* 2005; **11**: 94-99
- 40 **Anthony RM**, Schuitema AR, Bergval IL, Brown TJ, Oskam L, Klatser PR. Acquisition of rifabutin resistance by a rifampicin resistant mutant of *Mycobacterium tuberculosis* involves an unusual spectrum of mutations and elevated frequency. *Ann Clin Microbiol Antimicrob* 2005; **4**: 9
- 41 **Bakonyte D**, Baranauskaite A, Cicinaite J, Sosnovskaja A, Stakenas P. Mutations in the rpoB gene of rifampicin-resistant

- Mycobacterium tuberculosis clinical isolates from Lithuania. *Int J Tuberc Lung Dis* 2005; **9**: 936-938
- 42 **Cavusoglu C**, Karaca-Derici Y, Bilgic A. In-vitro activity of rifabutin against rifampicin-resistant Mycobacterium tuberculosis isolates with known rpoB mutations. *Clin Microbiol Infect* 2004; **10**: 662-665
- 43 **Jou R**, Chen HY, Chiang CY, Yu MC, Su IJ. Genetic diversity of multidrug-resistant Mycobacterium tuberculosis isolates and identification of 11 novel rpoB alleles in Taiwan. *J Clin Microbiol* 2005; **43**: 1390-1394
- 44 **Ma X**, Wang H, Deng Y, Liu Z, Xu Y, Pan X, Musser JM, Graviss EA. rpoB Gene mutations and molecular characterization of rifampin-resistant Mycobacterium tuberculosis isolates from Shandong Province, China. *J Clin Microbiol* 2006; **44**: 3409-3412
- 45 **McCammon MT**, Gillette JS, Thomas DP, Ramaswamy SV, Graviss EA, Kreiswirth BN, Vigg J, Quitugua TN. Detection of rpoB mutations associated with rifampin resistance in Mycobacterium tuberculosis using denaturing gradient gel electrophoresis. *Antimicrob Agents Chemother* 2005; **49**: 2200-2209
- 46 **Yuen LK**, Leslie D, Coloe PJ. Bacteriological and molecular analysis of rifampin-resistant Mycobacterium tuberculosis strains isolated in Australia. *J Clin Microbiol* 1999; **37**: 3844-3850
- 47 **Greenstein RJ**, Su L, Shahidi A, Brown ST. On the action of 5-amino-salicylic acid and sulfapyridine on M. avium including subspecies paratuberculosis. *PLoS ONE* 2007; **2**: e516

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## Mitochondrial protection by low doses of insulin-like growth factor- I in experimental cirrhosis

Raquel Pérez, María García-Fernández, Matías Díaz-Sánchez, Juan E Puche, Gloria Delgado, Marian Conchillo, Jordi Muntané, Inma Castilla-Cortázar

Raquel Pérez, Matías Díaz-Sánchez, Marian Conchillo, Departments of Human Physiology and Internal Medicine, University of Navarra, Pamplona 31080, Spain

María García-Fernández, Juan E Puche, Gloria Delgado, Inma Castilla-Cortázar, Department of Medical Physiology, School of Medicine, University of Málaga, Málaga 29080, Spain  
Juan E Puche, Inma Castilla-Cortázar, Department of Medical Physiology, School of Medicine, University USP-CEU, Madrid 28668, Spain

Jordi Muntané, Department of Internal Medicine, Liver Unit, University of Córdoba, Córdoba 14004, Spain

**Author contributions:** Pérez R worked on mitochondrial function tests, *in vivo* treatments; Díaz-Sánchez M worked on TUNEL assay; García-Fernández M contributed to analytical and mitochondrial function tests; Puche JE worked on experimental design and Western blot; Muntané J contributed to Western blot for caspase; Delgado G analyse the data; Conchillo M designed the study; Castilla-Cortázar I contributed to the histology, experimental design, *in vivo* treatment, statistical analysis and wrote the paper.

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Correspondence to: Inma Castilla de Cortázar Larrea, MD, Department of Medical Physiology, School of Medicine, University CEU-USP, Boadilla del Monte, Madrid 28668, Spain. [iccortazar@uma.es](mailto:iccortazar@uma.es)

Telephone: +34-91-3724765 Fax: +34-91-3724008

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### Abstract

**AIM:** To characterize the mitochondrial dysfunction in experimental cirrhosis and to study whether insulin-like growth factor- I (IGF- I) therapy (4 wk) is able to induce beneficial effects on damaged mitochondria leading to cellular protection.

**METHODS:** Wistar rats were divided into three groups: Control group, untreated cirrhotic rats and cirrhotic rats treated with IGF- I treatment (2 µg/100 g bw/d). Mitochondrial function was analyzed by flow cytometry in isolated hepatic mitochondria, caspase 3 activation was assessed by Western blot and apoptosis by TUNEL in the three experimental groups.

**RESULTS:** Untreated cirrhotic rats showed a mitochondrial dysfunction characterized by a significant reduction of mitochondrial membrane potential (in status 4 and 3); an increase of intramitochondrial reactive oxygen species (ROS) generation and a significant reduction of ATPase activity. IGF- I therapy normalized mitochondrial func-

tion by increasing the membrane potential and ATPase activity and reducing the intramitochondrial free radical production. Activity of the electron transport complexes I and III was increased in both cirrhotic groups. In addition, untreated cirrhotic rats showed an increase of caspase 3 activation and apoptosis. IGF- I therapy reduced the expression of the active peptide of caspase 3 and resulted in reduced apoptosis.

**CONCLUSION:** These results show that IGF- I exerts a mitochondrial protection in experimental cirrhosis leading to reduced apoptosis and increased ATP production.

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**Key words:** Insulin-like growth factor- I ; Cirrhosis; Mitochondrial protection; Caspases; Apoptosis; Oxidative damage

**Peer reviewer:** Richard A Rippe, Dr, Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7038, United States

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### INTRODUCTION

Insulin-like growth factor- I (IGF- I) is an anabolic hormone produced mainly in the liver in response to growth hormone (GH) stimulation<sup>[1]</sup>. In cirrhosis, the reduction of receptors for GH in hepatocytes and the reduced ability of synthesis of the hepatic parenchyma cause a progressive decrease in serum IGF- I levels<sup>[2,3]</sup>. We have shown previously that short courses of treatment with IGF- I in rats with carbon tetrachloride-induced cirrhosis induced many systemic beneficial effects<sup>[4-11]</sup> and showed hepatoprotective, antioxidant and antitubrogenic properties<sup>[12-15]</sup>. However, these mechanisms of IGF- I activities regarding the improvement of liver function and fibrosis are not fully understood. We have suggested previously that the described hepatoprotection by IGF- I therapy in rats with carbon tetrachloride-induced cirrhosis



could be related to mechanisms of mitochondrial protection<sup>[12]</sup>.

Mitochondria are a major source of reactive oxygen species (ROS) under physiologic conditions, because 2% to 3% of the O<sub>2</sub> consumed is converted to O<sub>2</sub><sup>•</sup>. Intramitochondrial ROS production increases after peroxidation of mitochondrial membrane lipids<sup>[16-20]</sup>. Mitochondria are particularly sensitive to ROS-induced injury in the pathogenesis of disease. Oxidative stress exerts deleterious effects on mitochondrial function by directly impairing oxidative phosphorylation through direct attack of proteins or membrane lipids. ROS can also induce mitochondrial DNA deletions and mitochondrial membrane permeability transition (MMPT)<sup>[21]</sup>. MMP pore opening activates caspases which is an endpoint to initiate cell death. Furthermore, it has been implicated in the pathogenesis of many fibroproliferative diseases including liver fibrogenesis<sup>[22]</sup>. Recently, a large number of studies have associated mitochondrial dysfunction caused by ROS to both accidental cell death (necrosis) and programmed cell death (apoptosis)<sup>[19,23,24]</sup>. In order to give a better insight into the mechanisms by which IGF- I exerts an hepatoprotective action on damaged liver and supposing that mitochondria could be one of the main cellular target of IGF- I, the aims of the present study were: (1) to characterize the mitochondrial dysfunction in experimental liver cirrhosis and (2) to study whether IGF- I (4 wk) induces beneficial effects on damaged mitochondria leading to cellular protection. Thus, we have extended our study to analyze the effect of IGF- I on mitochondrial function (Mitochondrial Potencial Membrane, intramitochondrial free radical production and “*in vitro*” complex activities), caspase 3 activation and apoptosis. In this series we included three experimental groups of Wistar rats: healthy control group (CO); untreated cirrhotic rats (CI) and cirrhotic rats treated with IGF- I at low doses (2 µg/100 g bw/d, CI + IGF- I).

## MATERIALS AND METHODS

### Animals, liver cirrhosis induction

All experimental procedures were performed in conformity with The Guiding Principles for Research Involving Animals<sup>[25]</sup>. Cirrhosis was induced as previously described<sup>[5,12]</sup>. Briefly, male Wistar rats (3 wk old, 130-150 g) were subjected to CCl<sub>4</sub> inhalation (Merck, Darmstadt, Germany) twice a week for 11 wk with a progressively increasing exposure time from 1 to 5 min. From that time since 12 wk until the 16 wk rats were exposed to CCl<sub>4</sub> once a week for 2 min (5 additional doses of CCl<sub>4</sub>).

During the whole period of cirrhosis induction animals received phenobarbital (Luminal, Bayer, Leverkusen, Germany) in the drinking water (400 mg/L). Both food (standard semipurified diet for rodents; B.K. Universal, Sant Vicent del Horts, Spain) and water were given *ad libitum*. Healthy, age and sex-matched control rats were maintained under the same conditions, but received neither CCl<sub>4</sub> nor phenobarbital.

### Study design

IGF- I therapy or saline was administered the last 4 wk

(13th-16th wk, 28 d), cirrhotic rats were randomly assigned to receive either vehicle (saline) (group CI, *n* = 6) or recombinant human IGF- I (Chiron Company, San Francisco, CA) (20 µg/kg per day in two divided doses, subcutaneously) (Group CI + IGF- I, *n* = 6) for 4 wk. Healthy control rats (Group CO, *n* = 6) received saline during the same period.

In the morning of the 29th day (after 4 wk of treatment), rats were weighed, blood was obtained from the retroocular plexus and animals were killed by decapitation. After the abdominal cavity was opened, the livers were dissected and weighed. No animals developed ascites in this series that was considered as compensated cirrhosis. A sample from the left major liver lobe and the testes was processed for histological examination (fixed in Bouin's solution) and the right lobe was placed in ice-cold isolation buffer. Bouin-fixed tissues were processed and sections (4 µm) were stained with haematoxylin and eosin and Masson's trichrome. Liver cirrhosis was confirmed in all animals treated with CCl<sub>4</sub> following the criteria previously reported<sup>[12,15]</sup>.

### Analytical methods

Liver function tests were determined by routine laboratory methods using a Hitachi 747 autoanalyzer (Boehringer Mannheim, Mannheim, Germany).

### 4-Hydroxynonenal determination

Lipid peroxidation is a well-established mechanism of cellular injury in animals and is used as an indicator of oxidative stress in cells and tissues. Lipid peroxides are unstable and decompose to form a complex series of compounds including reactive carbonyl compounds.

Polyunsaturated fatty acid peroxides generate malondialdehyde (MDA) and 4-hydroxynonenal (4-HNE) upon decomposition. Measurement of malondialdehyde and 4-hydroxyalkenals have been used as an indicator of lipid peroxidation<sup>[26]</sup>. The LPO-586 method (Biotech, Oxis International Inc, USA) is designed to assay MDA in combination with 4- hydroxynonenal.

The LPO-586<sup>TM</sup> assay is based on the reaction of a chromogenic reagent, N-methyl-2-phenylindole, with MDA and 4-HNE at 45°C. One molecule of either MDA or 4-hydroxyalkenal reacts with 2 molecules of reagent N-methyl-2-phenylindole to yield a stable chromophore with maximal absorbance at 586 nm. For simultaneous determination of MDA and 4-HNE, one must use the procedure utilizing methanesulfonic acid (MSA) as the acid solvent.

### Protein carbonyl content (PCC) determination

PCC was determined by the method of Levine *et al*<sup>[27]</sup>. The sample was divided into two portions containing 1-2 mg protein each. To one portion, an equal volume of 2 mol/L HCl was added and incubated at room temperature for one hour, and shaken intermittently. The other portion was treated with an equal volume of 10 mmol/L DNPH in 2 mol/L HCl and incubated for one hour at room temperature. After incubation, the mixture was precipitated with 10% TCA and centrifuged. The precipitate was washed with ethanol:ethyl acetate (1:1), twice dissolved in 1 mL of 6 mol/L guanidine HCl, centrifuged at low speed and

the supernatant was taken. The difference in absorbance between the DNPH-treated and HCl-treated samples was determined at 366 nm.

### Isolation of liver mitochondria

Liver mitochondrial fraction was prepared according to the method described by Schneider and Hogeboom with modifications. Liver samples were homogenized (1:10 w/v) in an ice-cold isolation buffer pH 7.4, containing sucrose 0.25 mmol/L,  $\text{KH}_2\text{PO}_4$  5 mmol/L, MOPS 5 mmol/L, and 0.1% BSA. The homogenate was centrifuged at 800 *g* for 10 min. The resulting supernatant was centrifuged at 8500 *g* for 10 min. The supernatant was discarded and the pellet was diluted in cold isolation buffer and centrifuged at 8500 *g* for 10 min three times. The final mitochondria pellet was resuspended in a minimal volume and aliquots were stored at -80°C until use in enzyme assays. All procedures were conducted at 4°C.

### Flow cytometry analysis

Mitochondrial membrane potential was evaluated using rhodamine 123 dye (RH123) obtained from Molecular Probes, Inc. (Eugene, OR). It has an absorbance maximum of 500 nm and an emission maximum of 523. Mitochondrial suspensions (40-50 µg mitochondrial prot/mL) were incubated in isolation buffer for 5 min in the dark with RH123 (0.5 µg/mL), after adding various agents. For state 4 conditions, mitochondrial samples were incubated with rotenone 25 µmol/L and energized with sodium succinate 5 mmol/L, and with rotenone 25 µmol/L, sodium succinate 5 mmol/L + ADP 200 µmol/L for state 3 conditions. The uncoupler CCCP 10 µmol/L was added to confirm that the uptake of RH123 was related to mitochondrial membrane potential.

The mitochondrial free radical production was measured by RH123 production, a fluorescent molecule derivated from DihydroRh123 without charge (DHR123) (Molecular Probes Inc.) employing the cytometry method performed by O'Connor<sup>[28]</sup> with a small modification.

The mitochondrial homogenates (100 µg/mL) were incubated with DHR123 8.2 nmol/L and radish peroxidase 7 U/mL for 5 min, at room temperature. Fluorescence values from the substrates were normalized with that obtained with peroxidase + uncoupler CCPC.  $\text{H}_2\text{O}_2$  1 mmol/L was employed as positive control.

After incubation, the suspensions were analyzed immediately. Gated mitochondrial population was chosen by flow cytometry, based on forward scatter (FS) and side scatter (SS) within mitochondria samples.

Cytofluorometric analysis was performed by using a flow cytometer EPICS XL (Beckman-Coulter, CA). Green fluorescence was detected with a wide band filter for RH123 centred in  $525 \pm 20$  nm (FL1).

The fluorescence intensity of dyes reflecting a minimum of 15000 individual mitochondria per sample were analyzed in an Epics XL flow cytometer (Beckman-Coulter, CA) using System II version 3.0. Software.

### In vitro mitochondrial activities

Mitochondrial suspensions were thawed and diluted with

potassium phosphate. Activities of the respiratory chain enzymes were measured at 37°C in Cobas Mira (ABXMicro, Alemania). *In vitro* mitochondrial activities were assessed as follow.

**Complex I NADH-ubiquinone oxidoreductase (E.C. 1.6.99.3.):** The activity was measured by following the decrease in absorbance due to oxidation of NADH to NAD at 340 nm<sup>[29]</sup>. The reaction mixture contained potassium phosphate 1.0 mol/L (pH 8),  $\text{NaN}_3$  0.1 mol/L, EDTA 1 mmol/L, NADH 10 mmol/L, Coenzyme Q1 1 mmol/L. The reaction was initiated by the addition of the mitochondrial suspensions and was monitored for 10 min.

**Complex II Succinate-ubiquinone oxidoreductase (E.C. 1.3.5.1.):** The activity was measured by following the decrease in absorbance due to coupled reduction of 2,6-dichlorophenolindophenol (DCPIP) at 600 nm. The reaction mixture contained sodium succinate 1.0 mol/L, potassium phosphate 1.0 mol/L (pH 7), 10% Triton X-100, EDTA 1 mmol/L, NADH 10 mmol/L, 0.1% DC-PIP, Coenzyme Q<sub>2</sub> 0.01 mg/mL. The reaction was initiated by the addition of the mitochondrial suspensions and was monitored for 10 min.

**Complex III Ubiquinol-ferricytochrome c oxidoreductase (E.C.1.10.2.2.):** The activity was measured by following the increase in absorbance due to the reduction ferricytochrome c at 550 nm. The reaction mixture contained sodium succinate 1.0 mol/L, potassium phosphate 0.04 mol/L (pH 7.4),  $\text{NaN}_3$  0.02 mol/L, EDTA 0.1 mmol/L, 0.1% bovine serum albumin, (CoQ<sub>2</sub>)  $\text{H}_2$  0.05 mg/mL. Mitochondrial sample was added and reaction was initiated by the addition of ferricytochrome c 3 mg/mL and was monitored for 2 min. Ferricytochrome c was prepared according to Trounce *et al*<sup>[30]</sup>.

**Complex IV Ferrocytochrome c-oxygen oxidoreductase (E.C.1.9.3.1.):** The activity was measured by following the decrease in absorbance due to the oxidation of ferrocytochrome c at 550 nm. The assay mixture consisted of potassium phosphate 0.1 mol/L (pH 7.4) and 1% ferrocytochrome c. The reaction was initiated by the addition of mitochondria and the reaction was monitored for 2 min. Ferrocytochrome c was prepared according to Trounce *et al*<sup>[30]</sup>.

**Complex V ATPase (E.C: 3.6.1.34.):** The activity was assayed by coupling the reaction to the pyruvate kinase and lactate dehydrogenase systems and measuring NADH oxidation at 340 nm. The assay system contained Tris-HCl buffer 65 mmol/L (pH 7.5), sucrose 300 mmol/L,  $\text{MgCl}_2$  4.75 mmol/L, ATP 4 mmol/L, NADH 0.4 mmol/L, phosphoenolpyruvate 0.6 mmol/L, KCN 5 mmol/L, PK 700 U/mL and LDH 1000 U/mL.

### Measurement of caspase-3 processing

Frozen livers (1 g) were homogenized (Ultra-turrax T25; Janke & Kunkel IKA-laboratory) in lysis solution (1% SDS, Tris-HCl 10 mmol/L, EDTA 50 mmol/L, PMSF

Table 1 Analytical data in the three experimental groups before and after treatment

		CO, healthy control group	CI, untreated cirrhotic group	CI + IGF- I, cirrhotic rats treated with IGF- I
Aspartate transaminase (IU/L)	d 0	75.0 ± 5.0	142.0 ± 35.0	150.0 ± 24.0
	d 30	86.0 ± 4.0	168.0 ± 29.0 <sup>b</sup>	128.0 ± 15.0 <sup>c</sup>
Alanine transaminase (IU/L)	d 0	34.0 ± 1.0	69.0 ± 10.0 <sup>b</sup>	73.0 ± 10.0 <sup>b</sup>
	d 30	33.0 ± 2.0	58.0 ± 7.0 <sup>b</sup>	58.0 ± 9.0 <sup>bc</sup>
Alkaline phosphatase (IU/L)	d 0	63.0 ± 4.0	110.0 ± 14.0 <sup>a</sup>	124.0 ± 21.0 <sup>a</sup>
	d 30	60.0 ± 2.0	100.0 ± 12.0 <sup>b</sup>	77.0 ± 8.0 <sup>c</sup>
Bilirubin (mg/dL)	d 0	0.1 ± 0.1	0.4 ± 0.2 <sup>a</sup>	0.4 ± 0.1 <sup>a</sup>
	d 30	0.1 ± 0.0	0.3 ± 0.1	0.2 ± 0.1 <sup>c</sup>
Cholesterol (mg/dL)	d 0	49.0 ± 4.0	76.0 ± 5.0 <sup>b</sup>	80.0 ± 7.0 <sup>b</sup>
	d 30	46.0 ± 2.0	61.0 ± 9.0	57.0 ± 5.0 <sup>c</sup>
Triglycerides (mg/dL)	d 0	151.0 ± 22.0	119.0 ± 6.0	101.0 ± 11.0
	d 30	102.0 ± 12.0	82.0 ± 11.0	70.0 ± 7.0
Glucose (mmol/L)	d 0	11.32 ± 0.63	6.97 ± 0.43 <sup>b</sup>	6.45 ± 0.51 <sup>b</sup>
	d 30	9.27 ± 0.34	6.4 ± 0.45 <sup>b</sup>	7.45 ± 0.32 <sup>bc</sup>
Albumin (g/dL)	d 0	3.6 ± 0.1	3.4 ± 0.1	3.4 ± 0.1
	d 30	3.8 ± 0.1	3.5 ± 0.2	3.7 ± 0.1
Total proteins (mmol/L)	d 0	6.9 ± 0.1	6.6 ± 0.2	6.5 ± 0.1
	d 30	6.8 ± 0.8	6.2 ± 0.3	6.6 ± 0.1

mean ± SEM; <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs CD groups; <sup>c</sup>*P* < 0.05 vs before treatment.

1 mmol/L, aprotinin 1 µg/mL and leupeptin 1 µg/mL) pH 7.4 at 4°C for 10 min, transferred to Eppendorf tubes and centrifuged at 20800 *g* at 4°C for 5 min. Proteins (100 µg) were separated by 12% SDS-PAGE and transferred to nitrocellulose. The membranes for measuring caspase-3 activation were incubated with anti-caspase-3 (dilution 1:150) rabbit polyclonal antibodies (Santa Cruz Biotechnology, Inc., California, USA) as primary antibodies and anti-rabbit-IgG-alkaline phosphatase (Sigma Chemical Co.) as secondary antibodies using BCIP-NBT as alkaline phosphatase substrate.

#### Apoptosis assessment by TUNEL at the Light-Microscopic Level

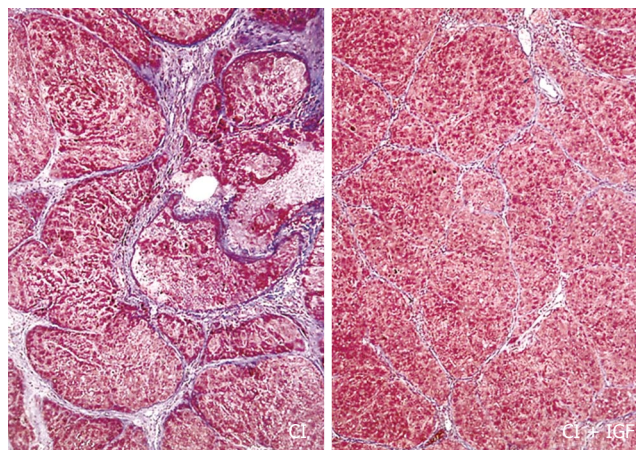
TUNEL was performed in deparaffinized 4-µm-thick sections of liver from the three experimental groups with an ApopTag kit (Oncor Gaithersburg, Maryland) according to the supplier's instructions. Sections were counterstained with hematoxylin. The number of TUNEL-positive hepatocytes was recorded in the whole preparation (at × 200 magnification). The results were expressed as stained cells per area (arithmetic mean of 4 areas).

#### Statistical analysis

Data are expressed as mean ± SEM. Statistical significance was estimated with paired or unpaired *t*-test as appropriate. A *P*-value of < 0.05 was considered significant. All analyses were performed by using the SPSS version 10.0. (SPSS Inc, Chicago, USA) statistical package.

## RESULTS

At baseline, before the onset of IGF- I treatment, groups CI and CI + IGF- I showed similar serum levels of alanine aminotransferase, aspartate aminotransferase, glucose, cholesterol, alkaline phosphatase, bilirubin, total protein and albumin which were all of them significantly abnormal when compared to those in control rats (Table 1). According to previous data, the biochemical results in this series



**Figure 1** Histopathological comparative study (4 µm sections; Masson's trichrome stain). An evident reduction of fibrosis was found in the cirrhotic group treated with IGF-I in agreement with previous studies<sup>[12,14,15]</sup>.

showed an improvement of liver function tests as it is summarized in Table 1.

In agreement with previous results, a reduction of fibrosis (Figure 1) and liver oxidative products were also observed in CI-IGF group: 4-hydroxynonenal, CO = 0.64 ± 0.05; CI = 0.78 ± 0.03; CI + IGF- I = 0.73 ± 0.04 (nmol/mg prot.); Protein carboxyl content (nmol/mg prot.): CO = 6.48 ± 0.71; CI = 8.15 ± 0.60; CI + IGF- I = 6.80 ± 0.64 (*P* < 0.05 CI vs CO), although the reduction was less notable than in previous studies because in this series (see Methods) cirrhotic animals received CCl<sub>4</sub> doses also during the 4 wk of treatment (with IGF- I or saline).

#### Mitochondrial membrane potential

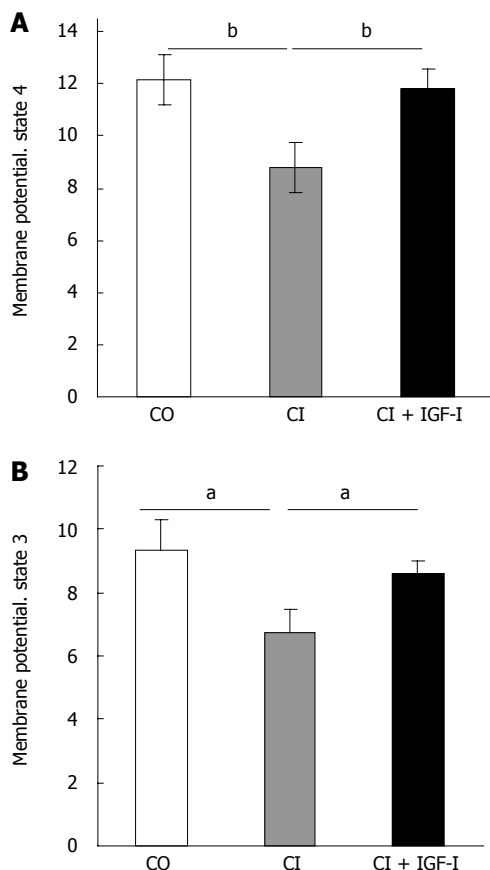
Gated mitochondrial population was chosen by flow cytometry (FCM), based on forward scatter (FS) and side scatter (SS) within mitochondria samples.

The membrane potential was monitored by fluorescence

Table 2 Activities of the electron transport complexes ( $n = 6$ )

	CO, healthy control group	CI, untreated cirrhotic group	CI + IGF, cirrhotic rats treated with IGF- I
I -NADH-ubiquinone oxidoreductase (nmol/min per mg prot)	7.68 ± 2.19	11.01 ± 1.60 <sup>c</sup>	16.10 ± 2.03
II -Succinate-ubiquinone oxidoreductase (μmol/min per mg prot)	0.38 ± 0.02	0.33 ± 0.04	0.35 ± 0.03
III -Ubiquinol-ferricytochrome oxidoreductase (nmol/min per mg prot)	89.57 ± 6.54	148.75 ± 23.26 <sup>a</sup>	163.15 ± 21.38 <sup>b</sup>
IV -Ferrocycytochrome c-oxygen oxidoreductase (pmol/min per mg prot)	3.91 ± 0.85	5.22 ± 1.01	5.66 ± 0.97

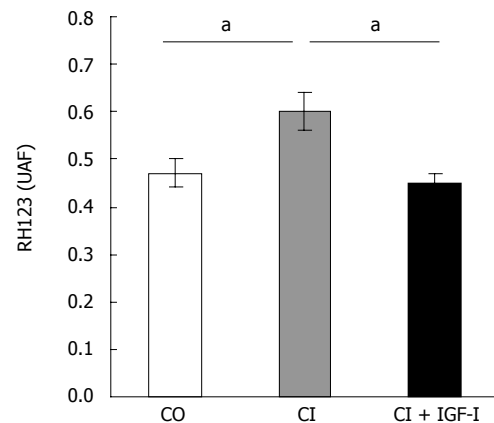
mean ± SEM; <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs CO groups; <sup>c</sup> $P < 0.05$  vs CI + IGF.



**Figure 2** Mitochondrial Membrane Potential (MMP): the MMP was evaluated by flow cytometry with Rhodamine 123 under respiratory conditions (with succinate), state 4 (A) and with ADP state 3 (B). MMP is expressed as fluorescence arbitrary units (FAU). mean ± SEM; <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ .

quenching of RH123 in mitochondria from liver rats under respiratory state 4. Under these conditions, the mitochondrial membrane potential is an index of mitochondrial energy status. Figure 2A shows that the membrane potential in state 4 was significantly decreased in the CI animals compared to control, and was restored upon IGF- I treatment (CO, healthy controls =  $12.18 \pm 0.96$ , CI, untreated cirrhotic rats =  $8.83 \pm 0.88$ , CI + IGF- I, cirrhotic rats treated with IGF- I =  $11.80 \pm 0.85$  Fluorescence Arbitrary Units -FAU-).

In a similar way, in the state 3 (addition of ADP as substrate), expressed as fluorescence arbitrary units, both the control rats and the CI + IGF- I rats showed significantly higher values than untreated cirrhotics rats treatment (CO =  $9.33 \pm 0.97$ , CI =  $6.71 \pm 0.58$ , CI + IGF- I =  $8.62 \pm 0.45$  FAU) (Figure 2B).



**Figure 3** Intramitochondrial ROS production in isolated mitochondria: mitochondria from untreated cirrhotic rats showed an increased production of free radicals as compared to healthy controls (CO) and cirrhotic rats treated with IGF- I (CI + IGF). mean ± SEM; <sup>a</sup> $P < 0.05$ .

### Free radical production

Mitochondria from untreated cirrhotic rats showed a significant increase in ROS generation (CI =  $0.58 \pm 0.03$  FAU,  $P < 0.05$  vs CO) when compared to controls (CO =  $0.47 \pm 0.02$  FAU) and cirrhotic rats treated with IGF- I (CI + IGF- I =  $0.45 \pm 0.01$  FAU,  $P < 0.05$  vs CI): see Figure 3.

### Activities of the electron transport complexes

Table 2 summarizes the activities of the electron transport complexes in mitochondria from the three experimental groups. As shown in Table 2, activities of complex I and III increased significantly in cirrhotic animals. This increase was significantly higher in the CI + IGF- I group. Therefore, activity of complex IV was slightly increased in CI and CI + IGF- I groups.

By contrast, complex II activity was slightly decreased in cirrhotic rats when compared to control group.

As shown in Figure 4, ATPase activity declined in untreated cirrhotics rats. However, there was no significant difference between the CO and CI + IGF- I groups in complex V activity treatment (CO =  $4.52 \pm 0.23$ , CI =  $3.43 \pm 0.21$ , CI + IGF- I =  $3.83 \pm 0.31$  nmol/min per mg prot).

### Measurement of caspase-3 activation

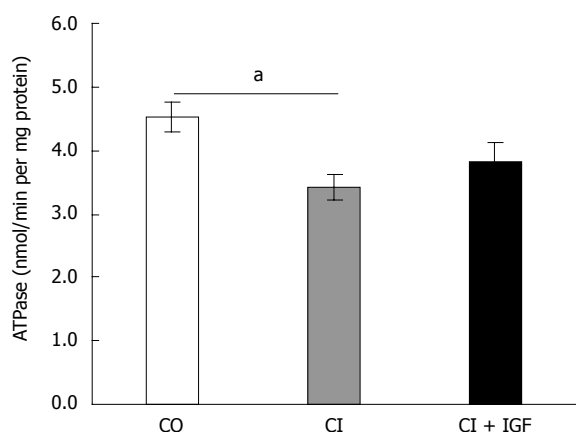
Western blotting for fragment-17 of caspase 3 showed a significant increase of caspase 3 activation in untreated cirrhotic rats when compared to healthy controls (Figure 5). However a notable reduction in the expression of this



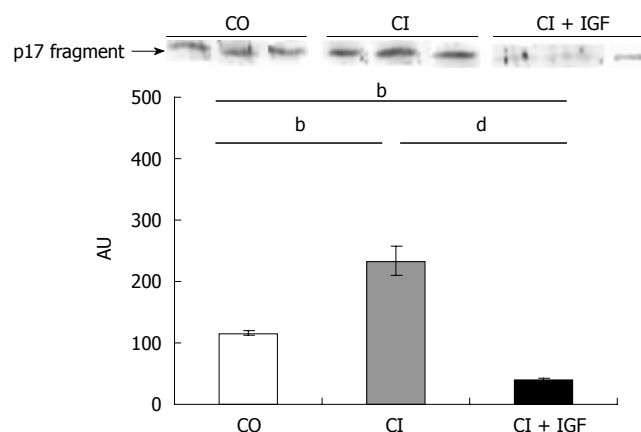
Table 3 TUNEL positive hepatocytes in the three experimental groups

	CO, healthy control group	CI, untreated cirrhotic group	CI + IGF, cirrhotic rats treated with IGF- I
TUNEL positive hepatocytes	1.1 ± 0.6 <sup>b</sup>	11.8 ± 3.7	3.6 ± 2.3 <sup>c</sup>

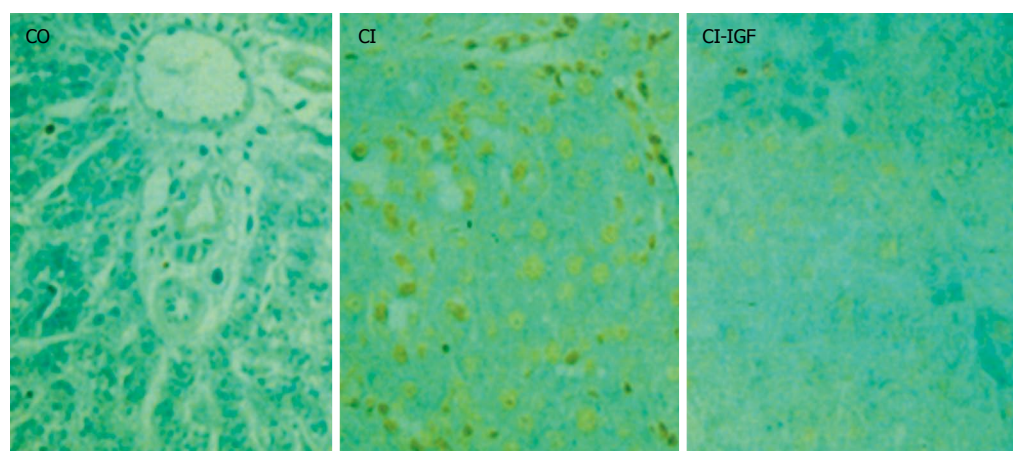
mean ± SEM; <sup>b</sup>*P* < 0.01 vs other groups; <sup>c</sup>*P* < 0.05 vs CI.



**Figure 4** Complex V ATPase activity: ATPase activity was significantly reduced in mitochondria from untreated cirrhotic rats (*P* < 0.05 vs CO group). IGF-therapy induced a little increase of ATPase activity (*P* = no significant vs CO group). mean ± SEM; <sup>a</sup>*P* < 0.05.



**Figure 5** Active fragment of caspase 3 by Western blot in liver homogenates: untreated cirrhotic rats showed an increment of caspase activation. IGF- I replacement therapy induced an inhibition of caspase 3 activation. <sup>b</sup>*P* < 0.01; <sup>d</sup>*P* < 0.001.



**Figure 6** Apoptosis by TUNEL in the liver from the three experimental groups: the number of TUNEL positive hepatocytes was increased in untreated cirrhotic rats (CI) as compared to healthy controls (CO). IGF- I therapy reduced significantly apoptosis in hepatocytes.

fragment was observed in cirrhotic animals treated with IGF- I.

### Apoptosis assessment by TUNEL

The number of TUNEL-positive hepatocytes was significant increased in untreated cirrhotic group as compared to controls. IGF- I treatment reduced apoptosis in hepatocytes as shown in Figure 6 and Table 3.

## DISCUSSION

This study analyzes the effect of IGF- I on mitochondrial damage, caspase 3 activation and hepatocyte apoptosis in rats with CCl<sub>4</sub>-induced cirrhosis. In previous studies, we have shown that low doses of IGF- I improved liver function and reduced oxidative liver injury and

fibrosis, suggesting a mitochondrial protection<sup>[12,14,15]</sup>. The mechanisms of this hepatoprotection are understood only partially; but, data in this paper give some insight.

When compared to healthy rats, untreated cirrhotic rats showed altered liver function tests and increased hepatic lipid peroxidation and protein carbonyl content (PCC) which was in agreement with previous data<sup>[12,14,15]</sup> and improved with IGF- I substitution.

The present work shows that mitochondrial dysfunction in rats with CCl<sub>4</sub>-induced cirrhosis is characterized by a significant depletion of the mitochondrial membrane potential (MMP), a reduction of ATPase activity, and a significant increase of intramitochondrial ROS generation.

In addition, these results indicate that the activities *in vitro* (in optimal conditions) of complex I and III were highest in cirrhotic animals when compared to control

animals. It would be an adaptive mechanism as in fact it is not possible to produce a normal proton gradient (Figure 2) in untreated cirrhotic rats. The increased mitochondrial production of  $O_2^{\cdot -}$  at complexes I and III and consequently of  $H_2O_2$  is a common finding in oxidative mitochondrial damage<sup>[31,32]</sup>. Uncontrolled mitochondrial formation of ROS promotes the activation of the mitochondrial membrane permeability transition<sup>[31]</sup>.

Several research groups have tried to characterize the effect of chronic ethanol consumption and CCl<sub>4</sub>-induced liver injury showing morphological alterations in mitochondria and deficient substrate oxidation, ADP phosphorylation, cytochrome c and enzyme content<sup>[16,31,33-37]</sup>. On the other hand, other authors have concluded that in chronic CCl<sub>4</sub>-treated rats, liver mitochondria are morphologically and functionally intact<sup>[38]</sup>. In the present work, the described alteration in mitochondrial function occurred at least after 16 wk of CCl<sub>4</sub> exposure.

The major finding of this work is that mitochondrial dysfunction leading to hepatocyte apoptosis is improved by IGF- I therapy. These results give new evidence of the hepatoprotective properties of IGF- I in experimental cirrhosis<sup>[12,15]</sup>.

In the present work, oxygen consumption by isolated mitochondria was not assessed and consequently proton leak data (oxygen consumption/MMP) are not available. However, the observed reduction of MMP with an increased generation of ROS suggests that oxygen is wasted by damaged mitochondria producing  $H_2O_2$  instead of a normal proton gradient, driving force of ATP synthesis through the proton pumping  $F_1F_0$  adenosine triphosphate synthase (ATP synthase)<sup>[31]</sup>.

The described mitochondrial dysfunction in this paper is corrected by IGF- I therapy since mitochondria from IGF- I treated cirrhotic rats showed a normal MMP and a recovered ATPase activity resulting in increased activity of complex I and III, but reduced ROS generation. Taken together, all of these data suggest an extramitochondrial protection of mitochondria by IGF- I.

Previously, we have reported that IGF- I treatment restored the expression of several protease inhibitors such as alpha-1-antichymotrypsin, the serine protease inhibitor 2<sup>[13]</sup>, that could contribute to the described mitochondrial protection. Further studies are necessary to elucidate all mechanisms involved in the hepatoprotection induced by IGF- I. In the previously mentioned work, we provided evidence that IGF- I treatment in cirrhotic rats modulates gene expression, reverting the global hypomethylation of the genomic DNA observed in cirrhosis<sup>[13]</sup>.

On the other hand, the mitochondrial dysfunction in the present study was associated with an overexpression of the active fragment of caspase 3 and an increased number of TUNEL positive hepatocytes in untreated cirrhotic rats. In accordance with the improvement of the mitochondrial function described in CI + IGF- I group, IGF- I treated animals showed a significant reduction of caspase 3 activation and apoptosis of hepatocytes.

In recent years, mitochondria have been recognized as regulators of cell death<sup>[39,40]</sup>. Cellular dysfunction induced by intra- or extracellular insults converge on mitochondria

and induce a sudden increase in permeability on the inner mitochondrial membrane, the so-called mitochondrial membrane permeability transition (MMPT). MMPT is caused by the opening of pores in the inner mitochondrial membrane, matrix swelling and outer membrane rupture. The MMPT is an endpoint to initiate cell death and a putative target for cellular protection since MMPT and the release of mitochondrial cytochrome c activate the apoptotic pathway by which initiator caspases (i.e. caspase 8 and 9) are converted to their active forms, which in turn activate downstream effector caspases (i.e. caspases 3, 6 and 7). Finally, cellular targets of the effector caspases include endonucleases and cytoskeletal proteins<sup>[41]</sup>.

These results show that IGF- I induces cell resistance to apoptosis by oxidative stress through mitochondrial protection. Mitochondria seem to be the main cellular targets of IGF- I action. Likewise, our data are in agreement with the observation that the effect of serum withdrawal on the autophagy of dysfunctional mitochondria is prevented by the addition of IGF- I<sup>[42]</sup>.

Accordingly, it has been reported that IGF- I inhibited the reduction of MMP, cytochrome c release, caspase 3 activity and apoptosis in several cell lines and experimental procedures<sup>[43-45]</sup>.

The described improvement of mitochondrial function in cirrhotic rats by IGF- I therapy resulted in an increment of the ATP synthesis. Interestingly, a recent trial with cirrhotic patients showed that the IGF- I therapy induced an improvement in albumin and metabolism increasing resting energy expenditure (REE) (kcal/d)<sup>[46]</sup>. Although the mechanism by which IGF- I influences REE in cirrhotic patients remains to be clarified, it could be related with an increment of ATP availability after IGF- I therapy.

In conclusion, these results show that IGF- I exerts a mitochondrial protection in experimental cirrhosis leading to reduced caspase activation and apoptosis and increasing ATP production. This work provides new evidence of the beneficial effect of IGF- I supplementation in experimental liver cirrhosis and experimental basis for further studies at exploring the potential of IGF- I in the treatment of human cirrhosis.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Liver cirrhosis is a condition of "IGF- I deficiency". The endogenous administration of low doses of insulin-like growth factor I (IGF- I) exerts a mitochondrial protection in experimental cirrhosis leading to reduced apoptosis and increased ATP production.

### Research frontiers

Liver cirrhosis. Antioxidant therapies. New strategies in chronic liver diseases. Axis GH/IGF- I in liver cirrhosis.

## Innovations and breakthroughs

Mitochondrial dysfunction in cirrhosis. Mitochondrial protection induced by IGF- I at low doses.

## Applications

This work provides new evidence of the beneficial effect of IGF- I supplementation in experimental liver cirrhosis and experimental basis for further studies at exploring the potential of IGF- I in the treatment of human cirrhosis.

## Peer review

This paper provides evidence in order to give a better insight into the mechanisms by which IGF- I exerts an hepatoprotective action on damaged liver and demonstrates that mitochondria are one of the main cellular targets of IGF- I . This study contributes to the characterization of mitochondrial dysfunction in experimental liver cirrhosis and shows the beneficial effects on damaged mitochondria leading to cellular protection induced by IGF- I therapy.

## REFERENCES

- 1 Sara VR, Hall K. Insulin-like growth factors and their binding proteins. *Physiol Rev* 1990; **70**: 591-614
- 2 Caufriez A, Reding P, Urbain D, Golstein J, Copinschi G. Insulin-like growth factor I: a good indicator of functional hepatocellular capacity in alcoholic liver cirrhosis. *J Endocrinol Invest* 1991; **14**: 317-321
- 3 Hattori N, Kurahachi H, Ikekubo K, Ishihara T, Moridera K, Hino M, Saiki Y, Imura H. Serum growth hormone-binding protein, insulin-like growth factor-I, and growth hormone in patients with liver cirrhosis. *Metabolism* 1992; **41**: 377-381
- 4 Picardi A, de Oliveira AC, Muguerza B, Tosar A, Quiroga J, Castilla-Cortazar I, Santidrian S, Prieto J. Low doses of insulin-like growth factor-I improve nitrogen retention and food efficiency in rats with early cirrhosis. *J Hepatol* 1997; **26**: 191-202
- 5 Castilla-Cortazar I, Prieto J, Urdaneta E, Pascual M, Nunez M, Zudaire E, Garcia M, Quiroga J, Santidrian S. Impaired intestinal sugar transport in cirrhotic rats: correction by low doses of insulin-like growth factor I. *Gastroenterology* 1997; **113**: 1180-1187
- 6 Castilla-Cortazar I, Picardi A, Tosar A, Ainzua J, Urdaneta E, Garcia M, Pascual M, Quiroga J, Prieto J. Effect of insulin-like growth factor I on in vivo intestinal absorption of D-galactose in cirrhotic rats. *Am J Physiol* 1999; **276**: G37-G42
- 7 Castilla-Cortazar I, Garcia M, Quiroga J, Diez N, Diez-Caballero F, Calvo A, Diaz M, Prieto J. Insulin-like growth factor-I reverts testicular atrophy in rats with advanced cirrhosis. *Hepatology* 2000; **31**: 592-600
- 8 Pascual M, Castilla-Cortazar I, Urdaneta E, Quiroga J, Garcia M, Picardi A, Prieto J. Altered intestinal transport of amino acids in cirrhotic rats: the effect of insulin-like growth factor-I. *Am J Physiol Gastrointest Liver Physiol* 2000; **279**: G319-G324
- 9 Cemborain A, Castilla-Cortazar I, Garcia M, Quiroga J, Muguerza B, Picardi A, Santidrian S, Prieto J. Osteopenia in rats with liver cirrhosis: beneficial effects of IGF-I treatment. *J Hepatol* 1998; **28**: 122-131
- 10 Cemborain A, Castilla-Cortazar I, Garcia M, Muguerza B, Delgado G, Diaz-Sanchez M, Picardi A. Effects of IGF-I treatment on osteopenia in rats with advanced liver cirrhosis. *J Physiol Biochem* 2000; **56**: 91-99
- 11 Castilla-Cortazar I, Aliaga-Montilla MA, Salvador J, Garcia M, Delgado G, Gonzalez-Baron S, Quiroga J, Prieto J. Insulin-like growth factor-I restores the reduced somatostatinergic tone controlling growth hormone secretion in cirrhotic rats. *Liver* 2001; **21**: 405-409
- 12 Castilla-Cortazar I, Garcia M, Muguerza B, Quiroga J, Perez R, Santidrian S, Prieto J. Hepatoprotective effects of insulin-like growth factor I in rats with carbon tetrachloride-induced cirrhosis. *Gastroenterology* 1997; **113**: 1682-1691
- 13 Mirpuri E, Garcia-Trevijano ER, Castilla-Cortazar I, Berasain C, Quiroga J, Rodriguez-Ortigosa C, Mato JM, Prieto J, Avila MA. Altered liver gene expression in CCl4-cirrhotic rats is partially normalized by insulin-like growth factor-I. *Int J Biochem Cell Biol* 2002; **34**: 242-252
- 14 Garcia-Fernandez M, Castilla-Cortazar I, Diaz-Sanchez M, Navarro I, Puche JE, Castilla A, Casares AD, Clavijo E, Gonzalez-Baron S. Antioxidant effects of insulin-like growth factor-I (IGF-I) in rats with advanced liver cirrhosis. *BMC Gastroenterol* 2005; **5**: 7
- 15 Muguerza B, Castilla-Cortazar I, Garcia M, Quiroga J, Santidrian S, Prieto J. Antifibrogenic effect in vivo of low doses of insulin-like growth factor-I in cirrhotic rats. *Biochim Biophys Acta* 2001; **1536**: 185-195
- 16 Bailey SM, Cunningham CC. Contribution of mitochondria to oxidative stress associated with alcoholic liver disease. *Free Radic Biol Med* 2002; **32**: 11-16
- 17 Bailey SM, Pietsch EC, Cunningham CC. Ethanol stimulates the production of reactive oxygen species at mitochondrial complexes I and III. *Free Radic Biol Med* 1999; **27**: 891-900
- 18 Kwong LK, Sohal RS. Age-related changes in activities of mitochondrial electron transport complexes in various tissues of the mouse. *Arch Biochem Biophys* 2000; **373**: 16-22
- 19 Kowaltowski AJ, Vercesi AE. Mitochondrial damage induced by conditions of oxidative stress. *Free Radic Biol Med* 1999; **26**: 463-471
- 20 Cardoso SM, Pereira C, Oliveira R. Mitochondrial function is differentially affected upon oxidative stress. *Free Radic Biol Med* 1999; **26**: 3-13
- 21 Kaplowitz N, Tsukamoto H. Oxidative stress and liver disease. *Prog Liver Dis* 1996; **14**: 131-159
- 22 Kaplowitz N. Biochemical and cellular mechanisms of toxic liver injury. *Semin Liver Dis* 2002; **22**: 137-144
- 23 Kiningham KK, Oberley TD, Lin S, Mattingly CA, St Clair DK. Overexpression of manganese superoxide dismutase protects against mitochondrial-initiated poly(ADP-ribose) polymerase-mediated cell death. *FASEB J* 1999; **13**: 1601-1610
- 24 Cortopassi GA, Wong A. Mitochondria in organismal aging and degeneration. *Biochim Biophys Acta* 1999; **1410**: 183-193
- 25 The Guiding Principles for Research Involving Animals. National Academy of Sciences. The National Institute of Health -NIH. 1991
- 26 Esterbauer H, Schaur RJ, Zollner H. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* 1991; **11**: 81-128
- 27 Levine RL, Garland D, Oliver CN, Amici A, Climent I, Lenz AG, Ahn BW, Shaltiel S, Stadtman ER. Determination of carbonyl content in oxidatively modified proteins. *Methods Enzymol* 1990; **186**: 464-478
- 28 O'Connor JE, Vargas JL, Kimler BF, Hernandez-Yago J, Grisolia S. Use of rhodamine 123 to investigate alterations in mitochondrial activity in isolated mouse liver mitochondria. *Biochem Biophys Res Commun* 1988; **151**: 568-573
- 29 Hatefi Y, Haavik AG, Griffiths DE. Studies on the electron transfer system. XL. Preparation and properties of mitochondrial DPNH-coenzyme Q reductase. *J Biol Chem* 1962; **237**: 1676-1680
- 30 Trounce I, Byrne E, Dennett X, Chen WW, Marzuki S. Affinity chromatography isolation of human cytochrome oxidase and small-scale Western immunoblot probing of the enzyme complex in mitochondrial cytopathy patients. *Biochem Med Metab Biol* 1991; **46**: 17-27
- 31 Hoek JB, Cahill A, Pastorino JG. Alcohol and mitochondria: a dysfunctional relationship. *Gastroenterology* 2002; **122**: 2049-2063
- 32 Sastre J, Serviddio G, Pereda J, Minana JB, Arduini A, Vendemiale G, Poli G, Pallardo FV, Vina J. Mitochondrial function in liver disease. *Front Biosci* 2007; **12**: 1200-1209
- 33 Jelski W, Chrostek L, Szmikowski M. [Biochemical basis of alcoholic liver injury]. *Pol Merkuri Lekarski* 2006; **21**: 376-380
- 34 Gramenzi A, Caputo F, Biselli M, Kuria F, Loggi E, Andreone P, Bernardi M. Review article: alcoholic liver disease--pathophysiological aspects and risk factors. *Aliment Pharmacol Ther* 2006; **24**: 1151-1161
- 35 Shukla SD, Aroor AR. Epigenetic effects of ethanol on liver and gastrointestinal injury. *World J Gastroenterol* 2006; **12**:

- 5265-5271
- 36 **Cunningham CC**, Bailey SM. Ethanol consumption and liver mitochondria function. *Biol Signals Recept* 2001; **10**: 271-282
  - 37 **Albano E**. Alcohol, oxidative stress and free radical damage. *Proc Nutr Soc* 2006; **65**: 278-290
  - 38 **Krahlentbuhl S**, Reichen J, Zimmermann A, Gehr P, Stucki J. Mitochondrial structure and function in CCl4-induced cirrhosis in the rat. *Hepatology* 1990; **12**: 526-532
  - 39 **Bras M**, Queenan B, Susin SA. Programmed cell death via mitochondria: different modes of dying. *Biochemistry (Mosc)* 2005; **70**: 231-239
  - 40 **Tsujimoto Y**. Cell death regulation by the Bcl-2 protein family in the mitochondria. *J Cell Physiol* 2003; **195**: 158-167
  - 41 **Earnshaw WC**, Martins LM, Kaufmann SH. Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem* 1999; **68**: 383-424
  - 42 **Gu Y**, Wang C, Cohen A. Effect of IGF-1 on the balance between autophagy of dysfunctional mitochondria and apoptosis. *FEBS Lett* 2004; **577**: 357-360
  - 43 **Leininger GM**, Russell JW, van Golen CM, Berent A, Feldman EL. Insulin-like growth factor-I regulates glucose-induced mitochondrial depolarization and apoptosis in human neuroblastoma. *Cell Death Differ* 2004; **11**: 885-896
  - 44 **Kondo T**, Kitano T, Iwai K, Watanabe M, Taguchi Y, Yabu T, Umehara H, Domae N, Uchiyama T, Okazaki T. Control of ceramide-induced apoptosis by IGF-1: involvement of PI-3 kinase, caspase-3 and catalase. *Cell Death Differ* 2002; **9**: 682-692
  - 45 **Ness JK**, Scaduto RC Jr, Wood TL. IGF-I prevents glutamate-mediated bax translocation and cytochrome C release in O4 + oligodendrocyte progenitors. *Glia* 2004; **46**: 183-194
  - 46 **Conchillo M**, de Knecht RJ, Payeras M, Quiroga J, Sangro B, Herrero JL, Castilla-Cortazar I, Frystyk J, Flyvbjerg A, Yoshizawa C, Jansen PL, Scharschmidt B, Prieto J. Insulin-like growth factor I (IGF-I) replacement therapy increases albumin concentration in liver cirrhosis: results of a pilot randomized controlled clinical trial. *J Hepatol* 2005; **43**: 630-636

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BASIC RESEARCH

## Hepatic reconstruction from fetal porcine liver cells using a radial flow bioreactor

Yuji Ishii, Ryota Saito, Hideki Marushima, Ryusuke Ito, Taro Sakamoto, Katsuhiko Yanaga

Yuji Ishii, Ryota Saito, Hideki Marushima, Ryusuke Ito, Taro Sakamoto, Katsuhiko Yanaga, Department of Surgery, the Jikei University School of Medicine, Tokyo 105-8461, Japan

Author contributions: Saito R, Marushima H, Ito R, Sakamoto T, and Yanaga K contributed equally to this work.

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Correspondence to: Yuji Ishii, MD, PhD, Department of Surgery, the Jikei University School of Medicine, 3-25-8 Nishi-shinbashi, Minato-ku, Tokyo 105-8461, Japan. [yujiyunayuta@jikei.ac.jp](mailto:yujiyunayuta@jikei.ac.jp)

Telephone: + 81-3-34331111 Fax: + 81-3-54724140

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### Abstract

**AIM:** To examine the efficacy of the radial flow bioreactor (RFB) as an extracorporeal bioartificial liver (BAL) and the reconstruction of liver organoids using embryonic pig liver cells.

**METHODS:** We reconstructed the liver organoids using embryonic porcine liver cells in the RFB. We also determined the gestational time window for the optimum growth of embryonic porcine liver cells. Five weeks of gestation was designated as embryonic day (E) 35 and 8 wk of gestation was designated as E56. These cells were cultured for one week before morphological and functional examinations. Moreover, the efficacy of pulsed administration of a high concentration hepatocyte growth factor (HGF) was examined.

**RESULTS:** Both cell growth and function were excellent after harvesting on E35. The pulsed administration of a high concentration of HGF promoted the differentiation and maturation of these fetal hepatic cells. Microscopic examination of organoids in the RFB revealed palisading and showed that bile duct-like structures were well developed, indicating that the organoids were mini livers. Transmission electron microscopy revealed microvilli on the luminal surfaces of bile duct-like structures and junctional complexes, which form the basis of the cytoskeleton of epithelial tissues. Furthermore, strong expression of connexin (Cx) 32, which is the main protein of hepatocyte gap junctions, was observed. With respect to liver function, ammonia detoxification and urea synthesis were shown to be performed effectively.

**CONCLUSION:** Our system can potentially be applied in the fields of BAL and transplantation medicine.

### INTRODUCTION

Although organ transplantation has become an established treatment, the shortage of donor organs remains a serious problem, for which tissue engineering is a possible solution. A bioartificial liver (BAL) system consists of three components: cells, a scaffold for the cells, and growth-regulating factors. Ideally, the cells for a BAL will exhibit similar functions to normal human mature hepatocytes while maintaining long-term viability and proliferative activity. However, a method of culturing human hepatocytes for a prolonged period has not been established so far.

We have developed a radial flow bioreactor (RFB), which is a 3-dimensional culture system that can be used for high-density culture<sup>[1-4]</sup>, achieving a cell density 10 times greater than that obtained with hollow-fiber culture<sup>[5,6]</sup>. A cylindrical bioreactor is filled with porous cellulose beads that act as microcarriers, and culture medium flows from the periphery toward the center of the reactor (Figure 1A)<sup>[2,7]</sup>. To achieve a high-density cell culture, it is essential to minimize any variations in the distribution of oxygen and nutrients between the culture medium at the inlet and outlet of the reactor. If medium flows from the periphery towards the center, a high perfusion rate can provide an adequate supply of oxygen and nutrients to cells at the center of the bioreactor even though oxygen and nutrients are consumed by cells at the periphery. Thus, there are similarities between the RFB and the anatomy of hepatic lobules (Figure 1B)<sup>[2,7]</sup>.

Because of the easy availability, recent reports point out a clinical use of BAL systems that utilize animal cells<sup>[8,9]</sup>. Fetal porcine hepatocytes have a high proliferative potential *in vitro*. Previous studies have shown that minimal immunogenicity is exhibited by tissues harvested at the

earliest available gestational age<sup>[10-12]</sup>. Thus, the earliest time after organogenesis is established appears to be preferable for human transplantation. In fact, Friedman *et al.*<sup>[13]</sup> have shown that maximal liver growth and function were achieved at the earliest teratoma-free gestational age of embryonic day (E) 28. In this study, the possibility of teratoma development was also taken into consideration, and construction of a BAL system was achieved with a RFB and fetal porcine livers obtained on E35 as the cell source, while E56 cells were also examined for comparison. Furthermore, this study also focused on the effect of hepatocyte growth factor (HGF), which was originally reported as a hepatocyte-specific mitogen with an important role in liver regeneration<sup>[14]</sup>.

## MATERIALS AND METHODS

### Isolation of fetal hepatocytes and nonparenchymal epithelial cells

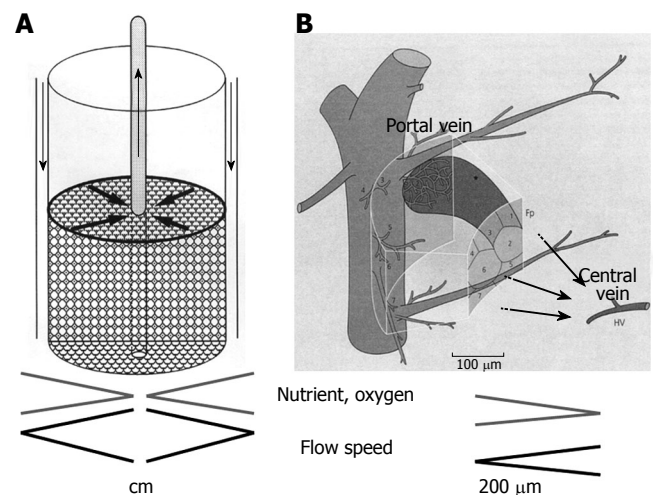
Pregnant mini swines (CSK-MS) weighing 30 to 35 kg were purchased from Chugai Institute of Medical Science (Nagano, Japan). Fetal hepatic cells were isolated from the porcine livers of embryos at 5 and 8 wk of gestation (NIBS strain) by the two-step liver perfusion method of Seglen with some modifications<sup>[15-17]</sup>. Five weeks of gestation was designated as E35 and 8 wk of gestation was designated as E56. For controls, mature porcine liver cells were used. After intraportal infusion of collagenase (0.05%) and dispase (1000 U/mL), the hepatic tissue was minced into pieces and shaken in collagenase solution. Then the collagenase-digested liver cell suspension was centrifuged at 50 *g* for 1 min. The E35 cells were divided into 3 fractions and the E56 cells were divided into 2 fractions. The hepatocyte fraction (lowest fraction) was maintained on ice in serum-free ASF 104 medium (Ajinomoto, Tokyo). The one and two upper fractions, respectively, were centrifuged once at 350 *g* for 5 min, and the cell pellet (containing non-parenchymal cells) was also maintained on ice in serum-free ASF 104 medium.

### Oxygen consumption in the RFB

An RFB system with a 15-mL capacity was placed in an aseptic room maintained at 37°C. The system was composed of an RFB and a conditioning vessel connected to a tank containing fresh medium and a recovery tank. The system was automatically controlled by a computer that monitored pH, glucose, and oxygen consumption. The oxygen tension in the culture medium was measured both within the reservoir and at the outlet of the bioreactor, and the oxygen consumption was monitored on the basis of the oxygen tension gradient ( $Do_{in} - Do_{out}$ :  $\Delta O_2$  ppm). Oxygen consumption was used as an index of the activity of the cells in the RFB system.

### Culture of cells in the RFB

The RFB (Biott, Tokyo, Japan) is a cylindrical bioreactor with a capacity of 15 mL that contains cellulose beads (Asahi Kasei, Tokyo, Japan). The RFB is attached to a reservoir containing culture medium and an automatic controller that maintains the oxygen content and pH of the



**Figure 1** The principle of RFB system. **A:** A cylindrical bioreactor is filled with porous cellulose beads that act as microcarriers and culture medium flows from the periphery toward the center of the reactor. Biases in distribution of oxygen and nutrients between the culture medium at the inlet and outlet of the reactor are minimized; **B:** The RFB system is similar to the organization of the hepatic primary lobe Figure 1B is reproduced from reference 7.

medium. Both hepatocytes ( $5 \times 10^7$ ) and non-parenchymal cells ( $5 \times 10^7$ ) were inoculated into the reservoir, which was filled with ASF104 medium containing 2% fetal bovine serum (FBS). The bioreactor was perfused in a closed circuit fashion for 2 h to maximize the efficiency of cell attachment to the cellulose beads, after which the reactor was switched to the open-circuit mode and the medium was changed to ASF104 without FBS. For this study, incubation in the RFB system was performed for one week before morphological and functional examinations.

### Factor mix and HGF

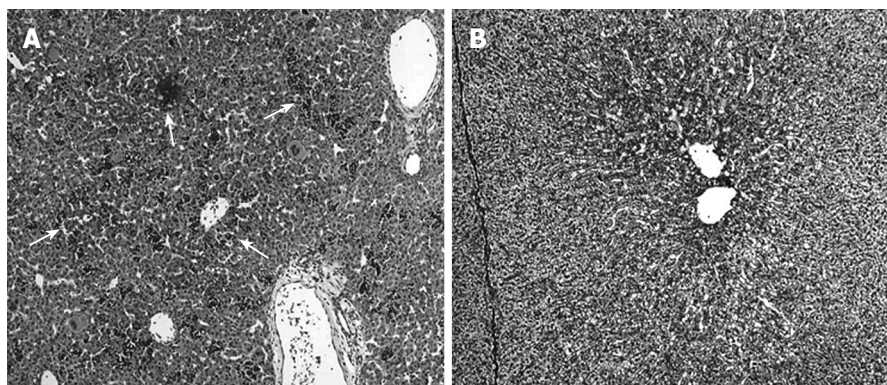
The factor mix (FM) that we formulated consists of  $10^{-7}$  mol/L insulin,  $10^{-7}$  mol/L dexamethasone, 100 ng/mL oncostatin M (OSM), 25 ng/mL epidermal growth factor (EGF), 1  $\mu$ g/mL L-ascorbic acid phosphate magnesium salt, 0.1 mmol/L nicotinamide, and antibiotics, which was added to the culture medium. HGF was also added at 20 ng/mL (low-dose group) or 100 ng/mL (high-dose group). HGF was administered three times (d 1, 3, and 5) by closed-circuit perfusion for 2 h.

### Experimental groups

The following groups were studied: FME35 group (ASF104 + FM), LFE35 group (ASF104 + FM + HGF 20 ng/mL: low-dose), and HFE35 group (ASF104 + FM + HGF 100 ng/mL: high-dose) for E35 cells, with corresponding groups for E56 cells. Two other groups, i.e., the FMH group (Adult: group H, ASF104 + FM) and the HFH group (Adult: group H, ASF104 + FM + HGF 100 ng/mL), were studied as control groups. Each group was set at  $n = 3$ .

### Ammonia loading test

In order to assess the performance of hepatocyte functions, the ammonia ( $NH_3$ ) loading test was performed. In consideration of the results of oxygen consumption and morphological features, the  $NH_3$  loading test compared the



**Figure 2** Histological findings of fetal porcine liver (HE staining). **A:** Extramedullary hematopoiesis was observed (arrow) and the cells are largely immature at embryonic d 35 (x 100); **B:** A definite lobular structures and a decrease of extramedullary hematopoiesis were observed at embryonic d 56 (x 100).

HFE35 group, FME35 group, and HFE56 group. After incubation for one week (d 7) in the RFB, ammonium chloride ( $\text{NH}_4\text{Cl}$ ) was added at three concentrations (1 mmol/L + 2 mmol/L + 3 mmol/L) every 8 h for 24 h, followed by 3 mmol/L for a further 24 h<sup>[4]</sup>. Then  $\text{NH}_3$  and urea levels were measured on d 8 and d 9 using an automatic high-speed amino acid analyzer JLC-300 (JEOL Ltd., Tokyo).

### Immunohistochemistry

For immunochemical study of cytokeratin 19 (CK19) (DakoCytomation), the streptavidin-biotin (SAB) technique was used<sup>[18,19]</sup>. Specimens of RFB cultures were fixed with Methacarn solution (methanol:chloroform:glacial acetic acid = 6:3:1), embedded in paraffin, cut into 3  $\mu\text{m}$  sections, and deparaffinized with graded xylene series. Endogenous peroxidase was inhibited by adding 0.3%  $\text{H}_2\text{O}_2$  methanol.

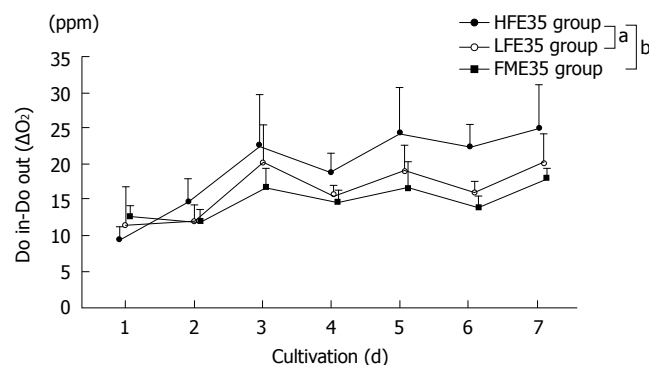
For immunofluorescence study of connexin 32 (Cx32), specimens of RFB cultures were fixed in cold absolute acetone for 10 min. Immunohistochemistry with a rabbit polyclonal anti-Cx32 (Zymed, South San Francisco, CA) was performed. Cx32 was visualized using Alexa Fluor 488-conjugated goat anti rabbit immunoglobulin G (Molecular Probes, Eugene, OR). For comparison, immunohistochemical staining as also performed on parenchymal cells and non-parenchymal cells in monolayer cell culture. Samples were examined with an epifluorescence microscope (Nikon, Tokyo, Japan) and a laser-scanning confocal microscope (MRC 1024; Bio-Rad, Hercules, CA).

### Transmission electron microscopy (TEM)

For TEM, cultured cells were fixed with 2.0% glutaraldehyde in 0.1 mol/L phosphate buffer (PB) for 1 h and postfixed with 1%  $\text{OsO}_4$  in 0.1 mmol/L PB for 1 h at 4°C. Specimens were dehydrated in ethanol and embedded in a mixture of Epon-Araldite. Thin sections (60 nm) were cut with a diamond knife mounted on an LKB ultratome, and stained with aqueous uranyl acetate. Then the sections were examined under a JEOL 1200EX electron microscope (JEOL Ltd., Tokyo, Japan).

### Statistical analysis

The oxygen consumption, the  $\text{NH}_3$  and urea levels were reported as a mean  $\pm$  SD. For individual parameters, differences between groups were assessed by repeated measures ANOVA<sup>[20,21]</sup> and Bonferroni's multiple



**Figure 3** Changes of the oxygen consumption in the HFE35, LFE35, and FME35 groups. HFE35 group vs FME35 group ( $P < 0.01$ ), HFE35 group vs LFE35 group ( $P < 0.05$ ), LFE35 group vs FME35 group (no significant). The data show the mean values, while error bars represent corresponding standard deviations. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ .

comparison test. All calculations were performed using Stat View-J statistical software (SAS Institute, Cary, NC) with  $P < 0.05$  considered significant.

## RESULTS

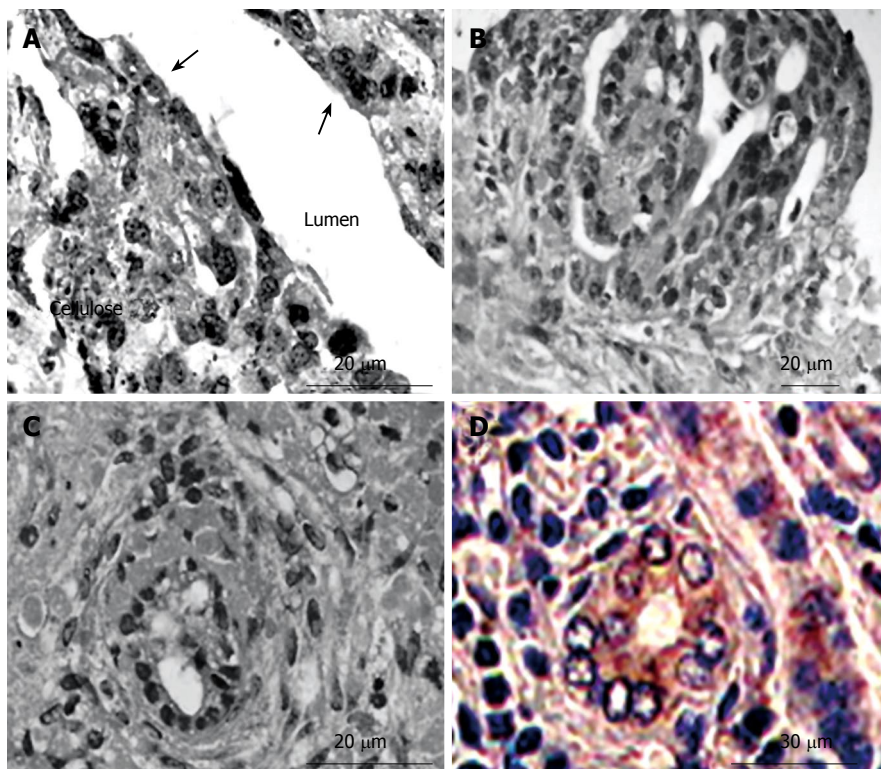
### Morphological features of fetal porcine liver cells

Figure 2A demonstrates the histological features of E35 porcine liver. Although the hepatic lobular structure is present, extramedullary hematopoiesis was also observed and the cells are largely immature. On the other hand, the E56 liver shows a definite lobular structure and a decrease of extramedullary hematopoiesis, so it more closely resembles the adult porcine liver (Figure 2B). Liver cells isolated on E56 were divided into two layers (parenchymatous cells and non-parenchymatous cells) after centrifugation of the cell suspensions in accordance with these histological findings; but, E35 liver cell suspensions were divided into three layers. The upper layer, the middle layer, and the lower layer consisted of parenchymal cells, extramedullary-hematopoiesis cells including immature hepatic cells, and non-parenchymal cells, respectively.

### Oxygen consumption

Changes in the oxygen consumption in each group, which is an index of cellular activity in RFB culture, are shown in Figure 3. Although viability of the FME35 group was maintained for one week, viability of the FMH group





**Figure 4** Histological findings of cultured cells in the RFB (hematoxylin-eosin staining and immunohistochemistry). **A:** Cultured cells formed layers on the cellulose beads. Non-parenchymal cells had a flat shape and existed on the surface of the perfused side (arrow); **B:** A palisading structure was observed in cell clumps; **C:** Bile duct-like structures were also observed in cell clumps; **D:** The expression of cytokeratin 19 (CK19) was observed in bile duct-like structures.

was difficult to maintain (data not shown). Although the LFE56 group did not show a significant change of oxygen consumption over time compared with the FME35 group ( $P = 0.2792$ ), the HFE35 group showed a significant change of oxygen consumption with time ( $P < 0.01$ ). Also compared to the LFE35 group, the HFE35 group showed a significant change over time ( $P < 0.05$ ). E56 cells gave the same results as E35 cells when cultured under the same conditions (data not shown). The HFH group, which was treated with a high concentration of HGF, also maintained viability for one week (data not shown).

#### Architecture of cells cultured in the RFB

In the bioreactor, cultured cells formed layers on the cellulose beads. Non-parenchymal cells had a flat shape and existed on the surface of the perfused side (Figure 4A). Moreover, cell clumps of 0.4 to 0.8 cm<sup>3</sup> separated from the cellulose beads to float in the RFB. In particular, these clumps were conspicuous in the HFE35 group. On histological examination of these clumps, a palisading structure (Figure 4B) and bile duct-like structures were also observed (Figure 4C), so reconstruction of a “mini-liver organoid” was achieved. Furthermore, CK19 which is the marker of a bile duct was also observed in bile duct-like structures (Figure 4D). TEM images revealed microvilli inside bile duct-like structures (Figure 5A). Moreover, junctional complexes were well-developed and tight junctions were also observed (Figure 5B). Immunohistochemical staining for Cx32, which is the main protein of gap junctions in hepatocytes of the organoid, showed expression in a whirl-shaped pattern (Figure 5C). These morphological characteristics were more notable in the HFE35 group. The expression of Cx32 in the parenchymal cells in monolayer culture was reduced

compared to that of the clump in RFB (Figure 5D). Furthermore, the expression of Cx32 in non-parenchymal cells in monolayer culture was not observed (Figure 5E).

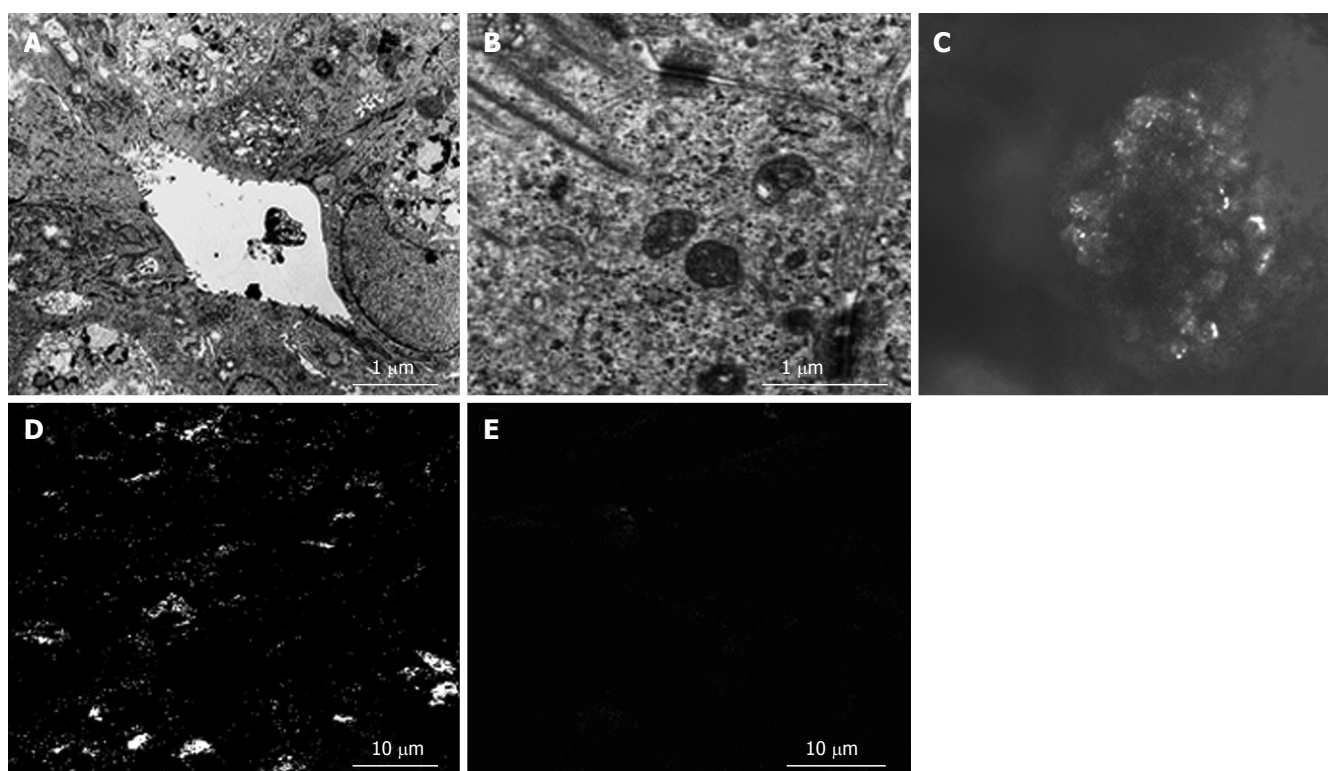
#### Ammonia loading test

The ammonia loading test was performed in the HFE35 group, which showed good oxygen consumption and morphological reconstruction of a mini liver, the FME35 group, and the HFE56 group. Compared with the FME35 group, the NH<sub>3</sub> concentration of the HFE35 group was decreased significantly ( $P < 0.01$ ) (Figure 6), and the urea concentration increased significantly along with a reduction of NH<sub>3</sub> ( $P < 0.01$ ) (Figure 7). Moreover, a significant reduction of the NH<sub>3</sub> concentration was seen in the HFE35 group compared with the HFE56 group ( $P < 0.05$ ) (Figure 6). On the other hand, the change of the urea concentration in the HFE35 group was marked, but there was no significant difference with the HFE56 group (Figure 7).

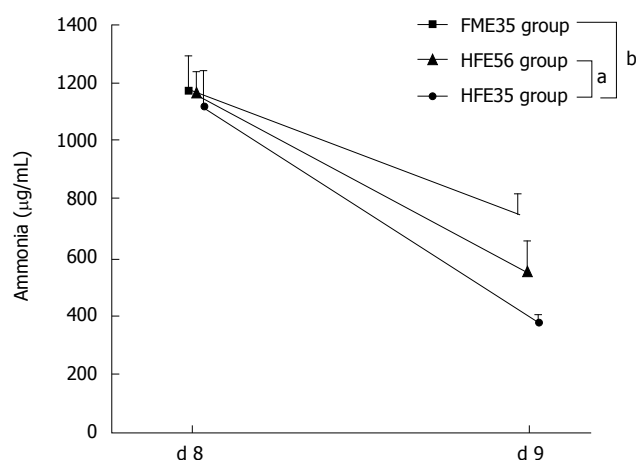
## DISCUSSION

In this study, fetal porcine hepatic cells with proliferative capacity were cultured in a RFB system that allowed high-density, three-dimension culture<sup>[1-4]</sup>, and the usefulness of the RFB system as a BAL and the possibility of liver reconstruction with implantable organoids were examined. Although the high proliferative capacity of embryonic cells is well known, it has been unclear at which gestational age the fetal liver cells show optimal proliferation and functional capacity when used as a source of cells for a BAL or for organoid reconstruction. In this study, fetal porcine livers (E35 and E56) were selected based on the findings of Friedman *et al.*, who examined the timing

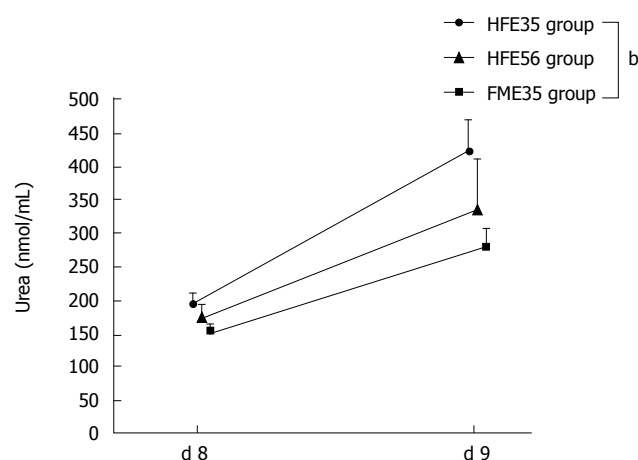




**Figure 5** A: Transmission electron microscopic images revealed microvilli inside bile duct-like structures; B: Junctional complexes were well developed and tight junctions were also observed; C: Connexin 32 (Cx32) in liver organoid, showed expression in a whirl-shaped pattern; D: Cx32 in parenchymal cells in monolayer culture was fewer than that of the organoid; E: Cx32 in non-parenchymal cells in monolayer culture was not observed.



**Figure 6** The  $\text{NH}_3$  loading test compared the HFE35 group, FME35 group, and HME56 group. HFE35 group vs FME35 group ( $P < 0.01$ ), HFE35 group vs HFE56 group ( $P < 0.05$ ). FME35 vs HFE56 (no significant). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ .



**Figure 7** Changes of urea concentration in the HFE35, HFE56, and FME35 groups. HFE35 group vs FME35 group ( $P < 0.01$ ), HFE35 vs HFE56 and HFE56 vs FME35 (no significant). <sup>a</sup> $P < 0.01$ .

of liver organogenesis in embryonic swines<sup>[13]</sup>. They determined distinct gestational time windows for the embryonic porcine liver and precursor cells, with maximal liver growth and function being achieved at the earliest teratoma-free gestational age of E28. In their tissue transplantation experiment, no development of teratomas was seen on E28 (0/23 transplants)<sup>[13]</sup>. Examination of hepatocyte function based on the serum albumin level also showed that E28 is the optimal gestational age for transplantation, and albumin secretion was decreased at E56 and E80<sup>[13]</sup>. Although this study was performed *in vitro*

using the RFB system, in consideration of the possibility of the teratoma development because the environment was similar to that *in vivo*, E35 was chosen as a safe gestational age and comparison with E56 was also performed.

At present, using cells from the fetal human liver is ethically problematic and great difficulties in obtaining such cells are expected. For this reason, fetal porcine liver cells were used in the present study. The following three points are also worth considering: (1) The anatomical and physiological characteristics of the porcine liver are similar to those of the human liver; (2) The gestation period of

pigs is comparatively short (about 112–116 d), and breeding is easy; (3) Breeding of specific-pathogen-free animals is also possible, so zoonoses can be avoided<sup>[22,23]</sup>. However, the swine is immunologically different from humans and hyperacute rejection of porcine organs by human recipients is mediated *via* antibodies directed against Gal $\alpha$ -1, 3-Gal on porcine cells, posing an immediate barrier to successful clinical xenotransplantation<sup>[24–26]</sup>. In addition, infection of the human host by porcine endogenous retroviruses poses a major problem<sup>[27]</sup>. It was reported that infection can be avoided by breeding GalT KO swines that show no infectivity for human cells *in vitro*<sup>[28,29]</sup>. Thus, porcine organs may be used for xenotransplantation in donors with no alternative.

With respect to growth factors, the factors that were considered to be necessary for organogenesis by inducing proliferation, differentiation, and maturation of co-cultured fetal hepatic parenchymal cells and non-parenchymal cells were added to the culture medium<sup>[30–35]</sup>. Hamazaki *et al.*<sup>[35]</sup> demonstrated that the important growth factors for hepatogenesis are fibroblast growth factor (FGF) at an early stage, HGF in the middle stage, and OSM, insulin, and dexamethasone at the late stage. OSM and dexamethasone are especially important for the maturation of hepatoblasts to hepatocytes<sup>[31,36]</sup>. On the other hand, it is generally accepted that HGF/scatter factor (SF) is an important paracrine mediator of epithelial-mesenchymal cell interactions, with potential involvement in organogenesis and angiogenesis<sup>[37,38]</sup>. In particular, a morphogenic effects of HGF/SF on the organization of focal contacts and cellular junctions has been documented<sup>[39]</sup>.

Morphological examination of each group showed that fetal hepatic parenchymal cells were adherent to the cellulose beads, while fetal hepatic non-parenchymal cells existed on side of perfusion (Figure 4A)<sup>[2]</sup>. Also, small organoids that had separated from the beads were observed floating in the RFB. In the HFE35 group, larger organoids were observed. Microscopic examination of the organoids from a high-HGF groups revealed a palisading architecture and bile duct-like structures with the expression of CK19, so the organoids could be called mini livers. Furthermore, TEM showed microvilli on the luminal surfaces of bile duct-like structures and junctional complexes, the basis of the cytoskeleton of epithelial tissue. Finally, strong expression of Cx32, which is the main protein of hepatocyte gap junctions, was observed<sup>[40,41]</sup>. These findings were considered to be the features of organoids which had adhered to the beads firmly until reaching a certain size.

Oxygen consumption was used as an index of cellular activity and it showed a significant change over time in the HFE35 group compared to the FME35 group, and a high level of oxygen consumption was maintained in the HFE35 group. Although the LFE35 group also showed a high consumption at the time of HGF addition compared to the FME35 group, there was no significant change over time. The E56 group also showed similar changes of oxygen consumption under the same conditions. The ammonia loading test was performed for evaluation of functionality of mature hepatocytes. Among 3 groups tested, the reduction of NH<sub>3</sub> and the increase of urea

production were highest in the HFE35 group, suggesting that the urea cycle was activated by elevation of the ammonia concentration. Although similar changes were observed in the HFE56 group, the extent of change was smaller than in the HFE35 group. These results showed that the organoids possessed some of the functions of mature hepatocytes.

Both proliferative activity and differentiation of cells are needed as an ideal source for a BAL. In this study, the combination of embryonic porcine liver (E35) cells and pulsing with a high concentration of HGF in our RFB provided a system for which clinical application may eventually be possible in the fields of BAL and transplantation medicine.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

As an alternative to liver transplantation, numerous researchers have been working towards the goal of development of a fully functional artificial liver. Researchers are, therefore, concentrating their efforts on hybrid systems incorporating human- or animal-derived cells. While research on bioartificial liver (BAL) system is still in its infancy, the urgent goal is to develop a sophisticated BAL suitable for clinical applications.

### Research frontiers

The flow bioreactor (RFB) system is a 3-dimensional culture system that can be used for high-density culture, achieving a cell density 10 times greater than that obtained with hollow-fiber culture. The culture medium flows from the periphery toward the center of the reactor. When medium flows from the periphery towards the center, a high perfusion rate can provide an adequate supply of oxygen and nutrients to cells at the center of the bioreactor even though oxygen and nutrients are consumed by cells at the periphery. This system simulates the anatomy of hepatic lobules. On the other hand, both proliferative activity and differentiation of cells are needed as an ideal source for a BAL. Fetal cells have a high proliferative potential *in vitro* and we selected the fetal porcine hepatocytes as a cell source. Furthermore, we examined the earliest teratoma-free gestational age of embryonic day. Furthermore, the efficacy of pulsed administration of a high concentration hepatocytes growth factor (HGF) was examined.

### Innovations and breakthroughs

In this study, the combination of embryonic porcine liver (embryonic d 35: E35) cells and pulsing a high concentration of HGF in our RFB provided a system for which clinical application may eventually be possible in the fields of BAL and transplantation medicine.

### Applications

Our RFB system may be able to perform clinical application as extracorporeal BAL for acute hepatic failure. Furthermore, the liver organoids in RFB have the potential future *in vivo* application (implantable bioartificial liver *etc.*).

### Peer review

The manuscript reports the hepatic reconstruction from fetal porcine liver cells using a radial flow bioreactor. The authors evidenced that cells organized in organoids with the presence of bile duct-like structure. They also showed that HGF favored differentiation and survival of cells in the bioreactor. Despite the fact that this bioreactor was described useful from maintenance of expression of CYP3A4

by human hepatocytes and that the use of pig cells is ethically problematic for tissue engineering and development of extracorporeal bioartificial liver, the results are interesting and well documented.

## REFERENCES

- Hiramoto A, Matsuura T, Aizawa M. Three-dimensional cell culture of hepatocytes using apatite-fiber scaffold and application to a radial-flow bioreactor. *Arch BioChem Res* 2006; **6**: 220-223
- Saito M, Matsuura T, Masaki T, Maehashi H, Shimizu K, Hataba Y, Iwahori T, Suzuki T, Braet F. Reconstruction of liver organoid using a bioreactor. *World J Gastroenterol* 2006; **12**: 1881-1888
- Iwahori T, Matsuura T, Maehashi H, Sugo K, Saito M, Hosokawa M, Chiba K, Masaki T, Aizaki H, Ohkawa K, Suzuki T. CYP3A4 inducible model for in vitro analysis of human drug metabolism using a bioartificial liver. *Hepatology* 2003; **37**: 665-673
- Nagamori S, Hasumura S, Matsuura T, Aizaki H, Kawada M. Developments in bioartificial liver research: concepts, performance, and applications. *J Gastroenterol* 2000; **35**: 493-503
- Sussman NL, Chong MG, Koussayer T, He DE, Shang TA, Whisennand HH, Kelly JH. Reversal of fulminant hepatic failure using an extracorporeal liver assist device. *Hepatology* 1992; **16**: 60-65
- Sussman NL, Kelly JH. Improved liver function following treatment with an extracorporeal liver assist device. *Artif Organs* 1993; **17**: 27-30
- Matsumoto T, Komori R, Magara T, Ui T, Kawakami M, Hano H. A study on the normal structure of human liver, with spectral reference to its angioarchitecture. *Jikei Med J* 1979; **26**: 1-40
- Demetriou AA, Brown RS Jr, Busuttill RW, Fair J, McGuire BM, Rosenthal P, Am Esch JS 2nd, Lerut J, Nyberg SL, Salizzoni M, Fagan EA, de Hemptinne B, Broelsch CE, Muraca M, Salmeron JM, Rabkin JM, Metselaar HJ, Pratt D, De La Mata M, McChesney LP, Everson GT, Lavin PT, Stevens AC, Pitkin Z, Solomon BA. Prospective, randomized, multicenter, controlled trial of a bioartificial liver in treating acute liver failure. *Ann Surg* 2004; **239**: 660-667; discussion 667-670
- Rozga J, Holzman MD, Ro MS, Griffin DW, Neuzil DF, Giorgio T, Moscioni AD, Demetriou AA. Development of a hybrid bioartificial liver. *Ann Surg* 1993; **217**: 502-509; discussion 509-511
- Dekel B, Burakova T, Ben-Hur H, Marcus H, Oren R, Laufer J, Reisner Y. Engraftment of human kidney tissue in rat radiation chimera: II. Human fetal kidneys display reduced immunogenicity to adoptively transferred human peripheral blood mononuclear cells and exhibit rapid growth and development. *Transplantation* 1997; **64**: 1550-1558
- Foglia RP, DiPreta J, Statter MB, Donahoe PK. Fetal allograft survival in immunocompetent recipients is age dependent and organ specific. *Ann Surg* 1986; **204**: 402-410
- Statter MB, Foglia RP, Parks DE, Donahoe PK. Fetal and postnatal testis shows immunoprivilege as donor tissue. *J Urol* 1988; **139**: 204-210
- Eventov-Friedman S, Katchman H, Shezen E, Aronovich A, Tchorsh D, Dekel B, Freud E, Reisner Y. Embryonic pig liver, pancreas, and lung as a source for transplantation: optimal organogenesis without teratoma depends on distinct time windows. *Proc Natl Acad Sci USA* 2005; **102**: 2928-2933
- Vigna E, Naldini L, Tamagnone L, Longati P, Bardelli A, Maina F, Ponzetto C, Comoglio PM. Hepatocyte growth factor and its receptor, the tyrosine kinase encoded by the c-MET proto-oncogene. *Cell Mol Biol (Noisy-le-grand)* 1994; **40**: 597-604
- Mitaka T, Mikami M, Sattler GL, Pitot HC, Mochizuki Y. Small cell colonies appear in the primary culture of adult rat hepatocytes in the presence of nicotinamide and epidermal growth factor. *Hepatology* 1992; **16**: 440-447
- Seglen PO. Preparation of isolated rat liver cells. *Methods Cell Biol* 1976; **13**: 29-83
- Zhou XD, Tokiwa T, Kano J, Kodama M. Isolation and primary culture of adult pig hepatocytes. *Meth Cell Soci* 1998; **19**: 277-284
- Guesdon JL, Ternynck T, Avrameas S. The use of avidin-biotin interaction in immunoenzymatic techniques. *J Histochem Cytochem* 1979; **27**: 1131-1139
- Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem* 1981; **29**: 577-580
- Chekaluk E, Hutchinson TP, Cairns D. Repeated measures ANOVA for responses developing over time. *Eur J Anaesthesiol* 1998; **15**: 381-382
- Vickers AJ. Analysis of variance is easily misapplied in the analysis of randomized trials: a critique and discussion of alternative statistical approaches. *Psychosom Med* 2005; **67**: 652-655
- Sachs DH. The pig as a potential xenograft donor. *Path Biol* 1994; **42**: 217-219
- Tojo H. Problem in the exploitation of transgenic farm animals for xenotransplantation. *Geka* 2001; **5**: 514-521
- Galili U. The alpha-gal epitope (Gal alpha 1-3Gal beta 1-4GlcNAc-R) in xenotransplantation. *Biochimie* 2001; **83**: 557-563
- Galili U, Mandrell RE, Hamadeh RM, Shohet SB, Griffiss JM. Interaction between human natural anti-alpha-galactosyl immunoglobulin G and bacteria of the human flora. *Infect Immun* 1988; **56**: 1730-1737
- Xu Y, Lorf T, Sablinski T, Gianello P, Bailin M, Monroy R, Kozlowski T, Awwad M, Cooper DK, Sachs DH. Removal of anti-porcine natural antibodies from human and nonhuman primate plasma in vitro and in vivo by a Galalpha1-3Galbeta1-4betaGlc-X immunoaffinity column. *Transplantation* 1998; **65**: 172-179
- van der Laan LJ, Lockey C, Griffeth BC, Frasier FS, Wilson CA, Onions DE, Hering BJ, Long Z, Otto E, Torbett BE, Salomon DR. Infection by porcine endogenous retrovirus after islet xenotransplantation in SCID mice. *Nature* 2000; **407**: 90-94
- Yamada K, Hirakata A. Xenotransplantation. *Sogorinsyo* (in Japanese) 2006; **55**: 2018-2023
- Wong BS, Yamada K, Okumi M, Weiner J, O'Malley PE, Tseng YL, Dor FJ, Cooper DK, Saidman SL, Griesemer A, Sachs DH. Allosensitization does not increase the risk of xenoreactivity to alpha1,3-galactosyltransferase gene-knockout miniature swine in patients on transplantation waiting lists. *Transplantation* 2006; **82**: 314-319
- Kinoshita T, Sekiguchi T, Xu MJ, Ito Y, Kamiya A, Tsuji K, Nakahata T, Miyajima A. Hepatic differentiation induced by oncostatin M attenuates fetal liver hematopoiesis. *Proc Natl Acad Sci USA* 1999; **96**: 7265-7270
- Kamiya A, Kinoshita T, Ito Y, Matsui T, Morikawa Y, Senba E, Nakashima K, Taga T, Yoshida K, Kishimoto T, Miyajima A. Fetal liver development requires a paracrine action of oncostatin M through the gp130 signal transducer. *EMBO J* 1999; **18**: 2127-2136
- Michalopoulos GK, Bowen WC, Zajac VF, Beer-Stolz D, Watkins S, Kostrubsky V, Strom SC. Morphogenetic events in mixed cultures of rat hepatocytes and nonparenchymal cells maintained in biological matrices in the presence of hepatocyte growth factor and epidermal growth factor. *Hepatology* 1999; **29**: 90-100
- Mitaka T, Sato F, Mizuguchi T, Yokono T, Mochizuki Y. Reconstruction of hepatic organoid by rat small hepatocytes and hepatic nonparenchymal cells. *Hepatology* 1999; **29**: 111-125
- Kano J, Noguchi M, Kodama M, Tokiwa T. The in vitro differentiating capacity of nonparenchymal epithelial cells derived from adult porcine livers. *Am J Pathol* 2000; **156**: 2033-2043
- Hamazaki T, Iiboshi Y, Oka M, Papst PJ, Meacham AM, Zon LI, Terada N. Hepatic maturation in differentiating embryonic stem cells in vitro. *FEBS Lett* 2001; **497**: 15-19
- Matsui T, Kinoshita T, Morikawa Y, Tohya K, Katsuki M, Ito Y, Kamiya A, Miyajima A. K-Ras mediates cytokine-induced

- formation of E-cadherin-based adherens junctions during liver development. *EMBO J* 2002; **21**: 1021-1030
- 37 **Sonnenberg E**, Meyer D, Weidner KM, Birchmeier C. Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. *J Cell Biol* 1993; **123**: 223-235
- 38 **Rosen EM**, Nigam SK, Goldberg ID. Scatter factor and the c-met receptor: a paradigm for mesenchymal/epithelial interaction. *J Cell Biol* 1994; **127**: 1783-1787
- 39 **Dowrick PG**, Warn RM. The effects of scatter factor on the cytoskeletal organization of epithelial cells. *Cancer Invest* 1990; **8**: 675-683
- 40 **Kojima T**, Fort A, Tao M, Yamamoto M, Spray DC. Gap junction expression and cell proliferation in differentiating cultures of Cx43 KO mouse hepatocytes. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G1004-G1013
- 41 **Kojima T**, Sawada N, Chiba H, Kokai Y, Yamamoto M, Urban M, Lee GH, Hertzberg EL, Mochizuki Y, Spray DC. Induction of tight junctions in human connexin 32 (hCx32)-transfected mouse hepatocytes: connexin 32 interacts with occludin. *Biochem Biophys Res Commun* 1999; **266**: 222-229

S- Editor Li DL L- Editor Kremer M E- Editor Ma WH





BASIC RESEARCH

## Enhanced expressions and activations of leukotriene C4 synthesis enzymes in D-galactosamine/lipopolysaccharide-induced rat fulminant hepatic failure model

Kui-Fen Ma, Hong-Yu Yang, Zhe Chen, Luo-Yang Qi, Dan-Yan Zhu, Yi-Jia Lou

Kui-Fen Ma, Hong-Yu Yang, Zhe Chen, Luo-Yang Qi, Dan-Yan Zhu, Yi-Jia Lou, Institute of Pharmacology & Toxicology and Biochemical Pharmaceutics, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, Zhejiang Province, China  
Author contributions: Ma KF and Lou YJ designed the research; Ma KF, Yang HY, Chen Z, Qi LY and Zhu DY performed the research.

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Correspondence to: Professor Yi-Jia Lou, Institute of Pharmacology & Toxicology and Biochemical Pharmaceutics, College of Pharmaceutical Sciences, Zhejiang University, 388 Yuhangtang Road, Hangzhou 310058, Zhejiang Province, China. [yijialou@zju.edu.cn](mailto:yijialou@zju.edu.cn)

Telephone: +86-571-88208403 Fax: +86-571-88208403

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### Abstract

**AIM:** To investigate the expression and activity of leukotriene C4 (LTC4) synthesis enzymes and their underlying relationship with cysteinyl leukotriene (cys-LT) generation in a rat fulminant hepatic failure (FHF) model induced by D-galactosamine/lipopolysaccharide (D-GalN/LPS).

**METHODS:** Rats were treated with D-GalN (300 mg/kg) plus LPS (0.1 mg/kg) for 1, 3, 6, and 12 h. Enzyme immunoassay was used to determine the hepatic cys-LT content. Reverse transcription-polymerase chain reaction (RT-PCR), Western blot or immunohistochemical assay were employed to assess the expression or location of LTC4 synthesis enzymes, which belong to membrane associated proteins in eicosanoid and glutathione (MAPEG) metabolism superfamily. Activity of LTC4 synthesis enzymes was evaluated by determination of the products of LTA4 after incubation with liver microsomes using high performance liquid chromatography (HPLC).

**RESULTS:** Livers were injured after treatment with D-GalN/LPS, accompanied by cys-LT accumulation at the prophase of liver injury. Both LTC4 synthase (LTC4S) and microsomal glutathione-S-transferase (mGST) 2 were expressed in the rat liver, while the latter was specifically located in hepatocytes. Their mRNA and protein expressions were up-regulated at an earlier phase after treatment with D-GalN/LPS. Meantime, a higher activity of LTC4 synthesis enzymes was detected, although the

activity of LTC4S played the main role in this case.

**CONCLUSION:** The expression and activity of both LTC4S and mGST2 are up regulated in a rat FHF model, which are, at least, partly responsible for cys-LT hepatic accumulation.

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**Key words:** Cysteinyl leukotriene; Microsomal glutathione S-transferase 2; Leukotriene C4 synthase; D-galactosamine/lipopolysaccharide; Fulminant hepatic failure

**Peer reviewer:** Yasuji Arase, MD, Department of Gastroenterology, Toranomon Hospital, 2-2-2Toranomonminato-ku, Tokyo 105-8470, Japan

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### INTRODUCTION

Leukotriene (LT) is a potent lipid mediator with biological activities related to inflammation and allergy<sup>[1]</sup>. It was reported LT is involved in a variety of diseases, such as allergic airway disease, dermatological disease, cardiovascular disease and liver injury<sup>[2-5]</sup>. Fulminant hepatic failure (FHF) is a severe liver injury accompanying hepatic encephalopathy<sup>[6]</sup> and causes multi-organ failure with an extremely high mortality rate, even if intensive care is provided. Treatment is directed at an early recognition of the complications and general supportive measures. The only proven therapy for those who are unlikely to recover is liver transplantation<sup>[7,8]</sup>. Although it has been considered to be caused by several hepatitis viruses, various drugs, toxins, and metabolic disorders, its pathogenesis is extremely complicated, with indeterminate mechanisms<sup>[9,10]</sup>. There is evidence that inflammatory response and microcirculatory disturbances contribute to FHF<sup>[11-14]</sup>. Suppression of the inflammatory process in the liver is an effective therapy for fulminant or severe acute hepatic failure<sup>[15]</sup>. An experimental model of FHF induced

by D-galactosamine (D-GalN)/lipopolysaccharide (LPS) well simulates the situation in clinical FHF<sup>[11]</sup>. D-GalN can deplete liver UTP, disrupt protein synthesis and sensitize hepatocytes to challenging agents such as LPS<sup>[10,16]</sup>. It was reported that arachidonate metabolism is changed and LT formation is stimulated in D-GalN/LPS-induced acute liver injury<sup>[17,18]</sup>. Inhibition of LT biosynthesis or blocking binding of LT at the receptor level could also prevent liver injury induced by D-GalN/LPS<sup>[19,20]</sup>. Therefore, LT as an inflammatory factor is probably involved in the pathogenesis of FHF.

LT is a metabolite of arachidonic acid synthesized *via* the 5-lipoxygenase pathway<sup>[21]</sup>. LTC4 synthase (LTC4S) catalyzes LTA4 and reduced glutathione (GSH) generating LTC4 is the first committed step in the synthesis of cysteinyl leukotrienes (cys-LT), including LTC4, LTD4, and LTE4<sup>[22]</sup>. Like LTC4S, members of membrane associated proteins in eicosanoid and glutathione metabolism (MAPEG) superfamily, namely microsomal glutathione-S-transferase 2 (mGST2) and mGST3 also catalyze LTC4 synthesis<sup>[23-25]</sup>. However, it was reported that rat mGST3 is unable to synthesize LTC4 even in the presence of the conserved Arg/Try catalytic pair<sup>[26]</sup>. LTC4S is a pivotal enzyme for cys-LT biosynthesis in human lung membranes and platelet homogenates where it is prevalently expressed<sup>[27,28]</sup>. A recent study in LTC4S knockout mice suggested that LTC4S is a predominant *in vivo* source for cys-LT synthesis<sup>[29]</sup>. Its crystal structure that was recently interpreted provides structural insights into the mechanism of LTC4 formation<sup>[30,31]</sup>. It is up-regulated in LPS-induced systemic inflammatory reactions<sup>[32]</sup>. Our previous study showed that hepatic ischemia/reperfusion (I/R) injury up-regulates the mRNA and protein expression of LTC4S and its activity<sup>[33]</sup>. However, other studies reported that mGST may account for LTC4S-like activity in non-inflammatory cells<sup>[24]</sup>. mGST2, 44% identical to LTC4S in primary structure, is the principal enzyme responsible for LTC4 production in human liver microsomes and endothelial cells<sup>[34,35]</sup>.

However, no reports are available on the alterations in both mRNA and protein of LTC4 synthesis enzymes including LTC4S and mGST2, their activities, and the cell types responsible for their expression during the FHF course. The mechanism underlying cys-LT generation at the early phase of FHF has not been extensively elucidated. Therefore, the present study aimed to investigate the expression and activity of LTC4 synthesis enzymes and the possible mechanism underlying cys-LT generation in a rat FHF model induced by D-GalN/LPS.

## MATERIALS AND METHODS

### Animals and experimental protocol

Male Sprague-Dawley rats, weighing  $200 \pm 20$  g, were obtained from Experimental Animal Center, Zhejiang University. The animal study protocol, in compliance with the Guidelines of China for Animal Care, was approved by the Ethics Committee of Zhejiang University and conformed to the internationally accepted principles in the care and use of experimental animals.

Rats were randomly divided into five groups. Vehicle rats (control) were injected with saline, and decapitated immediately after the second injection. Other animals were intraperitoneally injected with D-GalN (300 mg/kg), followed by LPS (0.1 mg/kg) 1 h later. Rats were decapitated at 1, 3, 6 and 12 h, respectively, after LPS administration. Sera and liver tissue were collected for further evaluation.

### Aminotransferase determination and histological evaluation

Serum ALT and AST were measured with an automatic analyzer (Hitachi 7600, Tokyo, Japan). The right lobe of liver was fixed in 4% paraformaldehyde for hematoxylin and eosin or immunohistochemistry staining. The left lobe of liver was snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until further preparation.

Paraffin-embedded liver tissue was cut into  $5\text{-}\mu\text{m}$  thick sections, deparaffinized in xylene, and rehydrated through a series of decreasing concentrations of ethanol. The sections were stained with hematoxylin and eosin and analyzed under a light microscope.

### Enzyme immunoassay

An aliquot of liver tissue (0.4 g) was individually homogenized in Dulbecco's phosphate-buffered saline and de-proteined with two volumes of ice-cold acetonitrile. Supernatant was collected before extraction with Sep-pak C18 cartridges (Water Corporation, USA). Methanol fraction was collected and evaporated to dryness under nitrogen for cys-LT measurement.

Cys-LT content in liver tissue was quantified with an enzyme immunoassay (EIA) kit (Cayman Chemical Company, USA) which recognizes LTC4, LTD4 and LTE4 for a combined quantitative value.

### RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated from rat liver tissues using the TRIzol reagent (Sanggon) according to its manufacturer's instructions and quantified by measuring the ultraviolet absorption at 260 nm. For the synthesis of cDNA, 3  $\mu\text{g}$  of total RNA from each sample was reverse-transcribed. Primers (Shanghai Sanggon, China) and application parameters for PCR analysis of LTC4S, mGST2 and  $\beta$ -actin are listed in Table 1. PCR products were analyzed by electrophoresis, visualized by ultraviolet transillumination of ethidium bromide-stained gels.

### Western blotting analysis

Total protein was harvested from liver tissues. An aliquot of protein (80  $\mu\text{g}$ ) was electrophoresed on 12.5% SDS-polyacrylamide gels. After separation, proteins were transferred onto a pure nitrocellulose blotting membrane (Pall, Dreieich, Germany). Membranes were incubated with their corresponding antibody (1:200 for LTC4 synthase antibody from Santa Cruz). Blots were then exposed to the corresponding secondary antibody (horseradish peroxidase-conjugated IgG, Beijing Zhongshan Biotechnology Company, 1:4000). Immunocomplexes

Table 1 Primers and application parameters for PCR analysis of the respective genes

Gene	Primer	Designed size (bp)	Annealing temperature (°C)	Mg <sup>2+</sup> (mmol/L)	Cycles
LTC4S	Sense 5'-GAAGAACTTTCCACGTGTCG-3' Antisense 5'-GTGCAGCCATTGCCACTAGC-3'	282	55	2.0	35
mGST2	Sense 5'-TTCAATCAAGTTTTTGCAACC-3' Antisense 5'-TCTTGGCAACATGAAAGTCC-3'	204	57	1.5	35
β-actin	Sense 5'-TGACGGGGTACCCACACTGTGCCCATCTA-3' Antisense 5'-CTAGAAGCAATTGCGGTGGACGATGGAGGG-3'	660	58	1.5	28

were visualized autoradiographically with an enhanced chemiluminescent substrate (ECL, Pierce, USA), and scanned using a bio-imaging analyzer (Bio-Rad, USA).

### Immunohistochemistry

Immunohistochemistry was performed to determine the localization and protein expression of LTC4S or mGST2. Liver tissue was cut into 5-μm thick sections with a microtome and the sections were then mounted on slides. Streptavidin-biotin-complex (SABC, Boster Co, China) immunohistochemistry was used to explore the expression of mGST2. Briefly, endogenous peroxidase was inactivated. After treated with 5% BSA for 30 min, specimens were incubated at 4°C overnight with primary antibody (mGST2 antibody from Abnova Corporation, Taiwan, final dilution 1:100; LTC4S antibody from Santa Cruz, USA, final dilution 1:200). Diaminobenzidine (DAB) was used for peroxidase reaction and the slides were counterstained with hematoxylin.

### Reverse-phase HPLC analysis of LTC4 synthesis enzyme activity

Activity of LTC4 synthesis enzymes was assayed by measuring the amount of LTC4 and its isomer produced after incubation with liver microsomal fraction and LTA4-free acid. LTA4 was saponified in acetone with 50 mmol/L NaOH (20% v/v) for 40 min at room temperature (RT) from LTA4 methyl ester (Cayman Chemical Co., USA). Microsomal fraction was harvested from liver tissue homogenates. An aliquot (100 μg) of microsomal fraction was incubated with 60 μmol/L LTA4-free acid, 10 mmol/L GSH and 0.2% BSA for 5 min at 37°C. The reaction was terminated by adding 100 μL stop solution (acetonitrile/methanol/acetic acid, 150:50:0.3, v/v) containing a defined amount of internal standard prostaglandin B2 (PGB2). Metabolites of LTA4 were resolved by isocratic RP-HPLC on an Agilent Zorbax SB-C18 column (4.6 mm × 250 mm, 5 μm). The mobile phase was at 1.0 mL/min comprised of acetonitrile: water: acetic acid (38:62:0.3, v/v, pH adjusted to 5.6 with NH<sub>3</sub>H<sub>2</sub>O). LTC4 and LTC4 isomer peaks were identified by comparing the retention time of LTC4 standard and on-line analysis of UV absorbance spectra. The amount of products formed was calculated by comparing the internal standard.

### LC/MS assay

LTC4 generation was further identified by LC/MS. Assay was performed on a HP 1100 HPLC system with an Esquire-LC 00075 quadrupole mass spectrometer

detection system. Agilent Zorbax SB-C18 column (4.6 mm × 250 mm, 5 μm) was used. The mass spectrometer was equipped with an electrospray ionization source. Typical source conditions were as follows: positive ionization mode, capillary 3.5 kV, skimmer 38.3 V, dry inert gas N<sub>2</sub> (10 L/min), temperature of the capillary 350°C, scan beginning at 200 m/z, scan ending at 700 m/z.

### Statistical analysis

All data were expressed as mean ± SD. The results in different groups were compared by one-way analysis of variance (ANOVA) with SPSS 10.0 for WINDOWS. *P* < 0.05 was considered statistically significant.

## RESULTS

### Serum ALT and AST examination and histological evaluation

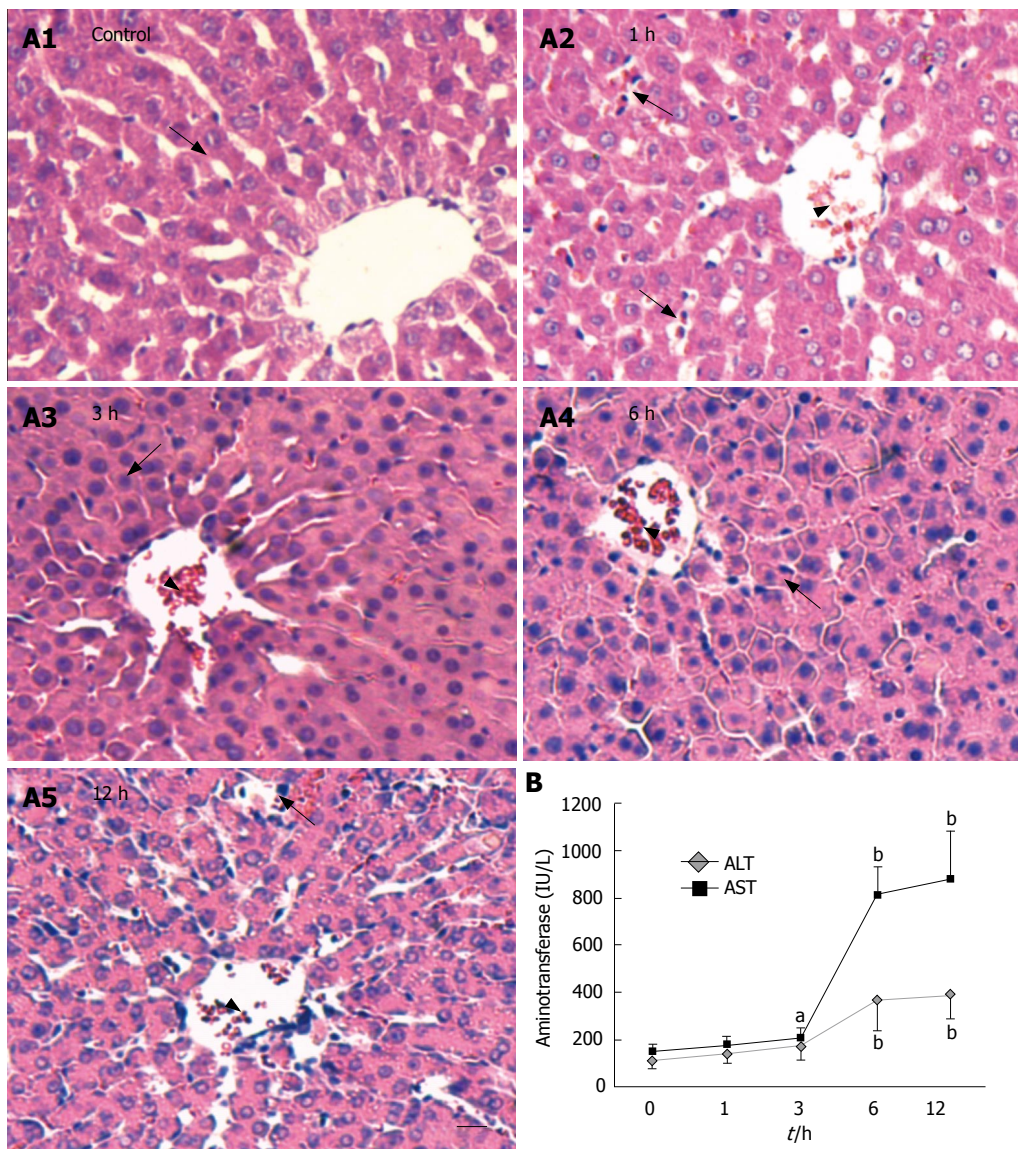
Rat liver injury was evaluated histologically and serologically after D-GalN/LPS treatment. As shown in Figure 1B, serum ALT and AST levels were continuously elevated and peaked at 12 h (392.2 ± 104.6 IU/L and 888.2 ± 190.8 IU/L respectively in the 12 h group, and 116.8 ± 39.4 IU/L and 156.2 ± 15.5 IU/L in the control group). ALT at 6, 12 h and AST at 3, 6, 12 h were significantly elevated in the D-GalN/LPS treatment group than those in the control group (*P* < 0.05).

Liver sections from the control group showed cord-like arrangement of hepatocytes with clear nucleus and intact endothelium in central veins without vascular congestion, observed with HE staining. Liver inflammation, found at 1 and 3 h after treatment with D-GalN/LPS, was characterized by congested central vein, infiltrated inflammatory cells and swollen hepatocytes. Dramatic injuries occurred in liver 6 and 12 h after treatment with D-GalN/LPS, and were characterized by disarranged hepatocytes, appearance of massive necrosis and broken cytolemma (Figure 1).

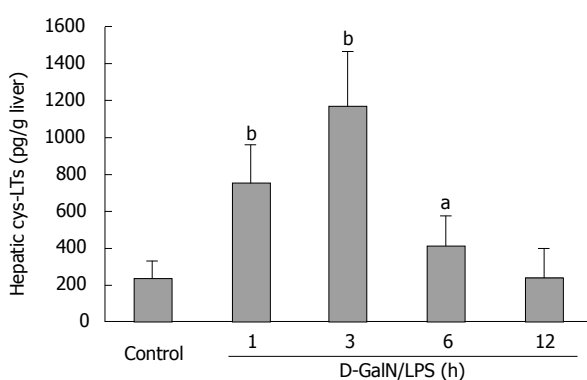
### Hepatic cys-LT accumulation after D-GalN/ LPS treatment

Cys-LT in liver, determined with a cys-LT EIA kit, was significantly higher at 1 h in the D-GalN/LPS treatment group than in the control group (1167.9 ± 340.5 pg/g *vs* 236.3 ± 88.5 pg/g, *P* < 0.05), increased to 4.9 folds at 3 h, and gradually returned to its basal values at 12 h (Figure 2), suggesting that D-GalN/LPS injection at an early stage could lead to accumulation of cys-LT, which may play an important role in the onset of FHF.





**Figure 1** Histology and enzyme evaluation of rat livers treated with D-galactosamine/lipopolysaccharide (D-GalN/LPS). **A:** Histological examination of the liver at indicated times after GalN/LPS treatment (hematoxylin-eosin staining, bar = 100  $\mu$ m). Arrow heads indicate congested central veins. The arrow in the control group **A1** indicates cord-like arrangement of hepatocytes with clear nucleoli. The arrows in the 1 h group **A2** indicate infiltrated inflammatory cells. The arrow in the 3 h group **A3** indicates swollen hepatocytes. The arrow in the 6 h group **A4** indicates disarranged hepatocytes. The arrow in the 12 h group **A5** indicates massive necrosis and broken cytolemma; **B:** Time course of serum ALT and AST in rats received intraperitoneal injection of D-GalN/LPS. Data are represented as mean  $\pm$  SD,  $n = 6$ ,  $^aP < 0.05$  and  $^bP < 0.01$  vs control group.



**Figure 2** Time course of hepatic cys-LT content in D-GalN/LPS-injured rats. Rats were treated with D-GalN/LPS as described. Cys-LT was quantified with an enzyme immunoassay kit which recognizes LTC<sub>4</sub>, LTD<sub>4</sub> and LTE<sub>4</sub> for a combined quantitative value. Each value represents the mean obtained from six rats in each group.  $^aP < 0.05$ ,  $^bP < 0.01$  vs control group.

### Expressions of LTC<sub>4</sub>S and mGST2 in liver tissues

mRNA and protein expressions of LTC<sub>4</sub>S and mGST2 were detected in D-GalN/LPS-injured liver tissues.

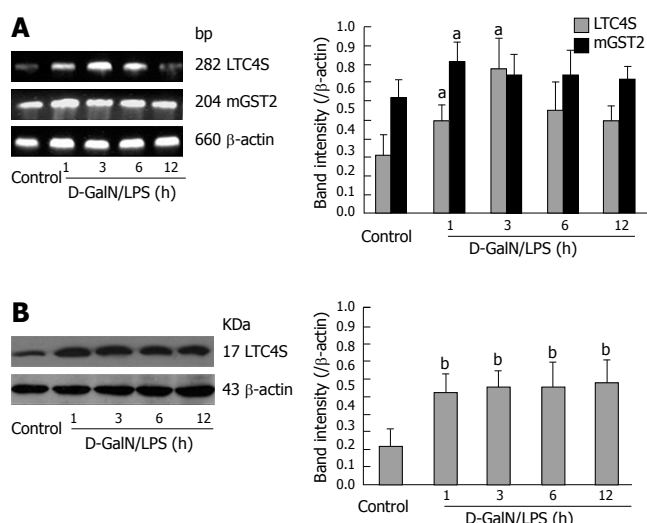
Figure 3A shows a representative of hepatic mRNA expression of LTC<sub>4</sub>S and mGST2 in rat liver during FHF. Densitometric analysis of their PCR products revealed that mRNA expressions of LTC<sub>4</sub>S and mGST2 were significantly increased at 1 h after treatment with D-GalN/LPS ( $P < 0.05$ ).

Considering the above changes in hepatic cys-LT content and the gene expressions of LTC<sub>4</sub> synthesis enzymes, we examined their protein levels in rat liver tissues by Western blotting analysis. The protein level of mGST2 was not detectable by Western blotting analysis because the proper antibody was not commercially available. As shown in Figure 3B, the protein expression of LTC<sub>4</sub>S was obviously increased after D-GalN/LPS treatment.

### Immunohistochemical localization of LTC<sub>4</sub> synthesis enzymes in liver tissue

To clarify what cell types increased the expression of LTC<sub>4</sub> synthesis enzymes in the rat FHF model, we performed immunohistochemical staining for paraffin-embedded liver sections from control and D-GalN/LPS treated rats.





**Figure 3** mRNA and protein expressions of LTC4S and mGST2 in D-GalN/LPS-treated liver tissues. **A:** mRNA expression of LTC4S and mGST2 detected by RT-PCR. cDNA synthesized from total RNA (3 μg) was used for PCR amplification with 35 cycles for LTC4S and mGST2. PCR products were electrophoresed on a 1.5% agarose gel. The intensity of each band was quantified by computer-assisted densitometry, and the data were compared to those of the corresponding band of β-actin (Right panel); **B:** Protein expression of LTC4S detected by Western blotting. An aliquot of total protein (80 μg) was subjected to immunoblot analysis as described in Methods. Left panel: Immunoreactive bands corresponding to LTC4S and β-actin in D-GalN/LPS-injured liver; right panel: Densities of the products quantified by computer-assisted densitometry, and the data were normalized to β-actin expression. Data are represented as mean ± SD, <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs control group.

In normal liver, liver cells exhibited a low immunological staining for LTC4S (Figure 4A). In D-GalN/LPS treated rats, LTC4S was detected in endothelial cells and most hepatocytes which were strongly stained, exhibiting a heterogeneous intralobular distribution. However, no positive infiltrated inflammatory cells or Kupffer cells were found. As shown in Figure 4B, mGST2 was mainly located in hepatocytes and its expression was increased in D-GalN/LPS-injured rat livers.

#### Activity of LTC4 synthesis enzymes in D-GalN/ LPS-treated rats

The activity of LTC4 synthesis enzymes in liver microsomal fraction were determined by RP-HPLC. A peak at the retention time of 6.06 min in the indicated mobile phase appeared using an LTC4 standard (Figure 5A), which was also detected at the same retention time in lung microsomes, as a positive control, when the microsomal fraction was incubated with LTA4 and GSH (Figure 5B). MS analysis of the peak displayed a protonated molecular ion at  $m/z$  625.8, corresponding to the LTC4 ion form (Figure 5C). We assessed the activity of mGST2 by identifying the isomer of LTC4, since mGST2 may produce not only LTC4, but also an isomer of LTC4 when incubated with LTA4 which is different from LTC4S<sup>[23,34]</sup>. A peak assessed as the LTC4 isomer was observed when the rat liver microsomal fraction was incubated with exogenous LTA4. The time course of LTC4 production in incubated samples is illustrated in Figure 5D. LTC4 production was increased after treatment with D-GalN/

LPS, suggesting that the activity of LTC4 synthesis was strengthened ( $P < 0.05$ ). The activity of isomer of LTC4 was also increased, indicating that the activity of mGST2 was also initiated, demonstrating that the activity of both mGST2 and LTC4S in rat liver microsomes was increased after D-GalN/ LPS treatment.

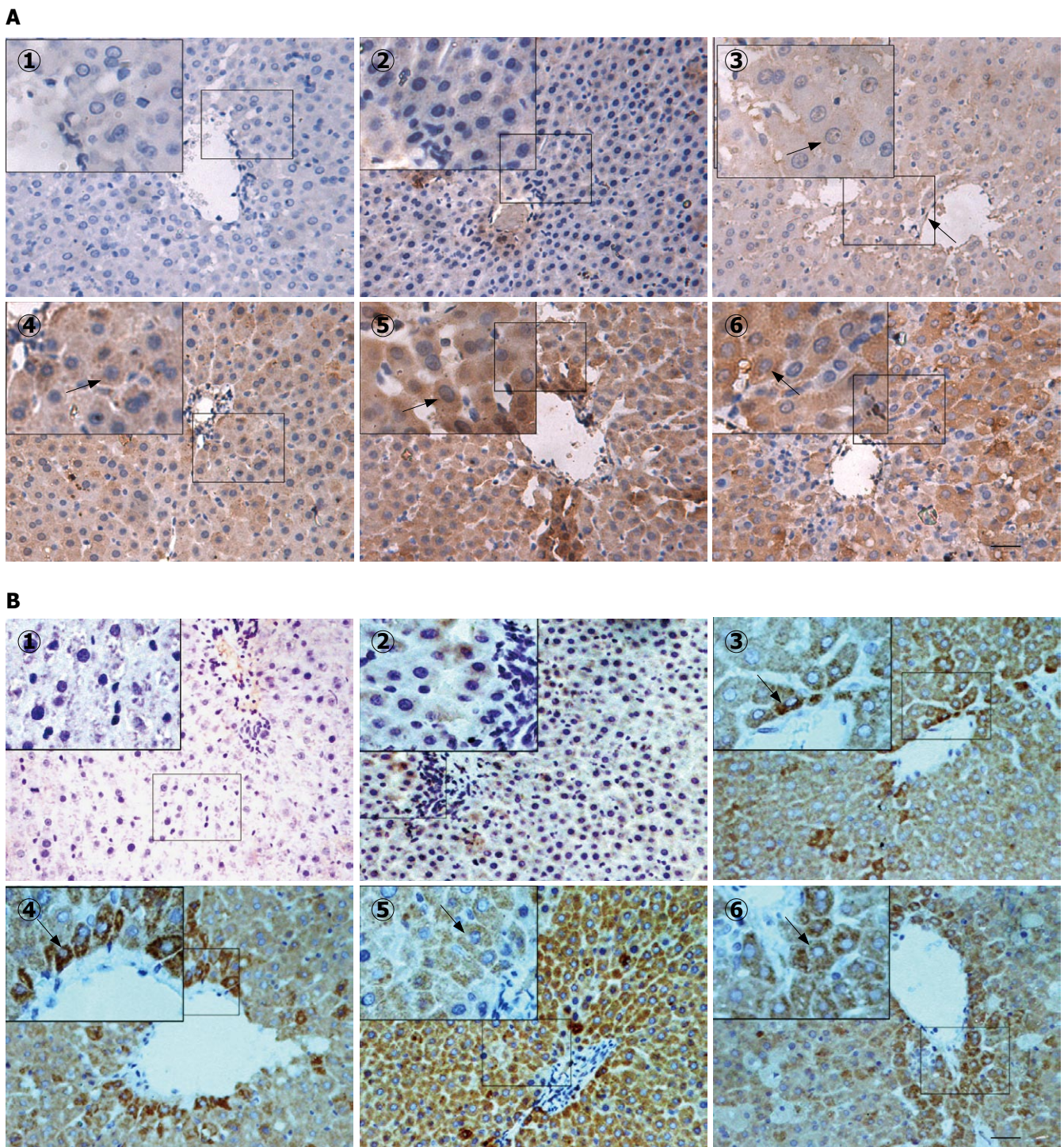
## DISCUSSION

Cys-LT has a wide variety of physiological and immunological effects on liver function and disease<sup>[36-39]</sup>. Cys-LT, as an inflammatory factor, is involved in the pathogenesis of FHF<sup>[17,18]</sup>, which has attracted the interest of clinicians<sup>[40]</sup>. However, the mechanism of cys-LT alteration during FHF has not been well elucidated yet. Thus, we employed a D-GalN/LPS-induced rat model of FHF to explore the possible reason of LTC4 alteration in the pathogenesis of FHF.

In the present study, we evaluated the inflammatory cell infiltration, hepatocytes damage and structure disorder after D-GalN/LPS treatment. Serological data further confirmed the impairment of liver function at a later phase of treatment. However, the hepatic cys-LT content was transiently increased before the occurrence of liver injuries. It was reported that inhibiting cys-LT biosynthesis or blocking binding of cys-LT at the receptor level can prevent liver injury<sup>[19,20]</sup>. D-GalN not only acts directly on hepatocytes by depleting liver UTP and disrupting protein synthesis, but also sensitizes hepatocytes to challenging agents such as LPS<sup>[10,16]</sup>. In addition, liver injury induced by D-GalN/LPS well simulates the situation in clinical FHF<sup>[11]</sup>. Our results suggested that cys-LT accumulation plays a role in D-GalN/LPS-induced FHF.

To elucidate whether cys-LT accumulation is related to LTC4 synthesis enzymes in the rat FHF model, we examined their expression and activity owing to lack of enough information in this model. The committed step in cys-LT biosynthesis in rats is catalyzed by LTC4 synthesis enzymes, but not by mGST3<sup>[22-24]</sup>. It was reported that mRNA expression of LTC4S in liver is so low that Northern blotting analysis is not sufficiently sensitive to detect it<sup>[29,41]</sup>. In the present study, the mRNA expression of mGST2 was significantly higher than that of LTC4S by RT-PCR. Protein expression of LTC4S was detected in rat liver instead of purified hepatocytes by Western blotting assay. Immunohistochemical staining was used to clarify which cell types expressed LTC4 synthesis enzymes and whether the expression was changed in the rat FHF model. The results showed that LTC4S was expressed in hepatocytes and sinusoidal endothelial cells, while mGST2 was only located in hepatocytes. Unlike the report of Schröder<sup>[32]</sup> that mGST2 remained unchanged after a single dose of LPS-induced systemic inflammatory reaction, our results showed that both LTC4S and mGST2 were increased in liver after D-GalN/LPS treatment. The result suggested that mGST2 may play a special role in the pathogenesis of FHF. The discrepancy may be due to the pretreatment with D-GalN and the different treatment time or dose of LPS<sup>[42]</sup>. D-GalN, as a specific hepatotoxic agent, renders hepatocytes sensitive to LPS.



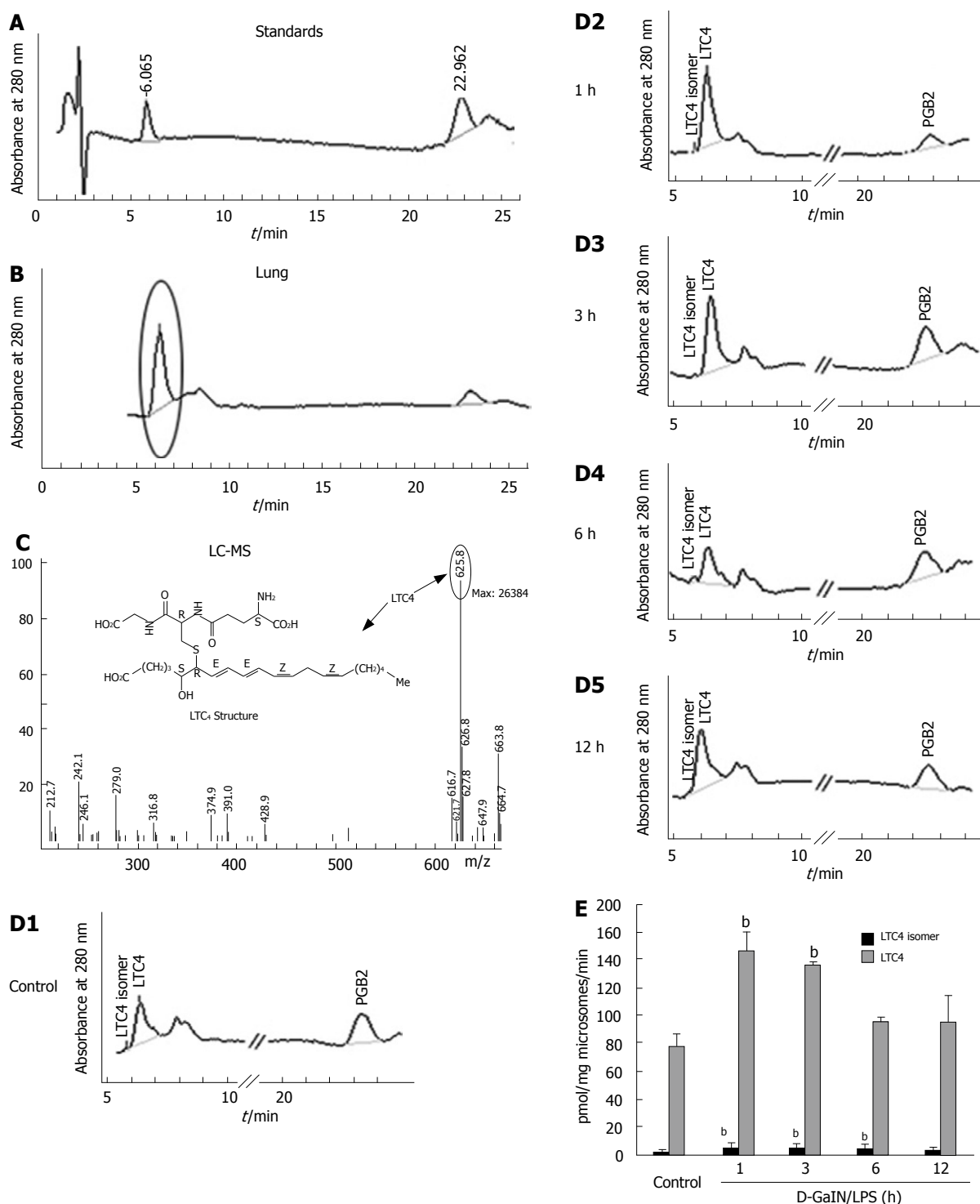


**Figure 4** Immunohistochemical assay of the expression and localization of LTC<sub>4</sub>S (A) and mGST2 (B) in control and D-GalN/LPS-treated liver tissues. Immunohistochemical staining for paraffin-embedded liver sections from the control and D-GalN/LPS-treated rats was examined as described in Materials and Methods. Arrows indicate the representative positive cells expressing the brown granules. 1: Absence of staining on omission of primary antibody; 2: Control; 3-6: Groups treated with D-GalN/LPS at 1, 3, 6 and 12 h, respectively. Bar = 100  $\mu$ m.

Pretreatment with D-GalN creates a different pathogenesis of liver injury. Different treatment time or dose of LPS could also produce different effects. For instance, LPS is usually thought to be a potent stimulus for LT production *in vivo*<sup>[43,44]</sup>. However, it was reported that LPS down-regulates cys-LT release and LTC<sub>4</sub>S gene expression in mononuclear phagocytes<sup>[45]</sup>. In the present study, LTC<sub>4</sub>S and mGST2 expressions were up-regulated after treatment with D-GalN (300 mg/kg) and LPS (0.1 mg/kg). Further

more, the liver not only plays a major role in metabolism and elimination of LT, but also produces cys-LT<sup>[46]</sup>. It was reported that hepatocytes generate cys-LT when co-cultured with Kupffer cells<sup>[47,48]</sup>. Therefore, increased expression of LTC<sub>4</sub> synthesis enzymes in liver may partly contribute to cys-LT accumulation.

The activity of LTC<sub>4</sub> synthesis enzymes was subsequently detected in D-GalN/LPS-induced rat FHF. The appearance of isomer of LTC<sub>4</sub> in HPLC traces



**Figure 5** LTC<sub>4</sub> synthesis enzyme activity in rat liver microsomal fraction. **A:** HPLC traces showing standard LTC<sub>4</sub> and internal standard PGB<sub>2</sub>; **B:** HPLC traces showing the main products when 100 000 × g pellets from lung incubated with LTA<sub>4</sub> (60 μmol/L) and glutathione (10 mmol/L); **C:** MS assay of the product showing its peak at the retention time of 6.06 min in HPLC; **D:** HPLC traces showing generated LTC<sub>4</sub> and isomer of LTC<sub>4</sub> by a microsomal fraction from D-GalN/LPS treated rat livers with indicated times; **E:** The amount of generated LTC<sub>4</sub> and isomer of LTC<sub>4</sub> was calculated by an area peak compared with the internal standard PGB<sub>2</sub> and then plotted. Each value represents the mean obtained from duplicate experiments with liver tissue from six rats in each group. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 vs control group.

suggested the activity of mGST2 in rat liver, since the isomer was used to distinguish between mGST2 and LTC<sub>4</sub>S activities involved in the biosynthesis of LTC<sub>4</sub><sup>[23,34]</sup>. According to Scoggan *et al.*<sup>[34]</sup>, a ratio of LTC<sub>4</sub>/LTC<sub>4</sub> isomer < 50 is typical for a system containing mostly mGST2 whereas a ratio > 50 is seen in tissues containing mostly LTC<sub>4</sub>S. The minimal ratio in our study was 32,

suggesting that mGST2 and LTC<sub>4</sub>S have activities in liver microsomes, but LTC<sub>4</sub>S takes the main responsibility in this case. The mGST2 activity was simultaneously increased, as demonstrated by the increased LTC<sub>4</sub> isomer. mGST2 in liver is a functional enzyme and plays a role in LTC<sub>4</sub> biosynthesis. Since mGST2 was not changed after a signal dose LPS treatment, the synergistic effect of D-GalN and



LPS may be the reason why the expression and activity of mGST2 were increased, which may partly contribute to the pathogenesis of FHF.

In conclusion, the expressions and activities of both LTC4S and mGST2 are up-regulated in a rat FHF model, which are, at least, partly responsible for cys-LT hepatic accumulation.

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## COMMENTS

### Background

Cysteinyl leukotriene (cys-LT), as an inflammatory factor, is probably involved in fulminant hepatic failure (FHF). LTC4 synthesis enzymes catalyze LTA4 to generate LTC4. However, no report on the alterations in mRNA and protein of LTC4S and mGST2 and their activities during FHF is available. The mechanism underlying cys-LT generation at an early phase of FHF has not been elucidated. Therefore, we focused on the expression and activity of LTC4 synthesis enzymes and their relationship with cys-LT generation in a rat FHF model induced by D-galactosamine/ lipopolysaccharide (D-GalN/LPS).

### Research frontiers

LTC4S is a pivotal enzyme for cys-LT biosynthesis in human lung membranes and platelet homogenates where it is prevalently expressed. Studies in the LTC4S knockout mice suggest that LTC4S is a predominant *in vivo* source for cys-LT synthesis. Its crystal structure was recently interpreted, which provides structural insights into the mechanism of LTC4 formation. It is up-regulated in LPS-induced systemic inflammatory reactions. Our previous study showed that hepatic infusion/reperfusion (I/R) injury can up-regulate mRNA and protein expression of LTC4S and enhance its activity. However, other studies reported the mGST may account for LTC4S-like activity in non-inflammatory cells. mGST2, 44% identical to LTC4S in primary structure, is the principal enzyme responsible for LTC4 production in human liver microsomes and endothelial cells.

### Innovations and breakthroughs

Increased expression of LTC4 synthesis enzymes may partly contribute to cys-LT accumulation and liver injury in a rat FHF model. mGST2 and LTC4S contribute to the pathological process of this model.

### Applications

The results provide a rationale for the pathophysiological process of FHF and potential drug molecular targets.

### Terminology

Cysteinyl leukotriene: A potent lipid mediator with biological activities related to inflammation and allergy. LTC4 synthesis enzymes include LTC4S, mGST2 and mGST3.

### Peer review

The authors showed that cys-LT accumulation in FHF was due to enhanced expression and activity of LTC4S and mGST2. The present study is interesting and instructive. The data are well described in this paper. The paper provides useful information on the daily management of patients with viral hepatitis.

## REFERENCES

- 1 Funk CD. Prostaglandins and leukotrienes: advances in eicosanoid biology. *Science* 2001; **294**: 1871-1875
- 2 Montuschi P, Sala A, Dahlen SE, Folco G. Pharmacological modulation of the leukotriene pathway in allergic airway disease. *Drug Discov Today* 2007; **12**: 404-412
- 3 Wedi B, Kapp A. Pathophysiological role of leukotrienes in dermatological diseases: potential therapeutic implications. *BioDrugs* 2001; **15**: 729-743
- 4 Funk CD. Leukotriene modifiers as potential therapeutics for cardiovascular disease. *Nat Rev Drug Discov* 2005; **4**: 664-672
- 5 Farzaneh-Far R, Moore K. Cysteinyl-leukotrienes and the liver. *Prostaglandins Other Lipid Mediat* 2003; **72**: 35-50
- 6 Bansal S, Dhawan A. Acute liver failure. *Indian J Pediatr* 2006; **73**: 931-934
- 7 MacQuillan G. Predicting outcome in acute liver failure: are we there yet? *Liver Transpl* 2007; **13**: 1209-1211
- 8 Stadlbauer V, Jalan R. Acute liver failure: liver support therapies. *Curr Opin Crit Care* 2007; **13**: 215-221
- 9 Galun E, Axelrod JH. The role of cytokines in liver failure and regeneration: potential new molecular therapies. *Biochim Biophys Acta* 2002; **1592**: 345-358
- 10 Newsome PN, Plevris JN, Nelson LJ, Hayes PC. Animal models of fulminant hepatic failure: a critical evaluation. *Liver Transpl* 2000; **6**: 21-31
- 11 Jalan R, Olde Damink SW, Hayes PC, Deutz NE, Lee A. Pathogenesis of intracranial hypertension in acute liver failure: inflammation, ammonia and cerebral blood flow. *J Hepatol* 2004; **41**: 613-620
- 12 O'Beirne JP, Chouhan M, Hughes RD. The role of infection and inflammation in the pathogenesis of hepatic encephalopathy and cerebral edema in acute liver failure. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 118-119
- 13 Shinoda M, Wakabayashi G, Shimazu M, Saito H, Hoshino K, Tanabe M, Morikawa Y, Endo S, Ishii H, Kitajima M. Increased serum and hepatic tissue levels of interleukin-18 in patients with fulminant hepatic failure. *J Gastroenterol Hepatol* 2006; **21**: 1731-1736
- 14 Song HL, Lu S, Liu P. Tumor necrosis factor-alpha induces apoptosis of enterocytes in mice with fulminant hepatic failure. *World J Gastroenterol* 2005; **11**: 3701-3709
- 15 Yamanouchi K, Eguchi S, Kamohara Y, Yanaga K, Okudaira S, Tajima Y, Kanematsu T. Glycine reduces hepatic warm ischaemia-reperfusion injury by suppressing inflammatory reactions in rats. *Liver Int* 2007; **27**: 1249-1254
- 16 Silverstein R. D-galactosamine lethality model: scope and limitations. *J Endotoxin Res* 2004; **10**: 147-162
- 17 Hagmann W, Denzlinger C, Keppler D. Role of peptide leukotrienes and their hepatobiliary elimination in endotoxin action. *Circ Shock* 1984; **14**: 223-235
- 18 Liu P, Kawada N, Mizoguchi Y, Morisawa S. Arachidonate metabolism in D-galactosamine or carbon tetrachloride-induced acute and chronic liver injuries in rats. *Gastroenterol Jpn* 1992; **27**: 624-631
- 19 Tiegs G, Wendel A. Leukotriene-mediated liver injury. *Biochem Pharmacol* 1988; **37**: 2569-2573
- 20 Hagmann W, Keppler D. Leukotriene antagonists prevent endotoxin lethality. *Naturwissenschaften* 1982; **69**: 594-595
- 21 Haeggstrom JZ, Wetterholm A. Enzymes and receptors in the leukotriene cascade. *Cell Mol Life Sci* 2002; **59**: 742-753
- 22 Lam BK. Leukotriene C(4) synthase. *Prostaglandins Leukot Essent Fatty Acids* 2003; **69**: 111-116
- 23 Jakobsson PJ, Mancini JA, Ford-Hutchinson AW. Identification and characterization of a novel human microsomal glutathione S-transferase with leukotriene C4 synthase activity and significant sequence identity to 5-lipoxygenase-activating protein and leukotriene C4 synthase. *J Biol Chem* 1996; **271**: 22203-22210
- 24 Jakobsson PJ, Mancini JA, Riendeau D, Ford-Hutchinson AW. Identification and characterization of a novel microsomal enzyme with glutathione-dependent transferase and peroxidase activities. *J Biol Chem* 1997; **272**: 22934-22939
- 25 Sjöström M, Jakobsson PJ, Juremalm M, Ahmed A, Nilsson G, Macchia L, Haeggström JZ. Human mast cells express two leukotriene C(4) synthase isoenzymes and the CysLT(1) receptor. *Biochim Biophys Acta* 2002; **1583**: 53-62
- 26 Schröder O, Sjöström M, Qiu H, Stein J, Jakobsson PJ,



- Haeggström JZ. Molecular and catalytic properties of three rat leukotriene C(4) synthase homologs. *Biochem Biophys Res Commun* 2003; **312**: 271-276
- 27 **Lam BK**, Penrose JF, Xu K, Baldasaro MH, Austen KF. Site-directed mutagenesis of human leukotriene C4 synthase. *J Biol Chem* 1997; **272**: 13923-13928
- 28 **Lam BK**, Austen KF. Leukotriene C4 synthase: a pivotal enzyme in cellular biosynthesis of the cysteinyl leukotrienes. *Prostaglandins Other Lipid Mediat* 2002; **68-69**: 511-520
- 29 **Kanaoka Y**, Maekawa A, Penrose JF, Austen KF, Lam BK. Attenuated zymosan-induced peritoneal vascular permeability and IgE-dependent passive cutaneous anaphylaxis in mice lacking leukotriene C4 synthase. *J Biol Chem* 2001; **276**: 22608-22613
- 30 **Ago H**, Kanaoka Y, Irikura D, Lam BK, Shimamura T, Austen KF, Miyano M. Crystal structure of a human membrane protein involved in cysteinyl leukotriene biosynthesis. *Nature* 2007; **448**: 609-612
- 31 **Martinez Molina D**, Wetterholm A, Kohl A, McCarthy AA, Niegowski D, Ohlson E, Hammarberg T, Eshaghi S, Haeggström JZ, Nordlund P. Structural basis for synthesis of inflammatory mediators by human leukotriene C4 synthase. *Nature* 2007; **448**: 613-616
- 32 **Schröder O**, Sjöström M, Qiu H, Jakobsson PJ, Haeggström JZ. Microsomal glutathione S-transferases: selective up-regulation of leukotriene C4 synthase during lipopolysaccharide-induced pyresis. *Cell Mol Life Sci* 2005; **62**: 87-94
- 33 **Yang SL**, Huang X, Chen HF, Xu D, Chen LJ, Kong Y, Lou YJ. Increased leukotriene c4 synthesis accompanied enhanced leukotriene c4 synthase expression and activities of ischemia-reperfusion-injured liver in rats. *J Surg Res* 2007; **140**: 36-44
- 34 **Scoggan KA**, Jakobsson PJ, Ford-Hutchinson AW. Production of leukotriene C4 in different human tissues is attributable to distinct membrane bound biosynthetic enzymes. *J Biol Chem* 1997; **272**: 10182-10187
- 35 **Sjöström M**, Jakobsson PJ, Heimbürger M, Palmblad J, Haeggström JZ. Human umbilical vein endothelial cells generate leukotriene C4 via microsomal glutathione S-transferase type 2 and express the CysLT(1) receptor. *Eur J Biochem* 2001; **268**: 2578-2586
- 36 **Samuelsson B**, Dahlen SE, Lindgren JA, Rouzer CA, Serhan CN. Leukotrienes and lipoxins: structures, biosynthesis, and biological effects. *Science* 1987; **237**: 1171-1176
- 37 **Titos E**, Claria J, Bataller R, Bosch-Marce M, Gines P, Jimenez W, Arroyo V, Rivera F, Rodes J. Hepatocyte-derived cysteinyl leukotrienes modulate vascular tone in experimental cirrhosis. *Gastroenterology* 2000; **119**: 794-805
- 38 **Takamatsu Y**, Shimada K, Chijiwa K, Kuroki S, Yamaguchi K, Tanaka M. Role of leukotrienes on hepatic ischemia/reperfusion injury in rats. *J Surg Res* 2004; **119**: 14-20
- 39 **Yang SL**, Chen LJ, Kong Y, Xu D, Lou YJ. Sodium nitroprusside regulates mRNA expressions of LTC4 synthesis enzymes in hepatic ischemia/reperfusion injury rats via NF-kappaB signaling pathway. *Pharmacology* 2007; **80**: 11-20
- 40 **Quiroga J**, Prieto J. Liver cytoprotection by prostaglandins. *Pharmacol Ther* 1993; **58**: 67-91
- 41 **Keppeler D**, Hagmann W, Rapp S. Role of leukotrienes in endotoxin action in vivo. *Rev Infect Dis* 1987; **9 Suppl 5**: S580-S584
- 42 **Pearson JM**, Bailie MB, Fink GD, Roth RA. Neither platelet activating factor nor leukotrienes are critical mediators of liver injury after lipopolysaccharide administration. *Toxicology* 1997; **121**: 181-189
- 43 **Shimada K**, Navarro J, Goeger DE, Mustafa SB, Weigel PH, Weinman SA. Expression and regulation of leukotriene-synthesis enzymes in rat liver cells. *Hepatology* 1998; **28**: 1275-1281
- 44 **Zaitu M**, Hamasaki Y, Matsuo M, Ichimaru T, Fujita I, Ishii E. Leukotriene synthesis is increased by transcriptional up-regulation of 5-lipoxygenase, leukotriene A4 hydrolase, and leukotriene C4 synthase in asthmatic children. *J Asthma* 2003; **40**: 147-154
- 45 **Serio KJ**, Johns SC, Luo L, Hodulik CR, Bigby TD. Lipopolysaccharide down-regulates the leukotriene C4 synthase gene in the monocyte-like cell line, THP-1. *J Immunol* 2003; **170**: 2121-2128
- 46 **Hagmann W**, Kaiser I, Jakschik BA. The sensitized liver represents a rich source of endogenous leukotrienes. *Hepatology* 1991; **13**: 482-488
- 47 **Fukai F**, Suzuki Y, Ohtaki H, Katayama T. Rat hepatocytes generate peptide leukotrienes from leukotriene A4. *Arch Biochem Biophys* 1993; **305**: 378-384
- 48 **Fukai F**, Suzuki Y, Nishizawa Y, Katayama T. Transcellular biosynthesis of cysteinyl leukotrienes by Kupffer cell-hepatocyte cooperation in rat liver. *Cell Biol Int* 1996; **20**: 423-428

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## Efficacy of current guidelines for the treatment of spontaneous bacterial peritonitis in the clinical practice

Stefania Angeloni, Cinzia Leboffe, Antonella Parente, Mario Venditti, Alessandra Giordano, Manuela Merli, Oliviero Riggio

Stefania Angeloni, Cinzia Leboffe, Antonella Parente, Mario Venditti, Manuela Merli, Oliviero Riggio, Department of Clinical Medicine, "Sapienza" University of Rome, Roma 00185, Italy

Alessandra Giordano, Department of Public Health, "Sapienza" University of Rome, Roma 00185, Italy

**Author contributions:** Angeloni S, Leboffe C, Venditti M and Parente A enrolled/collected the patients and collected and analysed the data; Giordano A performed bacteriological examinations; Angeloni S, Merli M and Riggio O designed the study and wrote the paper.

**Correspondence to:** Oliviero Riggio, MD, Department of Clinical Medicine, "Sapienza" University of Rome, Viale dell'Università 37, Roma 00185, Italy. [oliviero.riggio@uniroma1.it](mailto:oliviero.riggio@uniroma1.it)

Telephone: +39-64-9972001 Fax: +39-64-9972001

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evaluating its efficacy. The initial treatment with cefotaxime failed more frequently than expected. An increase in healthcare-related infections with antibiotic-resistant pathogens may explain this finding. A different first-line antibiotic treatment should be investigated.

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**Key words:** Spontaneous bacterial peritonitis; Cefotaxime; Antibiotic-resistant pathogens; Ascitic polymorphonuclear count; Cirrhosis

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### Abstract

**AIM:** To verify the validity of the International Ascites Club guidelines for treatment of spontaneous bacterial peritonitis (SBP) in clinical practice.

**METHODS:** All SBP episodes occurring in a group of consecutive cirrhotics were managed accordingly and included in the study. SBP was diagnosed when the ascitic fluid polymorphonuclear (PMN) cell count was  $> 250$  cells/mm<sup>3</sup>, and empirically treated with cefotaxime.

**RESULTS:** Thirty-eight SBP episodes occurred in 32 cirrhotics (22 men/10 women; mean age:  $58.6 \pm 11.2$  years). Prevalence of SBP, in our population, was 17%. Ascitic fluid culture was positive in nine (24%) cases only. Eleven episodes were nosocomial and 71% community-acquired. Treatment with cefotaxime was successful in 59% of cases, while 41% of episodes required a modification of the initial antibiotic therapy because of a less-than 25% decrease in ascitic PMN count at 48 h. Change of antibiotic therapy led to the resolution of infection in 87% of episodes. Among the cases with positive culture, the initial antibiotic therapy with cefotaxime failed at a percentage (44%) similar to that of the whole series. In these cases, the isolated organisms were either resistant or with an inherent insufficient susceptibility to cefotaxime.

**CONCLUSION:** In clinical practice, ascitic PMN count is a valid tool for starting a prompt antibiotic treatment and

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### INTRODUCTION

Spontaneous bacterial peritonitis (SBP) is a frequent and severe complication of cirrhotic patients with ascites<sup>[1]</sup>. It is defined as an infection of ascites in the absence of a contiguous source of infection, such as abdominal abscesses or intestinal perforations.

The prevalence of SBP in unselected, hospitalized, cirrhotic patients with ascites has been reported to range between 10% and 30%<sup>[1,2-6]</sup>. Following the first episode of SBP, the cumulative recurrence rate within one year of follow up is approximately 70%<sup>[7]</sup>.

In an initial series published in the 1970s, when SBP was first described, the mortality rate associated with an episode of SBP exceeded 80%<sup>[8]</sup>. This short-term prognosis has, however, considerably improved during the last decades. In more recent prospective studies, in fact, the mortality rate related to this complication was estimated to be around 20%-30%<sup>[9-11]</sup>. An early diagnosis and the promptness of an effective therapy are the most likely rea-

sons for this improvement in prognosis.

Symptoms of SBP may be insidious; in addition, by using conventional culture techniques, the ascitic fluid culture outcome is negative in up to 60% of patients with SBP. Since a rapid diagnosis and an early treatment have a crucial role, the antibiotic treatment cannot, therefore, be delayed to the moment when the microbiological results are available<sup>[12,13]</sup>.

In 2000, the International Ascites Club published the guidelines for the diagnosis and treatment of SBP in cirrhotic patients<sup>[12]</sup>. These guidelines suggested that a diagnosis of SBP should be based on polymorphonuclear (PMN) cell count in the ascitic fluid and that a PMN cell count greater than 250 cells/mm<sup>3</sup> should be considered highly suspicious of SBP, thus representing an indication to empirically initiate an antibiotic treatment. The gold standard treatment consists of third-generation cephalosporins, especially cefotaxime, given intravenously at a dose of 4-8 g/d for a minimum duration of 5 d. A repeat diagnostic paracentesis to document the response by a greater-than 25% decrease in ascitic fluid neutrophil count at 48 h after initiation of antibiotics is recommended. With this regimen, resolution of SBP is achieved in approximately 90% of patients and 30-d survival is at least 80%<sup>[12]</sup>. This recommendation is, however, based on the results of randomized controlled trials and its validity and applicability need to be verified in the clinical practice. Moreover, there have been suggestions that the type and etiology of bacterial infections in cirrhosis may have changed during recent years<sup>[14,15]</sup>. An increasing incidence of SBP caused by Gram-positive bacteria in cirrhotic patients with ascites has been observed by different authors<sup>[16,17]</sup>. In addition, an increased frequency of bacteria resistant to multiple antibiotics has been shown<sup>[18]</sup>. This may be due to the extensive use of quinolones, and, in particular, to the employment of norfloxacin for SBP prophylaxis, as well as an increasing use of invasive procedures for the complications of cirrhosis.

The recent changes in its microbial etiology may have several important implications for the management and treatment of SBP and suggest the need for verifying the efficacy of current guidelines.

The aim of our study was, therefore, to verify validity, applicability, and efficacy of the International Ascites Club guidelines for the treatment of SBP and assess the results of such approach in an unselected group of consecutive cirrhotic patients with SBP admitted to our Gastroenterology Unit during a three-year period (January 2004-January 2007).

## MATERIALS AND METHODS

### Materials

All the episodes of SBP occurring in cirrhotic patients admitted to our Gastroenterology Unit from January 2004 to January 2007 were managed according to the International Ascites Club guidelines<sup>[12]</sup> and included in the study.

The diagnosis of liver cirrhosis was based on clinical, biochemical, and/or histopathological data. The severity

of the liver disease was classified in each patient at entry according to the Child-Pugh's<sup>[19]</sup> and the model for end-stage liver disease's (MELD) scores<sup>[20]</sup>. The MELD score was assessed using the Mayo Clinic website calculator. The main cause of admission was recorded for each patient.

According to our routine clinical practice, a detailed medical history, a complete physical examination, standard laboratory tests (including a complete blood cell count, prothrombin time, biochemical tests of liver and kidney function, and fresh urine sediment), a chest x-ray film, a diagnostic paracentesis, and an ascitic fluid culture were performed in all the cirrhotic patients with ascites on the day of admission and whenever they developed symptoms and signs suspicious for SBP (i.e. fever, change in mental status, abdominal pain, peripheral leukocytosis, development of renal failure, hypotension, *etc.*) during hospitalization. In some patients re-admitted for recurrent ascites, a diagnostic paracentesis was also repeated.

The ascitic fluid samples were collected under aseptic conditions in tubes containing ethylenediamine tetraacetic acid anticoagulant and then tested to determine white blood cell (WBC) and PMN counts by automated cell blood counter (Technicon System H\*1; Bayer Diagnostics, Milan, Italy), as described elsewhere<sup>[21]</sup>. All the specimens were analyzed within one hour. Additional samples of ascitic fluid were collected for the determination of glucose, albumin, and total protein concentrations. Moreover, 10 mL of ascitic fluid were inoculated directly at the patient's bedside into aerobic and anaerobic blood culture bottles for bacteriological examination<sup>[22]</sup>. Bacterial identification and antimicrobial susceptibility testing were carried out by the VITEK2 system (bioMérieux SA, Marcy-l'Etoile, France). Double-disk synergy tests were used for the confirmation of extended-spectrum-lactamase (ESBL) producers.

### Patients' management

Those patients with a diagnosis of SBP (when the PMN cell count in the ascitic fluid was greater than 250 cells/mm<sup>3</sup>) were included in the study. Those with bacteriascites (i.e. positive ascitic fluid culture with < 250 neutrophils/mm<sup>3</sup>) or with clinical and laboratory data suggesting secondary peritonitis were excluded. All the patients were managed according to the International Ascites Club guidelines<sup>[12]</sup>. SBP was empirically treated with third-generation cephalosporins (intravenous cefotaxime, 2 g/8 h, for a minimum of 5 d), regardless of the positivity of the culture. The antibiotic dosage was adjusted to the renal function throughout the treatment period.

In those cases not responding to the initial antibiotic regimen, the therapy was appropriately changed, either according to the *in vitro* susceptibility of the isolated bacteria, or empirically. For this purpose, a further paracentesis was always performed 2 d after the beginning of the antibiotic treatment. Treatment failure was established when the condition of the patients rapidly deteriorated within the first hour of the antibiotic therapy (i.e. with development of shock), or when no significant decrease in the ascitic PMN count was observed in the follow-up paracentesis. A reduction in the PMN count of

less than 25% as compared with the pre-treatment value was considered as suggestive of failure of the antibiotic treatment.

Clinical signs and symptoms of infection (fever, abdominal pain, mental status change, hypotension, *etc.*) were recorded daily. Arterial pressure, heart rate, body temperature, and weight were measured daily. WBC count, serum urea, creatinine, and sodium levels were determined before the initiation of treatment, every 2 d during treatment, and 24 h after therapy completion.

Diuretics were routinely withdrawn at the time of diagnosis of SBP and therapeutic paracenteses were not allowed until the resolution of infection.

SBP was considered resolved when all the clinical signs of infection disappeared, the PMN count in the ascitic fluid decreased to less than 250 cells/mm<sup>3</sup>, total and differential WBC count normalized, and blood and ascitic fluid cultures were negative.

If signs or symptoms of infection developed after discontinuation of antibiotics, a paracentesis for PMN cell count was also repeated.

SBP was considered as “community-acquired” when it was present at admission, and as “nosocomial” when it developed during hospitalization in patients with a normal ascitic fluid at admission<sup>[14]</sup>.

SBP-related mortality was defined as a death caused by bacterial infection of the ascitic fluid, with clinical and bacteriologic evidence of uncontrolled infection.

After discharge from the hospital, most of the patients were followed as outpatients for continued care. All were prescribed norfloxacin for prophylaxis of SBP recurrence.

### Statistical analysis

Results are expressed as mean  $\pm$  SD. The differences between groups were determined by student's *t* test. The chi-square test was used when appropriate to determine the differences in proportions. The independent role of factors selected by the univariate analysis was assessed by stepwise regression analysis. The statistical significance was established at a *P* value of less than 0.05. Calculations were performed by using a statistical software program (Number Cruncher Statistical System 97, Kaysville, Utah, USA).

## RESULTS

From January 1, 2004 through December 31, 2006, 38 consecutive episodes of SBP occurred in 32 cirrhotic patients with ascites (22 men and 10 women, mean age 58.6-11.2 years) hospitalized in our Gastroenterology Unit.

In the same period, a total of 228 diagnostic paracenteses were performed in 129 cirrhotic patients with ascites consecutively admitted to our Unit. The prevalence of SBP, in our patient population, was therefore calculated as 17%.

Demographic and clinical characteristics of our patient population are reported in Table 1. The etiology of cirrhosis was alcoholic in 13 (41%) cases. All the patients had advanced cirrhosis with high serum bilirubin (9.5-10.5 mg/dL), low prothrombin activity (57%-18.8%), and high Child-Pugh's (10.2-1.9) and MELD's (19.4-8.5) scores.

SBP presented without symptoms and signs in most

**Table 1** Demographic, clinical, and biochemical characteristics of the patients (mean  $\pm$  SD)

	Patients
<i>n</i>	32
Age (yr)	58.6 $\pm$ 11.2
Gender (male/female)	22/10
Alcoholic origin (yes/no)	13/19
MELD score	19 $\pm$ 9
Child-Pugh class (A/B/C)	0/11/21
Bilirubin (mg/dL)	9.5 $\pm$ 10.5
Albumin (g/dL)	2.6 $\pm$ 0.5
Prothrombin time (%)	55.7 $\pm$ 18.8
Creatinine (mg/dL)	1.3 $\pm$ 0.5
Serum sodium (mEq/L)	133.4 $\pm$ 5.4
WBC (mm <sup>3</sup> )	8867.1 $\pm$ 6504

cases: at the time of hospital admission, fever was present in 12 cases, abdominal pain in six, and blood leukocyte counts were higher than 10 000/mm<sup>3</sup> in only 11 cases. At hospitalization, renal failure was recorded in 14 (37%) cases and hepatic encephalopathy in eight (21%).

In 16 of the 38 episodes, the presence of a risk factor for SBP occurrence was identified: there were seven cases with a previous episode of SBP (six of them were receiving antibiotic prophylaxis for SBP at inclusion), three patients had gastrointestinal bleeding, and six had undergone invasive procedures.

The ascitic fluid culture was positive in only nine (24%) of the 38 SBP episodes. The isolated organisms were Gram-negative bacilli in five cases (two *E. coli*, two *Klebsiella pneumoniae*, and one *Enterobacter*) and Gram-positive cocci in four (two *Enterococcus species*, one *Staphylococcus aureus*, and one *Streptococcus viridans*).

Eleven (29%) episodes were nosocomial and 71% were community-acquired. There was no significant difference in terms of resolution of infection and mortality between these two sub-groups.

Cefotaxime was used as an initial empirical therapy in 29 cases. In the remaining nine cases, a different antibiotic therapy was started for the following reasons: five developed SBP despite the antibiotic treatment with cephalosporins initiated for other reasons (i.e. as a prophylaxis before an invasive procedure or for bleeding), and four patients were allergic to cephalosporins. These nine patients were included only in the analysis for the identification of predictors of mortality.

The treatment was successful in 17 of the 29 episodes initially treated with cefotaxime (59%), while in 12 (41%) episodes the initial antibiotic therapy with cefotaxime was changed because of a less-than 25% decrease in the ascitic fluid of PMN cell count at paracentesis performed after 48 h. In these patients, the antibiotic therapy was changed to imipenem-cilastatin in six cases, piperacillin-tazobactam in two, ampicillin-sulbactam in two and amoxicillin-clavulanic acid in one. The last patient died 48 h after the beginning of the antibiotic therapy. In five cases, a further change in the antibiotic treatment was necessary.

Among the nine episodes of SBP in whom the culture of ascitic fluid was positive, the initial antibiotic therapy



with cefotaxime failed in four cases, a percentage (44%) similar to that of the whole series. In these four episodes, the isolated organisms were either resistant to cefotaxime (ESBL-positive *E. coli*, *Enterobacter*, and *Enterococcus*), or had an inherent insufficient susceptibility to cefotaxime (*Staphylococcus aureus*).

SBP resolved in 26/29 episodes initially treated with cefotaxime, while three patients died with signs of active infection. Two further deaths due to SBP occurred among the nine patients not treated initially with cefotaxime. At multivariate analysis, the only variables that showed an independent relationship with mortality for SBP were the presence, at entry, of renal failure (defined as an increase in serum urea and/or creatinine to greater-than 30 mg/dL or 1.2 mg/dL respectively) and a mean arterial pressure < 70 mmHg (R-squared = 0.35).

## DISCUSSION

The aim of our study was to verify the validity, applicability, and efficacy of the guidelines proposed in 2000 by the International Ascites Club for the treatment of SBP. The applicability in the clinical practice of a guideline derived from randomized controlled investigations is very important. Moreover, such analysis is also justified by the evidence that the type and etiology of bacterial infections in cirrhosis may have changed during recent years<sup>[14-18]</sup>. For this purpose, the results of patients' management according to these guidelines were evaluated in an unselected group of cirrhotic patients with SBP consecutively observed in the last three-year period.

Our study suggests that, in clinical practice, an approach to SBP based on ascitic fluid PMN cell count is correct and valid for starting the antibiotic treatment and evaluating its efficacy as well. On the other hand, the suggestion of using third-generation cephalosporins (cefotaxime) as the first-line antibiotic treatment is not equally valid, since a switch to another antibiotic was necessary in more than 40% of our cases.

Infections may frequently occur in patients with liver cirrhosis, especially when decompensated, and may be a cause of death *per se*; but, they can also act as a trigger for a number of severe complications, such as hepatic encephalopathy and renal failure<sup>[23]</sup>. Moreover, infection has been related to variceal bleeding both in terms of pathogenesis of portal pressure increment and severity of bleeding episodes<sup>[24,25]</sup>, since the related mortality was reduced by prompt antibiotic therapy<sup>[26]</sup>. SBP is one of the most frequent infections in patients with cirrhosis<sup>[6]</sup>. Fever, leukocytosis, and abdominal symptoms are rare (recorded in 20% only of our series); the identification of the infection of ascitic fluid is, therefore, based only on the result of the diagnostic paracentesis. A PMN cell count > 250 cell/mm<sup>3</sup> has been proposed as the most important parameter for the diagnosis of SBP, as we isolated responsible bacteria in the ascitic fluid culture very infrequently (recorded in only 24% of the episodes observed in our cohort). The low proportion of positive ascitic fluid cultures is probably due to the relatively low concentration of bacteria in the ascitic fluid as compared with the infections in other organic flu-

ids (e.g. urine)<sup>[12]</sup>. For the same reason, a therapy based on the isolation of the responsible bacteria is seldom achievable and the antibiotic treatment cannot be delayed to the moment when microbiological results are available<sup>[12,13]</sup>. In these conditions, the efficacy of the empiric antibiotic treatment can rarely be based on the amelioration of the symptoms or on microbiological results. Therefore, a reduction of PMN cell count below 250 cell/mm<sup>3</sup> or of 25% of the initial value has been suggested as the main criterion for establishing the efficacy of the antibiotic and the need for switching the therapy. Our study confirms the validity of such an approach. Based on PMN cell count, we were, in fact, able to identify the failure of the initial therapy on time, and the consequent change of the antibiotic therapy allowed us to control the infection in the majority of cases, with in-hospital mortality rate of less than 15%. This result is similar to that reported in the literature and is particularly good when considering the severe conditions of our patients.

In our study, the presence of arterial hypotension or renal failure at admission was the only independent predictor of mortality for SBP. To our knowledge, this observation is similar to the findings of other studies<sup>[27]</sup>. According to the 2000 Ascites Club guidelines the use albumin, as suggested by the paper of Sort *et al*<sup>[28]</sup>, was not included in our protocol for SBP management. Even the most recent guidelines for the prevention and treatment of HRS<sup>[29]</sup> suggest that albumin administration may reduce the incidence of renal failure and mortality in patients with SBP, but recommended further studies to define the optimal doses and the subgroup of patients for whom albumin is highly indicated. In our study, renal dysfunction was independently related with mortality and this finding supports the importance of improving the systemic hemodynamics and, thus, of renal function during the treatment of SBP.

According to the PMN cell count carried out at 48-h diagnostic paracentesis, cefotaxime-suggested as the first-line empiric antibiotic treatment-failed in more than 40% of SBP episodes. The need for changing antibiotic treatment is higher than that reported in previous studies<sup>[30-35]</sup>.

Although the cases with a positive culture were few (only 9 out of 29 patients), in these episodes the percentage of treatment failure of the initial therapy with cefotaxime was similar to that of the entire series (44%). In these patients, cefotaxime failed because the isolated organisms were intrinsically resistant to cefotaxime (as enterococci) or capable of degrading the expanded-spectrum cephalosporins (as ESBL-producing *E. coli* or Amp C  $\beta$ -lactamase producing *Enterobacter* species) or bacteria with a inherent insufficient susceptibility to cefotaxime (as *Staphylococcus aureus*). If this small subgroup is to be considered representative of the organisms currently involved in the development of SBP, our study supports the possibility that, in recent years, the microbial etiology of SBP is changing, as it seems to have more generally occurred for bacterial infections of these kinds of patients<sup>[14]</sup>. In our hospital, a recent survey<sup>[36]</sup> on 4769 samples collected for bacterial isolation from April to September 2006, showed a high prevalence of ESBL-producing enterobacteriaceae as an emergent cause of infections. In particular, among the ESBL-positive

*E. coli*, strains with CTX-M  $\beta$ -lactamases, specifically able to hydrolyze cefotaxime, were the most diffused. It is interesting to note that, although in the majority of our patients SBP was defined as “community-acquired” and nosocomial infections-defined as an infection of ascitic fluid diagnosed after a first negative ascitic fluid analysis-were not prevalent in the group resistant to cefotaxime, the above organisms are typically nosocomial. In other words, the episodes of SBP resistant to cefotaxime may be considered as healthcare-related infections<sup>[37]</sup>, probably due to the fact that compromised patients, as the cirrhotic patients included in the present study, have the frequent need of hospital assistance including outpatient visits, diagnostic invasive examinations, day-hospital admissions, *etc.*, which may facilitate contact with nosocomial antibiotic-resistant pathogens. These considerations should induce a change in our approach aimed not only at changing the first line antibiotic therapy in SBP, but also at reducing and making the patients’ access to the hospital more appropriate and rational.

In conclusion, an approach to SBP based on ascitic fluid PMN cell count is correct and valid in the clinical practice for both starting promptly the antibiotic treatment and evaluating its efficacy. However, the initial treatment with cefotaxime failed more frequently than expected. These results should promote investigations aimed at identifying different approaches. The antibiotics used for the empiric initial treatment should be chosen among those able to control infections which are often healthcare-related and thus sustained by antibiotic-resistant bacteria. The characteristics of bacterial infection in a given geographical area and community should be taken into account. Therefore, the generalization of our findings, which are derived by a monocentric study, deserves further investigations.

## COMMENTS

### Background

Spontaneous bacterial peritonitis (SBP) is a severe complication of cirrhotic patients with ascites. A prompt diagnosis and an early treatment are crucial for the outcome of these patients.

### Research frontiers

In 2000, the International Ascites Club published the guidelines for the diagnosis and treatment of SBP in cirrhotic patients. This recommendation is, however, based on the results of randomized controlled trials and its validity and applicability need to be verified in clinical practice.

### Innovations and breakthroughs

In agreement with the International Ascites Club guidelines, the study confirms the importance of performing a second paracentesis at 48 h after the initiation of the antibiotic therapy as well as the utility of the cut-off of PMN count in ascitic fluid for establishing treatment failure. The suggestion of using third-generation cephalosporins (cefotaxime) as the first-line antibiotic treatment is arguable, since a switch to another antibiotic was necessary in more than 40% of cases.

### Applications

The study supports the possibility that, in recent years, the microbial etiology of SBP is changing as well as the sensibility of pathogens to antibiotic therapy. The characteristics of bacterial infection in a given geographical area and community should, therefore, be taken into account and each hospital should performed studies on bacterial sensibility and design their antibiotic strategy for SBP.

## Peer review

It is a well-designed and well-written paper. The study adds important information on the management of cirrhotic patients with SBP.

## REFERENCES

- 1 Caly WR, Strauss E. A prospective study of bacterial infections in patients with cirrhosis. *J Hepatol* 1993; **18**: 353-358
- 2 Pinzello G, Simonetti RG, Craxi A, Di Piazza S, Spano C, Pagliaro L. Spontaneous bacterial peritonitis: a prospective investigation in predominantly nonalcoholic cirrhotic patients. *Hepatology* 1983; **3**: 545-549
- 3 Almdal TP, Skinhoj P. Spontaneous bacterial peritonitis in cirrhosis. Incidence, diagnosis, and prognosis. *Scand J Gastroenterol* 1987; **22**: 295-300
- 4 Llach J, Rimola A, Navasa M, Gines P, Salmeron JM, Gines A, Arroyo V, Rodes J. Incidence and predictive factors of first episode of spontaneous bacterial peritonitis in cirrhosis with ascites: relevance of ascitic fluid protein concentration. *Hepatology* 1992; **16**: 724-727
- 5 Gilbert JA, Kamath PS. Spontaneous bacterial peritonitis: an update. *Mayo Clin Proc* 1995; **70**: 365-370
- 6 Garcia-Tsao G. Bacterial infections in cirrhosis: treatment and prophylaxis. *J Hepatol* 2005; **42** Suppl: S85-S92
- 7 Tito L, Rimola A, Gines P, Llach J, Arroyo V, Rodes J. Recurrence of spontaneous bacterial peritonitis in cirrhosis: frequency and predictive factors. *Hepatology* 1988; **8**: 27-31
- 8 Conn HO, Fessel JM. Spontaneous bacterial peritonitis in cirrhosis: variations on a theme. *Medicine (Baltimore)* 1971; **50**: 161-197
- 9 Thuluvath PJ, Morss S, Thompson R. Spontaneous bacterial peritonitis--in-hospital mortality, predictors of survival, and health care costs from 1988 to 1998. *Am J Gastroenterol* 2001; **96**: 1232-1236
- 10 Llovet JM, Planas R, Morillas R, Quer JC, Cabre E, Boix J, Humbert P, Guilera M, Domenech E, Bertran X. Short-term prognosis of cirrhotics with spontaneous bacterial peritonitis: multivariate study. *Am J Gastroenterol* 1993; **88**: 388-392
- 11 Toledo C, Salmeron JM, Rimola A, Navasa M, Arroyo V, Llach J, Gines A, Gines P, Rodes J. Spontaneous bacterial peritonitis in cirrhosis: predictive factors of infection resolution and survival in patients treated with cefotaxime. *Hepatology* 1993; **17**: 251-257
- 12 Rimola A, Garcia-Tsao G, Navasa M, Piddock LJ, Planas R, Bernard B, Inadomi JM. Diagnosis, treatment and prophylaxis of spontaneous bacterial peritonitis: a consensus document. International Ascites Club. *J Hepatol* 2000; **32**: 142-153
- 13 Hoefs JC. Diagnostic paracentesis. A potent clinical tool. *Gastroenterology* 1990; **98**: 230-236
- 14 Fernandez J, Navasa M, Gomez J, Colmenero J, Vila J, Arroyo V, Rodes J. Bacterial infections in cirrhosis: epidemiological changes with invasive procedures and norfloxacin prophylaxis. *Hepatology* 2002; **35**: 140-148
- 15 Singh N, Wagener MM, Gayowski T. Changing epidemiology and predictors of mortality in patients with spontaneous bacterial peritonitis at a liver transplant unit. *Clin Microbiol Infect* 2003; **9**: 531-537
- 16 Cholongitas E, Papatheodoridis GV, Lahanas A, Xanthaki A, Kontou-Kastellanou C, Archimandritis AJ. Increasing frequency of Gram-positive bacteria in spontaneous bacterial peritonitis. *Liver Int* 2005; **25**: 57-61
- 17 Campillo B, Dupeyron C, Richardet JP, Mangeney N, Leluan G. Epidemiology of severe hospital-acquired infections in patients with liver cirrhosis: effect of long-term administration of norfloxacin. *Clin Infect Dis* 1998; **26**: 1066-1070
- 18 Park YH, Lee HC, Song HG, Jung S, Ryu SH, Shin JW, Chung YH, Lee YS, Suh DJ. Recent increase in antibiotic-resistant microorganisms in patients with spontaneous bacterial peritonitis adversely affects the clinical outcome in Korea. *J Gastroenterol Hepatol* 2003; **18**: 927-933
- 19 Pugh RN, Murray-Lyon IM, Dawson JL, Pietroni MC,

- Williams R. Transection of the oesophagus for bleeding oesophageal varices. *Br J Surg* 1973; **60**: 646-649
- 20 **Malinchoc M**, Kamath PS, Gordon FD, Peine CJ, Rank J, ter Borg PC. A model to predict poor survival in patients undergoing transjugular intrahepatic portosystemic shunts. *Hepatology* 2000; **31**: 864-871
- 21 **Angeloni S**, Nicolini G, Merli M, Nicolao F, Pinto G, Aronne T, Attili AF, Riggio O. Validation of automated blood cell counter for the determination of polymorphonuclear cell count in the ascitic fluid of cirrhotic patients with or without spontaneous bacterial peritonitis. *Am J Gastroenterol* 2003; **98**: 1844-1848
- 22 **Runyon BA**, Canawati HN, Akriviadis EA. Optimization of ascitic fluid culture technique. *Gastroenterology* 1988; **95**: 1351-1355
- 23 **Ruiz-del-Arbol L**, Urman J, Fernandez J, Gonzalez M, Navasa M, Monescillo A, Albillos A, Jimenez W, Arroyo V. Systemic, renal, and hepatic hemodynamic derangement in cirrhotic patients with spontaneous bacterial peritonitis. *Hepatology* 2003; **38**: 1210-1218
- 24 **Goulis J**, Patch D, Burroughs AK. Bacterial infection in the pathogenesis of variceal bleeding. *Lancet* 1999; **353**: 139-142
- 25 **Goulis J**, Armonis A, Patch D, Sabin C, Greenslade L, Burroughs AK. Bacterial infection is independently associated with failure to control bleeding in cirrhotic patients with gastrointestinal hemorrhage. *Hepatology* 1998; **27**: 1207-1212
- 26 **Bernard B**, Grange JD, Khac EN, Amiot X, Opolon P, Poynard T. Antibiotic prophylaxis for the prevention of bacterial infections in cirrhotic patients with gastrointestinal bleeding: a meta-analysis. *Hepatology* 1999; **29**: 1655-1661
- 27 **Follo A**, Llovet JM, Navasa M, Planas R, Forns X, Francitorra A, Rimola A, Gassull MA, Arroyo V, Rodes J. Renal impairment after spontaneous bacterial peritonitis in cirrhosis: incidence, clinical course, predictive factors and prognosis. *Hepatology* 1994; **20**: 1495-1501
- 28 **Sort P**, Navasa M, Arroyo V, Aldeguez X, Planas R, Ruiz-del-Arbol L, Castells L, Vargas V, Soriano G, Guevara M, Ginos P, Rodes J. Effect of intravenous albumin on renal impairment and mortality in patients with cirrhosis and spontaneous bacterial peritonitis. *N Engl J Med* 1999; **341**: 403-409
- 29 **Salerno F**, Gerbes A, Ginos P, Wong F, Arroyo V. Diagnosis, prevention and treatment of hepatorenal syndrome in cirrhosis. *Gut* 2007; **56**: 1310-1318
- 30 **Felisart J**, Rimola A, Arroyo V, Perez-Ayuso RM, Quintero E, Gines P, Rodes J. Cefotaxime is more effective than is ampicillin-tobramycin in cirrhotics with severe infections. *Hepatology* 1985; **5**: 457-462
- 31 **Rimola A**, Salmeron JM, Clemente G, Rodrigo L, Obrador A, Miranda ML, Guarner C, Planas R, Sola R, Vargas V. Two different dosages of cefotaxime in the treatment of spontaneous bacterial peritonitis in cirrhosis: results of a prospective, randomized, multicenter study. *Hepatology* 1995; **21**: 674-679
- 32 **Navasa M**, Follo A, Llovet JM, Clemente G, Vargas V, Rimola A, Marco F, Guarner C, Forne M, Planas R, Banares R, Castells L, Jimenez De Anta MT, Arroyo V, Rodes J. Randomized, comparative study of oral ofloxacin versus intravenous cefotaxime in spontaneous bacterial peritonitis. *Gastroenterology* 1996; **111**: 1011-1017
- 33 **Ricart E**, Soriano G, Novella MT, Ortiz J, Sabat M, Kolle L, Sola-Vera J, Minana J, Dedou JM, Gomez C, Barrio JL, Guarner C. Amoxicillin-clavulanic acid versus cefotaxime in the therapy of bacterial infections in cirrhotic patients. *J Hepatol* 2000; **32**: 596-602
- 34 **Runyon BA**, McHutchison JG, Antillon MR, Akriviadis EA, Montano AA. Short-course versus long-course antibiotic treatment of spontaneous bacterial peritonitis. A randomized controlled study of 100 patients. *Gastroenterology* 1991; **100**: 1737-1742
- 35 **Chen TA**, Lo GH, Lai KH, Lin WJ. Single daily amikacin versus cefotaxime in the short-course treatment of spontaneous bacterial peritonitis in cirrhotics. *World J Gastroenterol* 2005; **11**: 6823-6827
- 36 **Carattoli A**, Garcia-Fernandez A, Varesi P, Fortini D, Gerardi S, Penni A, Mancini C, Giordano A. Molecular epidemiology of *Escherichia coli* producing extended-spectrum beta-lactamases isolated in Rome, Italy. *J Clin Microbiol* 2008; **46**: 103-108
- 37 **Friedman ND**, Kaye KS, Stout JE, McGarry SA, Trivette SL, Briggs JP, Lamm W, Clark C, MacFarquhar J, Walton AL, Reller LB, Sexton DJ. Health care--associated bloodstream infections in adults: a reason to change the accepted definition of community-acquired infections. *Ann Intern Med* 2002; **137**: 791-797

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## Role of colonoscopy in patients with persistent acute diverticulitis

Adi Lahat, Henit Yanai, Emad Sakhnini, Yoram Menachem, Simon Bar-Meir

Adi Lahat, Henit Yanai, Emad Sakhnini, Yoram Menachem, Simon Bar-Meir, Department of Gastroenterology, Chaim Sheba Medical Center and Sackler School of Medicine, Tel Aviv University, Tel Aviv, Israel

**Author contributions:** Yanai H, Sakhnini E, Menachem Y performed supportive work; Lahat A and Bar-Meir S performed the research and wrote the paper.

**Correspondence to:** Simon Bar-Meir, MD, Department of Gastroenterology, Chaim Sheba Medical Center, Tel Hashomer 52651, Israel. [barmeirs@yahoo.com](mailto:barmeirs@yahoo.com)

Telephone: +972-3-5302679 Fax: +972-3-5303070

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**Peer reviewer:** Ian D Wallace, MD, Shakespeare Specialist Group, 181 Shakesperare Rd, Milford, Auckland 1309, New Zealand

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### Abstract

**AIM:** To identify patients with persistent acute diverticulitis who might benefit from an early colonoscopy during their first hospitalization.

**METHODS:** All patients hospitalized between July 2000 and December 2006 for acute diverticulitis who underwent colonoscopy were included in the study. Patients were followed during hospitalization and after discharge. Patients were considered to have a persistent course of acute diverticulitis if symptoms continued after 1 wk of conventional treatment with IV antibiotics, or if symptoms recurred within 2 mo after discharge. Patients were considered to benefit from an early colonoscopy if the colonoscopy was therapeutic or if it changed a patient's outcome.

**RESULTS:** Three hundred and six patients were hospitalized between July 2000 and December 2006 with the diagnosis of acute diverticulitis. Two hundred and twenty four of these were included in the study group. Twenty three patients (10.3%) fulfilled the criteria for a persistent course of acute diverticulitis. Of them, four patients (17.4%) clearly benefited from an early colonoscopy; these patients' clinical course is described. None of the patients with a regular non-persistent course demonstrated any benefit from colonoscopy.

**CONCLUSION:** Early colonoscopy detected other significant pathology, which accounted for the clinical presentation in 17% of patients with persistent acute diverticulitis. Therefore, we believe an early colonoscopy should be considered in all patients with a persistent clinical course.

### INTRODUCTION

Diverticular disease of the colon is a common condition in Western societies. The incidence rises with age. By the age of 85 two thirds of the population in developed countries will have developed colonic diverticula<sup>[1,2]</sup>. Most patients will remain asymptomatic. Only a minority will have complications. The most common complication is acute diverticulitis which occurs in 10%-25% of patients<sup>[2-6]</sup>. In most patients, the disease is mild, responds well to antibiotic therapy and does not recur<sup>[7]</sup>. However, there are patients who suffer from a persistent disease course. This relatively small group of patients does not respond well to conventional therapy, usually suffers from longer disease duration and might experience recurrent exacerbations. These patients often require a more aggressive therapeutic approach.

Colonoscopy is advised after an attack of acute diverticulitis in order to completely evaluate the colonic lumen and exclude a potential malignancy. However, it is a common practice to postpone colonoscopy until symptoms have fully subsided and perform it at least six wk after discharge, in order to avoid the potential risk of converting a sealed perforation into a free perforation<sup>[8-12]</sup>.

In two previous studies, we have shown that performing an early colonoscopy during hospitalization for acute diverticulitis is feasible and safe<sup>[13,14]</sup>. Early in-hospital colonoscopy was associated with better patient compliance, but a lower rate of cecal intubation. Patients with persistent diverticulitis seemed to benefit most from having early colonoscopy. The aim of this study was, therefore, to assess the yield of performing an early colonoscopy on patients with persistent acute diverticulitis, and to try to identify the subgroup of patients with persistent acute diverticulitis who might benefit from an early colonoscopy during their first hospitalization.



## MATERIALS AND METHODS

### Patients

All patients who were hospitalized between July 2000 and December 2006 for acute lower abdominal pain, fever above 38°C and leukocytosis above 12000, and who were suspected of having acute diverticulitis, were prospectively studied. All patients underwent abdominal CT and only those with CT findings compatible with acute diverticulitis were included in the study group. CT criteria for acute diverticulitis included the presence of colonic diverticuli, thickening of the colonic wall at the site of the diverticuli and peri-colic fat infiltration.

During hospitalization, patients were treated with IV antibiotics suitable to cover abdominal flora (usually ampicillin and garamycin) at the appropriate dosages.

Patients were considered to have a persistent course of acute diverticulitis if symptoms continued after 1 wk of conventional treatment with IV antibiotics, or if symptoms recurred within 1 mo after discharge.

All patients enrolled in the study underwent colonoscopy. Early colonoscopy was defined as colonoscopy performed during hospitalization on symptomatic patients or within 6 wk of the initial presentation. Late colonoscopy was defined as when the procedure was performed at least 6 wk after discharge, and when the patient was asymptomatic. The timing of the colonoscopy was based upon randomization protocols, as described in our previous two studies<sup>[13,14]</sup>.

All patients were closely followed during hospitalization and after discharge. Their follow-up after discharge was done by regular phone calls, conducted by the study coordinator as to their well being.

Patients enrolled in this study belong to a database of patients with acute diverticulitis. This database was used in our previous publications as well<sup>[13-15]</sup>.

Patients were considered to benefit from an early colonoscopy if the colonoscopy was therapeutic or if it changed the patient's outcome. Excluded from the study group were patients who refused to undergo colonoscopy (either early or late), patients with a history of colonoscopy within one year prior to the current episode of acute diverticulitis, patients operated on due to colonic perforation and patients lost to follow-up. The study was approved by the local ethics committee.

### Statistical analysis

Patients' characteristics were analyzed by the  $\chi^2$  test with Yates correction. Statistical analyses were conducted by using Statsoft software (Tulsa, OK, USA).

## RESULTS

Three hundred and six patients were hospitalized at the Chaim Sheba Medical Center between July 2000 and December 2006 with the diagnosis of acute diverticulitis. Eighty two patients did not undergo colonoscopy and, therefore, only 224 patients were included in the study group (Figure 1).

Twenty three patients (10.3%) fulfilled the criteria for a persistent course of acute diverticulitis. Fourteen patients underwent an early colonoscopy and the other

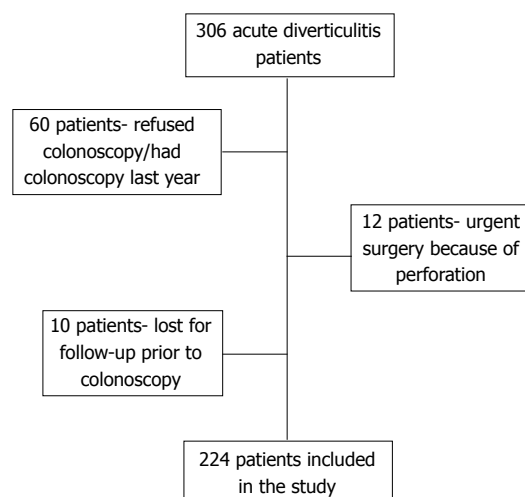


Figure 1 Study scheme.

Table 1 Beneficial and non-beneficial colonoscopies

Colonoscopy	Protracted course (n = 23)	Non-protracted course (n = 201)
Beneficial	4 (17.4%) <sup>b</sup>	0
Non-beneficial	19 (82.6%)	201

<sup>b</sup>P < 0.01 vs non-beneficial colonoscopies.

nine a late colonoscopy. Four patients (17.4%) clearly benefited from an early colonoscopy, whereas the remaining 19 patients underwent colonoscopy with no significant findings apart from the detection of diverticula and occasionally inflammation. Twelve of these patients eventually underwent sigmoidectomy because of recurrent episodes of diverticulitis, and their symptoms resolved. An additional two patients were offered sigmoidectomy, but refused. They continued to experience recurrent episodes of acute diverticulitis. The remaining five patients recovered completely with no further symptoms during a mean follow up period of 2.2 years. Interestingly, none of the patients with a regular non-persistent course demonstrated any benefit from colonoscopy (Table 1). Thus, colonoscopy was found to be beneficial only in patients with a persistent course of diverticulitis. The 4 patients in whom early colonoscopy seemed to be beneficial are described here.

### Patient No. 1

A 55-year-old man was admitted to another hospital 2 mo earlier with a clinical picture of acute diverticulitis. The patient was treated conservatively with IV antibiotics and was discharged after his symptoms subsided. Due to recurrent abdominal pain and fever he was readmitted to our hospital. In-hospital colonoscopy detected an obstructive mass in the sigmoid colon. Histology showed an adenocarcinoma. The patient underwent a sigmoidectomy.

### Patient No. 2

A 50-year-old woman was hospitalized due to left lower quadrant pain, fever and weight loss of 6 kg over a 4 mo period. CT was interpreted as compatible with acute

diverticulitis (Figure 2). After conservative treatment with IV antibiotics her symptoms improved and she was discharged. However, her symptoms did not resolve and she continued to have left lower quadrant pain. A colonoscopy, performed 2 mo later, revealed an obstructing mass in the sigmoid colon. Histology confirmed the diagnosis of adenocarcinoma. While waiting for her operation, she developed a free perforation and underwent emergency surgery with tumor resection and a temporary colostomy. Earlier colonoscopy could probably have enabled her to undergo elective surgery with primary closure of the colon.

#### **Patient No. 3**

A 60-year-old man was transferred from another hospital where he was hospitalized because of acute diverticulitis with an abscess in the left lower abdominal quadrant. The abscess was treated with IV antibiotics, but with no clinical response. A CT-guided drainage of the abscess was performed. Symptoms persisted, and the patient underwent colonoscopy. On colonoscopy, a 2-cm polyp was detected at the beginning of the inflamed area, 15 cm from the anus. Histology demonstrated a moderately differentiated adenocarcinoma arising in a tubulovillous adenoma. The tumor infiltrated the submucosa as well. No diverticuli were seen. The mucosa of the colonic segment between 15–30 cm looked irregular and biopsies showed nonspecific chronic inflammation. The patient underwent sigmoidectomy with resolution of his symptoms.

#### **Patient No. 4**

A 73-year-old woman was admitted with symptoms suggestive of acute diverticulitis. CT showed a thickened bowel wall of the sigmoid colon with multiple diverticula. The patient was treated with IV antibiotics for 10 d, with no improvement. On the 10th d of her hospitalization she underwent colonoscopy. On colonoscopy, a small chicken bone was found trapped in the diverticulum, with a purulent discharge from that diverticulum.

Removal of the bone during the colonoscopy produced complete recovery, and the patient was discharged asymptomatic and afebrile 1 d following the procedure.

## **DISCUSSION**

In this prospective study, we assessed the value of performing an early colonoscopy in a selective group of patients with a persistent course of acute diverticulitis.

Our data showed 17.4% of such patients will benefit from an early colonoscopy. We believe non-responders to conventional therapy form a special subgroup of patients with acute diverticulitis. These patients have a more severe disease and worse prognosis, as demonstrated by the fact that sixty-five percent of these patients eventually underwent a sigmoidectomy.

In our previous publications, we showed the main disadvantage of performing an early colonoscopy during hospitalization is the higher rate of incomplete examinations (18%)<sup>[13,14]</sup>. However, in this small but important group of patients with a persistent disease course, an early colonoscopy seemed to be beneficial. Therefore, the main



**Figure 2** Abdominal CT of patient No. 2. The arrow indicates the tumor misinterpreted as acute diverticulitis.

question is whether in cases of persistent course of acute diverticulitis the benefit of performing an early colonoscopy prevails over the risk of an incomplete examination. Identifying the patients from this group in whom a benefit is expected from an early colonoscopy would probably contribute to decision making. Therefore, in this study we focused on patients with a persistent course of acute diverticulitis in whom colonoscopy was beneficial. Three of the four patients (patient No. 1, 3, 4) were briefly mentioned in our previous publications<sup>[8,9]</sup>. In this paper we concentrated on those patients and added an additional patient to our series.

However, we were unable to determine predictors for the subgroup of patients who may benefit from early colonoscopy. The reasons for this might be the small number of patients involved, or, simply, because there are no such predictors.

Nevertheless, in the light of the significant findings in this small group of patients, we believe colonoscopy should be offered to all patients with a protracted disease course. However, it is not advocated for patients with a non-protracted course.

In conclusion, early colonoscopy should be considered in all patients with a protracted clinical course. In this small group of patients, 17% of the patients will benefit from the procedure with no additional risk of complications.

## **COMMENTS**

### **Background**

Patients with persistent acute diverticulitis have longer disease duration and do not respond to conventional therapy. In previous studies we showed that early in-hospital colonoscopy is feasible and safe in patients with acute diverticulitis.

### **Research frontiers**

This study was designed to investigate the benefit from performing an early colonoscopy during hospitalization in patients with persistent acute diverticulitis.

### **Applications**

Persistent acute diverticulitis affects about 10% of acute diverticulitis patients. This study showed that in this special group of patients, performing an early colonoscopy had a therapeutic benefit of 17%. Considering that performing an early colonoscopy during an attack of acute diverticulitis was shown to be safe in previous studies, this study suggests an early in-hospital colonoscopy should be considered in all patients with a persistent course of acute diverticulitis.

**Peer review**

In this prospective study, the authors focused on the yield of performing an early colonoscopy in a well defined group of patients with acute diverticulitis - patients who suffer from a persistent disease course. Study results suggest that in this small group of patients early colonoscopy will detect other significant pathology which will change patient's management in 17% of cases. Therefore, performing an early colonoscopy in patients with persistent diverticulitis is well advised.

**REFERENCES**

- 1 **Hughes LE**. Postmortem survey of diverticular disease of the colon. I. Diverticulosis and diverticulitis. *Gut* 1969; **10**: 336-344
- 2 **Garcia G**. Diverticulitis. In: Blaser MT, Smith DD, Ravdin JI. Eds. Infections of the gastrointestinal tract, 2nd ed. Philadelphia: Lippincott Williams & Wilkins, 2002: 306-316
- 3 **Parks TG**. Natural history of diverticular disease of the colon. *Clin Gastroenterol* 1975; **4**: 53-69
- 4 **Painter NS**, Burkitt DP. Diverticular disease of the colon, a 20th century problem. *Clin Gastroenterol* 1975; **4**: 3-21
- 5 **Farrell RJ**, Farrell JJ, Morrin MM. Diverticular disease in the elderly. *Gastroenterol Clin North Am* 2001; **30**: 475-496
- 6 **Wong WD**, Wexner SD, Lowry A, Vernava A 3rd, Burnstein M, Denstman F, Fazio V, Kerner B, Moore R, Oliver G, Peters W, Ross T, Senatore P, Simmang C. Practice parameters for the treatment of sigmoid diverticulitis--supporting documentation. The Standards Task Force. The American Society of Colon and Rectal Surgeons. *Dis Colon Rectum* 2000; **43**: 290-297
- 7 **Kaiser AM**, Jiang JK, Lake JP, Ault G, Artinyan A, Gonzalez-Ruiz C, Essani R, Beart RW Jr. The management of complicated diverticulitis and the role of computed tomography. *Am J Gastroenterol* 2005; **100**: 910-917
- 8 **Almy TP**, Howell DA. Medical progress. Diverticular disease of the colon. *N Engl J Med* 1980; **302**: 324-331
- 9 **Penfold JC**. Perforation of the colon complicating colonoscopy: report of a case. *Dis Colon Rectum* 1975; **18**: 626-627
- 10 **Forde KA**. Colonoscopy in complicated diverticular disease. *Gastrointest Endosc* 1977; **23**: 192-193
- 11 **Dean AC**, Newell JP. Colonoscopy in the differential diagnosis of carcinoma from diverticulitis of the sigmoid colon. *Br J Surg* 1973; **60**: 633-635
- 12 **Panish JF**. Limitations and complications of colonoscopy. *Gastrointest Endosc* 1980; **26**: 20S-21S
- 13 **Sakhnini E**, Lahat A, Melzer E, Apter S, Simon C, Natour M, Bardan E, Bar-Meir S. Early colonoscopy in patients with acute diverticulitis: results of a prospective pilot study. *Endoscopy* 2004; **36**: 504-507
- 14 **Lahat A**, Yanai H, Menachem Y, Avidan B, Bar-Meir S. The feasibility and risk of early colonoscopy in acute diverticulitis: a prospective controlled study. *Endoscopy* 2007; **39**: 521-524
- 15 **Lahat A**, Menachem Y, Avidan B, Yanai H, Sakhnini E, Bardan E, Bar-Meir S. Diverticulitis in the young patient--is it different? *World J Gastroenterol* 2006; **12**: 2932-2935

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## Precautions in caudate lobe resection: Report of 11 cases

Zeng-Qing Wen, Yi-Qun Yan, Jia-Mei Yang, Meng-Chao Wu

Zeng-Qing Wen, Yi-Qun Yan, Jia-Mei Yang, Meng-Chao Wu, First Department of Hepatic Surgery, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China

Author contributions: Wen ZQ, Yan YQ, Yang JM and Wu MC contributed equally to this work; Wu MC designed the study; Wen ZQ, Yan YQ, Yang JM performed the resection; Wen ZQ wrote the paper.

Correspondence to: Dr. Zeng-Qing Wen, First Department of Hepatic Surgery, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438,

China. [wenzq188@sina.com](mailto:wenzq188@sina.com)

Telephone: +86-21-25070770 Fax: +86-21-25070770

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### Abstract

**AIM:** To find the precautions against the safety in caudate lobe resection.

**METHODS:** The clinical data obtained from 11 cases of primary liver cancer in caudate lobe who received hepatectomy successfully were retrospectively analyzed. Four safe procedures were used in resection of primary liver cancer in caudate lobe: (1) selection of appropriate skin incision to obtain excellent exposure of operative field; (2) adequate mobilization of the liver to allow the liver to be displaced upwards to the left or to the right; (3) preparatory placement of tapes for total hepatic vascular exclusion, so that this procedure could be used to control the fatal bleeding of the liver when necessary; (4) selection of the ideal route for hepatectomy based on the condition of the tumor and the combined removal of multiple lobes if necessary. Among the 11 cases, simple occlusion of vessels of porta hepatis was used in caudate lobectomy for 6 cases, while in the other cases, the vessels were intermittently occluded several times or total hepatic vascular isolation was used in the caudate lobectomy. Combined partial right hepatectomy was done for 3 cases, combined left lateral lobectomy for 2 cases and caudate lobectomy alone for 6 cases.

**RESULTS:** Operation was successful for all the 11 cases. Intermittent inflow occlusion was performed for all patients for 15 min at 5-min intervals. Blockade was performed twice in 3 patients and total hepatic vascular exclusion was performed in one of the three patients. Blockade was performed three times in one patient, including a total hepatic vascular exclusion. Total hepatic vascular exclusion was performed only in one patient. The mean blood loss was 300 mL. Ascites and pleural effusion occurred in 4 patients, jaundice in 1 patient. Six

patients died of tumor recurrence in 6, 11, 12, 13, 15, 19 mo after operation, respectively. The other 5 patients have survived more than 16 mo since the operation.

**CONCLUSION:** Caudate lobectomy for liver cancer in candidate lobe can be safely performed with the above procedures.

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**Key words:** Caudate lobe; Primary liver cancer; Hepatectomy; Porta hepatis; Vascular exclusion

**Peer reviewer:** Leonidas G Koniaris, Professor, Alan Livingstone Chair in Surgical Oncology, 3550 Sylvester Comprehensive Cancer Center (310T), 1475 NW 12th Ave, Miami, FL 33136, United States

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### INTRODUCTION

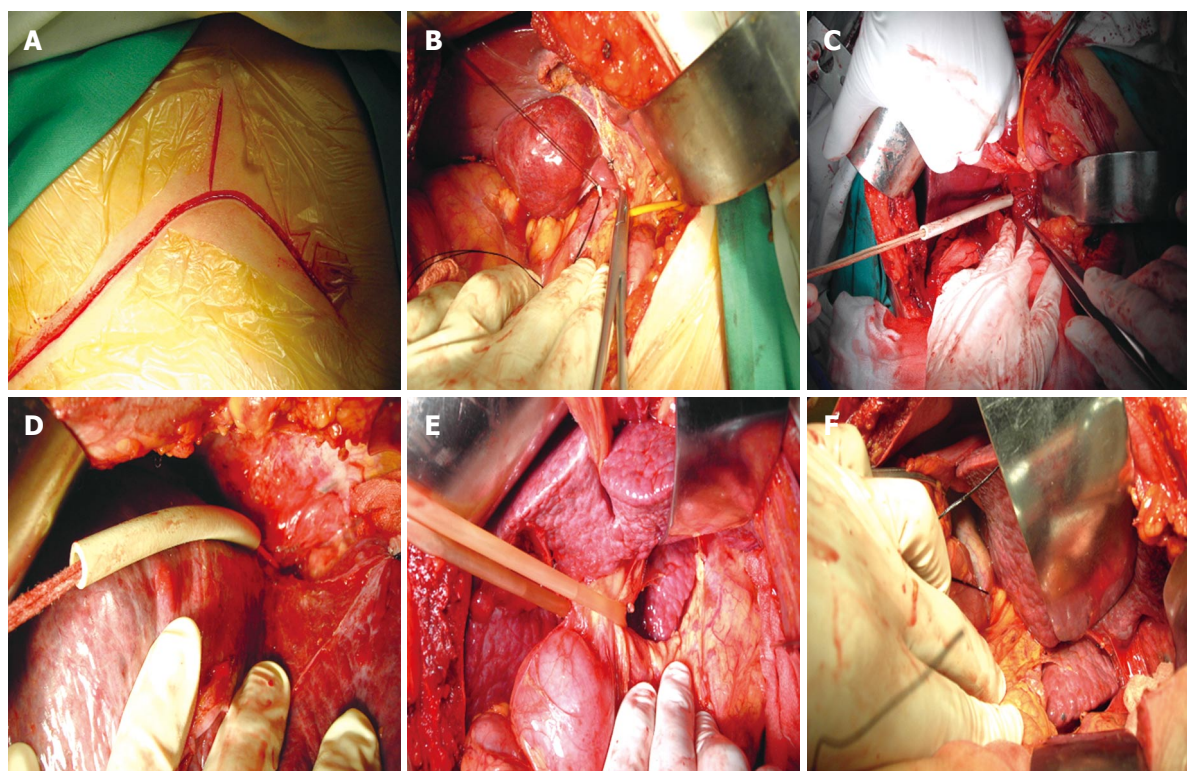
Caudate lobe is the first segment of liver in the Couinaud's classification<sup>[1-3]</sup>, and because of its unique anatomical site, its resection has a high risk<sup>[4-7]</sup>. If appropriate surgical procedures are used, it can be safely and successfully resected<sup>[8]</sup>.

### MATERIALS AND METHODS

#### Patients

From 2003 to 2005, four safe procedures were used in resection of primary liver cancer in caudate lobe. Eleven cases of primary liver cancer in caudate lobe received resection successfully at the First Department of Hepatic Surgery of the Eastern Hepatobiliary Surgery Hospital, Second Military Medical University. Among the 11 cases, 9 were males and 2 females (age range, 32-65 years, and mean age 53 years). All the patients were diagnosed with primary liver cancer. Nine patients were positive for hepatitis B surface antigen and their liver cirrhosis was confirmed by histologic examination of the resected specimens. The primary liver cancer was originated from the caudate lobe in 9 cases and from the adjacent lobe in 2 cases. Three cases received liver lobe resection ago. Nine cases were diagnosed with hepatocellular carcinoma and 2 cases with biliary cell carcinoma.





**Figure 1** A reversed T-shaped skin incision (A), ligated short hepatic veins (B), tapes around the infrahepatic IVC (C), tapes around the suprahepatic IVC (D), hepatic hilum pulled aside (E), inversed left lobe (F). IVC: Inferior vena cava.

### Surgical procedures

**Skin incision:** A reversed T-shaped skin incision was made (bilateral oblique incision under costal arch) (Figure 1A). The costal arch was hauled and secured with a special liver hook. The whole abdominal cavity was explored to rule out intra-abdominal metastasis.

**Dividing ligaments:** The falciform ligament was divided up to the front of the suprahepatic inferior vena cava (IVC), then the coronary, triangular, hepatorenal and hepatogastric ligaments were cut off, making the whole liver moveable.

**Dissecting blood vessels of caudate lobe:** The liver was pulled upward to exposure the infrahepatic IVC. The short hepatic veins of the caudate lobe were dissected and ligated (Figure 1B). Three thin and short hepatic veins were divided. The caudate portal triads were ligated and divided. Thus, the caudate lobe was isolated completely. The caudate lobe was detached from the neighboring liver parenchyma where large branches must be ligated if there are any.

**Preparing tapes:** As a safety precaution, tapes were preplaced around the infrahepatic and suprahepatic IVC (total hepatic vascular exclusion) (Figure 1C and 1D).

**Pringle maneuver:** The resection was begun with the occlusion of the first hepatic hilum (Pringle maneuver). The hepatic hilum was pulled aside to obtain the excellent exposure of caudate lobe (Figure 1E). Total vascular exclusion was used only when the patient had laceration

of the infra vena cava or liver vein. Such a maneuver could achieve hemodynamic instability.

**Resection:** In our series, the left lobe was removed in 9 patients and the liver was inversed without removing the left lobe in 9 patients (Figure 1F), the right lobe was removed in 2 patients. The anterior entry approach was not used in any patients. The caudate lobe was resected in 6 patients. However, additional right hemihepatectomy was performed for 3 patients and left hemihepatectomy for 2 patients. The left lateral lobe or the right lateral lobe was resected to release the space, and then the caudate lobe was resected.

### RESULTS

Intermittent inflow occlusion at the hepatoduodenal ligament was performed for all patients for 15 min at 5-min intervals. Six patients finished the operation. Blockade was performed twice in 3 patients and total hepatic vascular exclusion was performed in one of the three patients. Blockade was performed three times in one patient, including a total hepatic vascular exclusion. Total hepatic vascular exclusion was performed only in one patient. Among the patients undergoing total hepatic vascular exclusion, the infra vena cava was repaired in one patient, the liver vein was repaired in one patient, and the caudate lobe was resected with the right suprarenal gland in one patient.

It was reported that there are more intraoperative blood loss, longer operation time and more postoperative

complications in resection of the caudate lobe than for other lobes<sup>[9,10]</sup>. In our study, blood loss varied from 100 to 800 mL. The mean blood loss was 300 mL. Ascites and pleural effusion occurred in 4 patients, jaundice in 1 patient. Two patients with severe cirrhosis were cured and drainage occurred in the right thoracic cavity of 2 patients with hydrothorax.

Intrahepatic recurrence was noted in 5 patients and lung metastasis was found in one patient. The 6 patients died of tumor recurrence 6, 11, 12, 13, 15, 19 mo after operation, respectively. The other 5 patients have survived more than 16 mo since the operation.

## DISCUSSION

### *Unique anatomy and resection risk of caudate lobectomy*

The first segment of caudate lobe is divided into three sub-segments: Spiegel lobe, paracaval portion, and caudate process<sup>[11,12]</sup>. The Spiegel lobe is located behind the lesser omentum, just to the left of intrahepatic IVC. The paracaval portion is in front of the intrahepatic IVC, just to the right of the Spiegel lobe, and is closely attached to the right and middle hepatic veins. The caudate process is a tongue-like projection between the IVC and the adjacent portal vein, just to the right of the paracaval portion.

There are three porta hepatis in a liver<sup>[13,14]</sup>. The first porta hepatis denotes the hilum in a general sense; the second porta hepatis denotes the confluence of major hepatic veins, and the third porta hepatis denotes the segment of retrohepatic IVC with a series of short hepatic veins. Caudate lobe is, thus, surrounded by the three porta hepatis, all of which consist of important and potentially dangerous structures in terms of performing operation<sup>[15,16]</sup>. In view of the unique anatomical location, caudate lobe resection is technically challenging, especially for isolated caudectomy because it is easy to damage the hilum and bile duct in dissecting the anterior of caudate lobe, the inferior vena cava and cause uncontrolled bleeding when the posterior of caudate lobe is dissected as well as most difficult to remove the tumor near the protruded portion or inferior vena cava portion.

### *Clinical significance of caudate lobectomy*

Carcinoma, originating from the caudate lobe, cannot be treated effectively with trans-arterial chemotherapy embolization (TACE)<sup>[17,18]</sup>, because the supply vessels of caudate lobe are different from those of other lobes. Caudate lobe has many supply blood vessels from the right and left portal veins and hepatic arteries, many veins outflow the caudate lobe into the inferior vena cava and hepatic veins. It is not easy to completely embolize so many arteries. Percutaneous ethanol injection therapy (PEIT)<sup>[19]</sup> and percutaneous radiofrequency ablation (PRFA)<sup>[20]</sup> are also limited in the treatment of caudate lobe tumor, because injection or penetration can easily damage the adjacent blood vessels or other important structures. Therefore, caudate lobectomy may be the only effective method to cure the cancer.

### *Safe surgical precautions*

**Surgical approach to caudate lobe:** The choice

of procedures is essential to the success of caudate lobectomy<sup>[21]</sup>. There are different procedures of caudate lobectomy, but only three are effective. (1) Anterior procedure: resection of the hepatic parenchyma from the middle line with the right and left porta hepatis pulled aside to expose the caudate lobe. This method is complicated and causes huge injuries. We do not consider anterior procedure in caudate lobectomy resection. (2) Right procedure: resection of the 6 segments to expose the caudate lobe. This procedure resects more normal tissues. It is thus, not suitable for cirrhosis. If partial right lobe is not resected, it is difficult to dissect the protruded right portion of caudate lobe. (3) Left procedure: if the carcinoma is close to the left and upside of the lobe, resection of the left lateral lobe can expose the caudate lobe. It is better not to dissect the left lateral lobe. Otherwise, it causes liver failure if cirrhosis is severe. After the left lateral lobe is removed and the liver is inversed to the right and upwards, it is easy to perform the procedure. This procedure is commonly used to resect the caudate lobe.

**Preparation of infra vena cava occlusion belt:** Preparation of infra vena cava belt is to block intraoperative vital bleeding caused by laceration of the infra vena cava and hepatic vein<sup>[22]</sup>. If bleeding occurs, the mortality would be high. We think that infra vena cava block belt should be prepared for all patients<sup>[23]</sup>. In practice, only few patients need infra vena cava block<sup>[24]</sup>. We stress that infra vena cava block belt secures the safety of operation. When total hepatic vascular exclusion is used, the block time should be controlled. The authors think that the preferable block time is less than 15 min in order to reduce complications after operation, because patients with liver cirrhosis have insufficient liver function.

### *Repair of laceration of infra vena cava or hepatic vein:*

Once much blood emerges through the wound, laceration of the infra vena cava is diagnosed<sup>[25]</sup>. We should make a prompt decision without any hesitation to perform total hepatic vascular exclusion, in order to remove the tumor quickly and repair the laceration. However, if the laceration is small and can be located, it is not necessary to perform total hepatic vascular exclusion. We can use the finger press method to stanch bleeding and repair the vessels quickly<sup>[26]</sup>.

## COMMENTS

### *Background*

Primary liver cancer originating from the caudate lobe is not rare. It is difficult to remove it because of its unique anatomical location. We presented a safe technique of caudate lobectomy for liver cancer.

### *Applications*

Caudate lobectomy can be safely performed when appropriate skin incision is made, liver ligaments are adequately cut off, tapes for total hepatic vascular exclusion are placed and the ideal section route is selected.

### *Peer review*

This is an interesting paper describing caudate lobe resection and the precautions against the safety in caudate lobe resection. The authors suggest that caudate lobectomy for liver cancer in caudate lobe can be safely performed when proper procedures are used.

## REFERENCES

- 1 **Wu MC.** Liver surgery. Shanghai: Scientific and technical documents publishing house, 2000: 14-15
- 2 **Niu CX, Li CL, Yang Y.** Applied anatomy of the hepatic caudate lobectomy. *Zhongguo Linchuang Jiepouxue Zazhi* 2001; **19**: 151-154
- 3 **Couinaud C.** Surgical Anatomy of the Liver Revisited. Paris, France: Couinaud, 1989: 123-134
- 4 **Fan J, Wu ZQ, Tang ZY, Zhou J, Qiu SJ, Ma ZC, Zhou XD, Yu YQ.** Complete resection of the caudate lobe of the liver with tumor: technique and experience. *Hepatogastroenterology* 2001; **48**: 808-811
- 5 **Pol B, Campan P, Hardwigsen J, Botti G, Pons J, Le Treut YP.** Morbidity of major hepatic resections: a 100-case prospective study. *Eur J Surg* 1999; **165**: 446-453
- 6 **Hu JX, Miao XY, Zhong DW, Dai WD, Liu W.** Anterior approach for complete isolated caudate lobectomy. *Hepatogastroenterology* 2005; **52**: 1641-1644
- 7 **Shimada M, Matsumata T, Maeda T, Yanaga K, Taketomi A, Sugimachi K.** Characteristics of hepatocellular carcinoma originating in the caudate lobe. *Hepatology* 1994; **19**: 911-915
- 8 **Fukada T, Kimura F, Takayasiki T, Ito H, Shimizu H, Togawa A, Ohtsuka M, Yoshidome H, Kato A, Miyazaki M.** Anterior transhepatic approach for hepatocellular carcinoma located in deep positions of segment VIII. *Hepatogastroenterology* 2007; **54**: 536-538
- 9 **Tanaka S, Shimada M, Shirabe K, Maehara S, Tsujita E, Taketomi A, Maehara Y.** Surgical outcome of patients with hepatocellular carcinoma originating in the caudate lobe. *Am J Surg* 2005; **190**: 451-455
- 10 **Li AJ, Zhou WP, Wu MC, Luo XJ.** Hepatectomy after primary repair of ruptured liver cancer. *Hepatobiliary Pancreat Dis Int* 2007; **6**: 267-270
- 11 **Bartlett D, Fong Y, Blumgart LH.** Complete resection of the caudate lobe of the liver: technique and results. *Br J Surg* 1996; **83**: 1076-1081
- 12 **Kumon M.** Anatomy of the caudate lobe with special reference to the portal vein and bile duct. *Acta Hepatol Jpn* 1985; **26**: 1193-1199
- 13 **Shibata T, Maetani Y, Ametani F, Kubo T, Itoh K, Konishi J.** Efficacy of nonsurgical treatments for hepatocellular carcinoma in the caudate lobe. *Cardiovasc Intervent Radiol* 2002; **25**: 186-192
- 14 **Wang Y, Chen Han, Sun YF, Wei GT, Wu MC.** Discussion of the surgical method of hepatic caudate lobe. *Zhongguo Putong Waiké Zazhi* 2005; **20**: 36-40
- 15 **Xing X, Li H, Liu WG.** Clinical studies on inferior right hepatic veins. *Hepatobiliary Pancreat Dis Int* 2007; **6**: 579-584
- 16 **Peng SY, Liu YB, Xu B, Cai XJ.** Role and significance of extrahepatic control of hepatic vein and inferior vena cava in difficult hepatectomies for patients with liver tumor (in Chinese). *Zhonghua Waiké Zazhi* 2004; **42**: 260-264
- 17 **Shibata T, Maetani Y, Ametani F, Kubo T, Itoh K, Konishi J.** Efficacy of nonsurgical treatments for hepatocellular carcinoma in the caudate lobe. *Cardiovasc Intervent Radiol* 2002; **25**: 186-192
- 18 **Yamamoto T, Hirohashi K, Kubo S, Uenishi T, Ogawa M, Hai S, Sakabe K, Tanaka S, Shuto T, Tanaka H.** Hepatectomy with transcatheter arterial embolization for large hepatoma in the caudate lobe. *Hepatogastroenterology* 2003; **50**: 2173-2175
- 19 **Matsumoto T, Iwaki K, Hagino Y, Kawano K, Kitano S, Tomonari K, Matsumoto S, Mori H.** Ethanol injection therapy of an isolated bile duct associated with a biliary-cutaneous fistula. *J Gastroenterol Hepatol* 2002; **17**: 807-810
- 20 **Yamakado K, Nakatsuka A, Akeboshi M, Takaki H, Takeda K.** Percutaneous radiofrequency ablation for the treatment of liver neoplasms in the caudate lobe left of the vena cava: electrode placement through the left lobe of the liver under CT-fluoroscopic guidance. *Cardiovasc Intervent Radiol* 2005; **28**: 638-640
- 21 **Peng SY, Li JT, Mou YP, Liu YB, Wu YL, Fang HQ, Cao LP, Chen L, Cai XJ, Peng CH.** Different approaches to caudate lobectomy with "curettage and aspiration" technique using a special instrument PMOD: a report of 76 cases. *World J Gastroenterol* 2003; **9**: 2169-2173
- 22 **Smyrniotis V, Farantos C, Kostopanagiotou G, Arkadopoulos N.** Vascular control during hepatectomy: review of methods and results. *World J Surg* 2005; **29**: 1384-1396
- 23 **Dixon E, Vollmer CM Jr, Bathe OF, Sutherland F.** Vascular occlusion to decrease blood loss during hepatic resection. *Am J Surg* 2005; **190**: 75-86
- 24 **Zhou WP, Wu MC, Chen Han, Yao XP, Yang GS, Wu BW.** Resection of the hepatic caudate lobe. *Zhonghua Gandan Waiké Zazhi* 2001; **7**: 43-44
- 25 **Chao J, Tang WM, Ou QJ.** Surgical management of posterohepatic vena cava injury during hepatectomy. *Zhonghua Gandan Waiké Zazhi* 2002; **8**: 352-353
- 26 **Li A, Wu M, Yang G, Chen H, Shen F.** [Management of retrohepatic inferior vena cava injuries in hepatectomy for neoplasm]. *Zhonghua Waiké Zazhi* 1999; **37**: 14-17

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## Protective effect of inactivated hepatitis A vaccine against the outbreak of hepatitis A in an open rural community

Yue-Gen Shen, Xie-Jun Gu, Jian-Hong Zhou

Yue-Gen Shen, Xie-Jun Gu, Jian-Hong Zhou, Xiuzhou District Center for Disease Control and Prevention, Jiaxing 314001, Zhejiang Province, China

Author contributions: Shen YG designed the research; Shen YG, Gu XJ, and Zhou JH performed the research; Gu XJ analyzed the data; Shen YG wrote the paper.

Correspondence to: Yue-Gen Shen, Xiuzhou District Center for Disease Control and Prevention, Jiaxing 314001, Zhejiang Province, China. [jxshenyuegen@126.com](mailto:jxshenyuegen@126.com)

Telephone: +86-573-82050505 Fax: +86-573-82051498

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### Abstract

**AIM:** To evaluate the protective effect of inactivated hepatitis A vaccine (Healive®) against hepatitis A outbreak in an emergency vaccination campaign.

**METHODS:** During an outbreak of hepatitis A in Honghe Town, Xiuzhou District, Jiaxing City, Zhejiang Province, two nonrandomized controlled trials were conducted in September 2006. The first trial was to vaccinate 108 anti-HAV negative individuals with close contacts of the patients from September with 1 dose of an inactivated hepatitis A vaccine, Healive®. The control group comprised of 115 individuals with close contacts of the patients before September. The second trial was to vaccinate 3365 primary and secondary school students who volunteered to receive a dose of Healive® and 2572 students who did not receive Healive® serving as its controls. An epidemiological survey was conducted to evaluate the protective efficacy of the vaccine.

**RESULTS:** A total of 136 hepatitis A cases were reported during an outbreak that started in June, peaked in August and September, and ended after December of 2006. After a massive vaccination of school children in September, the number of cases declined significantly. No hepatitis A was detected in the 108 vaccinated individuals with close contacts of patients, whereas 4 cases of hepatitis A were found in the controls. The infection rate of hepatitis A was not significantly different in the individuals with close contacts of patients whether or not they received the vaccine ( $P = 0.122$ ). No hepatitis A was detected in the 3365 students who received the vaccine, four cases of hepatitis A were found in the controls. The infection rate of students with or without vaccination was significantly different in the students who received the vaccine (0/3365 vs 4/2572,  $P = 0.035$ ). The protective efficacy of the vaccine was 100%.

**CONCLUSION:** Inactivated hepatitis A vaccine demonstrates a good protective effect against an outbreak of hepatitis A.

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**Key words:** Hepatitis A; Outbreak; Inactivated hepatitis A vaccine; Emergency vaccination; Protective efficacy

**Peer reviewers:** Jaime Guardia, Professor, Internal Medicine and Liver Unit, Hospital Universitari 'Vall d'Hebron', Universitat Autònoma de Barcelona, Barcelona 08035, Spain; Yasuji Arase, Dr, Department of Gastroenterology, Toranomon Hospital, Tokyo 105-8470, Japan

Shen YG, Gu XJ, Zhou JH. Protective effect of inactivated hepatitis A vaccine against the outbreak of hepatitis A in an open rural community. *World J Gastroenterol* 2008; 14(17): 2771-2775 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2771.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2771>

### INTRODUCTION

Hepatitis A is an acute, usually self-limited disease of the liver caused by hepatitis A virus (HAV). Although it is often a benign infection, up to 15% of the infected persons have a protracted relapsing disease course lasting up to 6 mo<sup>[1]</sup>. Hepatitis A infection may lead to acute liver failure with a mortality rate of 0.43‰-0.58‰ in 2005 and 2006 according to China Center for Disease Control and Prevention<sup>[2]</sup>. An estimated 1.5 million clinical cases of hepatitis A occur globally each year. Since HAV is transmitted from person to person, primarily through the faecal-oral route, the incidence of hepatitis A is closely related to the socioeconomic development and hygienic conditions. In China, the incidence of hepatitis A was above 100/100 000 in the 1980s. With the economic growth in the past decade, especially with the usage of live attenuated hepatitis A vaccine and inactivated hepatitis A vaccine<sup>[3,4]</sup>, its incidence has decreased from 20/10 000 in 1996 to 5/10 000 in 2006<sup>[5]</sup>. Local outbreaks, however, are often reported in small cities and rural areas. In a previous study to investigate the prophylactic use of attenuated hepatitis A vaccine during an outbreak in a village, the live vaccine did not show protective effect, as the infection rate was not significantly different between the vaccinated and control groups<sup>[6]</sup>. The reason for this might be that the antibody induction period of the attenuated hepatitis A



vaccine is relatively long and the seroconversion reaches a peak 2 or 3 mo after injection<sup>[7]</sup>. In contrast, the antibody induction period of inactivated hepatitis A vaccine is as short as 2 wk<sup>[8]</sup>, and the seroconversion reaches a peak one month after delivery<sup>[9]</sup>. It is generally recognized that inactivated hepatitis A vaccine could provide good protection against hepatitis A after exposure to HAV<sup>[10,11]</sup>. Until now, the protective efficacy of inactivated hepatitis A vaccine has not been documented as an emergency vaccination in Chinese population.

Honghe Town, which belongs to the Xiuzhou District, Jiaxing City, Zhejiang Province, is an open rural community in southeastern China. Hepatitis A has been periodically epidemic in this area before vaccines were commonly used, and its incidence has been brought down to the national level with the introduction of hepatitis A vaccination programs. A serological survey in 2002 showed that the anti-HAV positive rate is 67.13% in this area<sup>[12]</sup>. Great changes have taken place in recent years, as the number of migrants now exceeds that of the local population. Many immigrants live in cramped dwellings and have limited access to appropriate hygienic conditions. Their sewage is drained directly into farmlands and streams without any proper treatment. Moreover, food safety is not guaranteed, as many people get food from unauthorized street stands. Infectious diseases are, thus, very likely to develop and get circulated.

Honghe Town consists of 9 villages with a population of 59700, including a local population of 27000 and a migrant population of 32700. In August and September 2006, a hepatitis A outbreak was detected by the Xiuzhou District Center for Disease Control and Prevention. An emergency vaccination program was implemented for those with close contacts of patients and all school children in Honghe Town to interrupt the outbreak. Here, we report the protective effect of vaccination with Healive<sup>®</sup>, an inactivated hepatitis A vaccine developed and manufactured in China, on the control of hepatitis A in an open rural community.

## MATERIALS AND METHODS

### *Surveillance of hepatitis A cases*

Routine surveillance data on hepatitis A cases were obtained from the reports of physicians, laboratories, and hospitals. The major diagnostic criteria for hepatitis A were nausea, abdominal discomfort, jaundice, dark urine, presence of IgM antibody, and elevated serum alanine aminotransferase (ALT) level.

Considering two weeks needed for antibody induction with inactivated hepatitis A vaccine<sup>[6]</sup> and a 15-50 d incubation period of hepatitis A, we defined 15 d post-vaccination as the window period. Any case occurring within the window period was excluded from the vaccine protective efficacy analysis.

### *Composition of the vaccine*

The inactivated hepatitis A vaccine, Healive<sup>®</sup>, developed and manufactured by Sinovac Biotech Co. Ltd. (Beijing, China), was licensed in China in 2002. The vaccine is

prepared from the TZ84 strain of HAV. The virus used for vaccine production can grow in human fetal lung diploid fibroblast 2BS cells. Whole viruses were extracted from tissue culture, purified, formalin inactivated, and then adsorbed onto aluminum hydroxide. The pediatric dose contains 250 U/0.5 mL HAV antigen, and the adult dose contains 500 U/1.0 mL HAV antigen. Other ingredients include aluminium hydroxide, sodium dihydrogen phosphate, disodium hydrogen phosphate, sodium chloride, and water for injection.

### *Study populations and vaccination programs*

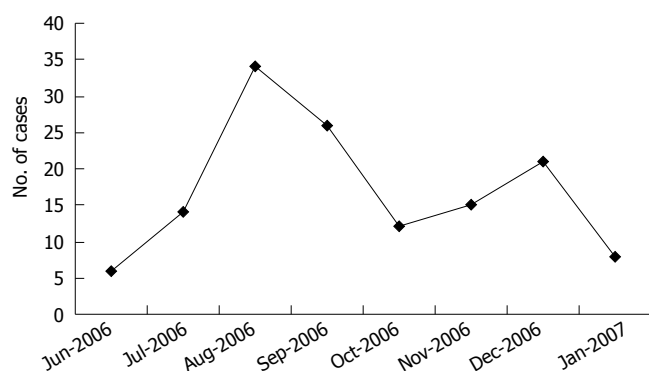
During the outbreak of hepatitis A in Honghe Town, Xiuzhou District, Jiaxing City, Zhejiang Province, two nonrandomized controlled trials were conducted. Both trials were approved by the Medical Ethics Committee of Xiuzhou District.

Starting in September 2006, anti-HAV tests were performed in individuals with close contacts of hepatitis A patients, of them 108 were negative for HAV. In the first trial, inactivated hepatitis A vaccine, Healive<sup>®</sup> was emergently delivered to the 108 individuals with close contacts of hepatitis A patients as a post-prophylactic measure after informed consent was obtained. Subjects younger than 16 years old received one injection of a pediatric dose of vaccine containing 250 U/0.5 mL hepatitis A virus antigen and those older than 16 years received one injection of an adult dose of vaccine containing 500 U/1.0 mL hepatitis A virus antigen. These 108 subjects served as the intervention group. Meanwhile, a retrospective investigation and follow-up study were conducted among 44 patients from June to August, who had 115 close contacts and none of them had a previous hepatitis A vaccination. Because the vaccination program was not implemented until September, these 115 close contacts formed a natural non-intervention group. All those with close contacts in the intervention and non-intervention groups were observed for 60 d.

The second trial of the vaccination program was carried out from September 2 to 8 in primary and secondary school students. A written consent form (which provided information about the burden of the disease, the role of vaccine, and the cost of vaccine) was given to each student in the primary and secondary schools in Honghe Town. After the consent forms were signed by the students' parents, 3365 students who consented to receive the vaccination served as the vaccine group, and 2572 students who did not choose to receive served as the control group. Students having either a history of hepatitis A infection or contraindications for hepatitis A vaccination were excluded from inoculation. One dose of Healive<sup>®</sup>, containing 250 U/0.5 mL hepatitis A virus antigen, was delivered to all students in the vaccine group.

### *Data analysis*

The protective rate was calculated from the formula: (incidence of non-intervention group-incidence of intervention group)/incidence of non-intervention group. Fisher's exact test was used to compare the incidence between the intervention and non-intervention groups. Z-test was used to analyze the number of cases. A two-tailed



**Figure 1** Number of hepatitis A cases by month in Honghe Town during the outbreak of hepatitis A in 2006.

*P* value less than 0.05 was considered statistically significant. Statistical analysis was carried out using SPSS 11.5 software.

## RESULTS

### Epidemiology of the hepatitis A outbreak

The outbreak of hepatitis A started in June 2006 and ended in January 2007 (Figure 1). A total of 136 cases of hepatitis A were reported. The first wave peaked in August and September in 67.65% (92/136) of the total cases. Forty-four cases of hepatitis A occurred in the second wave of outbreak, accounting for 32.35% (44/136) of the cases. The incidence was 227.86/100 000. Among the 136 reported cases, 64 patients were immigrating population while 72 patients were local population.

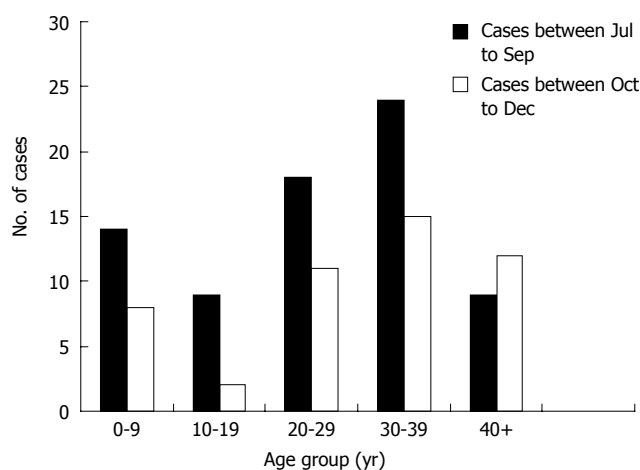
The cases had a different age distribution within the two populations. In the local population, most of the cases [81.9% (59/72)] were found at the age of 20-49 years, while 37.5% (24/64) and 32.8% (21/64) of the cases were found in the migrant population at the ages of 0-10 and 30-39 years, respectively.

### Protective efficacy of the vaccine in those with close contacts of the patients

Within the 60-d observation period, 4 subjects in the non-intervention group during the first trial developed clinical symptoms and were diagnosed with hepatitis A. In the intervention group, the earliest and latest emergency injection of the vaccine was given 1 d and 31 d post-contact (the median was 12 d). No hepatitis A clinical symptoms were observed in the intervention group during the 60-d observation period. The statistical test showed the difference in the infection rates between those with close contacts who received vaccination (0/108) and those who did not receive vaccination (4/115) was not significant ( $P = 0.122$ ). The protective efficacy was 100% in those with close contacts.

### Protective efficacy of the vaccine in students

To evaluate the ability of a massive vaccination of students to prevent the outbreak, students were enrolled in the vaccine group or the control group, according to their willingness to receive the vaccine. After the vaccination for 3365 students in early September 2006, one hepatitis



**Figure 2** Hepatitis A cases by age group in Honghe Town before and after intervention. The vaccination was implemented in early September 2006.

**Table 1** Hepatitis A incidence in vaccination and control groups

Group	No. of subjects	No. of cases	Incidence (1/100 000)
Vaccine	3365	0	0
Control	2572	4	155.5

A case was reported 3 d after injection. Another 5 cases were detected between September and December 2006 in the control group of 2572 students, on September 16 and 30, October 5, November 1, and December 10. The single case in the vaccine group and September 16 case in the control group were excluded from the incidence analysis. The incidence of hepatitis A in the vaccine and control groups was calculated (Table 1). Fisher's exact test indicated that the incidence of hepatitis A in the control group was significantly higher than that in the vaccine group ( $P = 0.035$ ). The protective efficacy was 100% in students who received vaccination.

### Effect of vaccination program on reducing the number of cases

After vaccination, the number of cases was reduced dramatically in the student group (5-19 years old) and in other age groups (Figure 2). Compared with the number of cases before the vaccination program, the number of cases in the student group (5-19 years old) decreased by 70% after the vaccination program (20 cases in July-September *vs* 6 cases in October-December) ( $Z = 2.746$ ,  $P < 0.01$ ). The number of cases decreased by 22.22% (54 cases in July-September *vs* 42 cases in October-December, ( $Z = 1.224$ ,  $P > 0.05$ ) among those outside the student group.

## DISCUSSION

We report a vaccination program in certain populations of an open rural community using an inactivated hepatitis A vaccine as an intervention to a hepatitis A outbreak. This is the first study to demonstrate the protective

**Table 2** Anti-HAV sero-positive rate in the community of Honghe Town

	Age group (yr)							Total
	0-	5-	10-	20-	30-	40-	50-	
Number of tested persons	8	19	97	136	160	71	41	532
Number of positive results	4	16	78	103	120	59	34	414
Positive rate (%)	50	84.2	80.4	75.7	75	83.1	82.9	77.8

effect of inactivated hepatitis A vaccine (Healive<sup>®</sup>) on a Chinese population, and the vaccine was shown to have a 100% protective efficacy, suggesting that the vaccination program reduced the incidence of new hepatitis A cases after a massive vaccination in school children. The precise contribution of the vaccine campaign to the control of the outbreak of hepatitis A is difficult to quantify because the outbreaks are often on the wane over time.

The outbreak of hepatitis A showed a typical epidemic mode: a weak second wave appeared a couple of months after the first wave. One possible reason for the second wave is that 30% of the patients were not hospitalized, and the HAV transmission might have resulted from a delayed quarantine. The appearance of the second wave could also be explained by the fact that the vaccinated population was not large enough to form immunity. In this vaccination campaign, 3365 students and 108 individuals with close contacts of hepatitis A patients were inoculated, leading to a vaccine coverage of 56.7% [ $3365/(3365 + 2572)$ ] in students and an average vaccine coverage of 5.82% [ $(3365 + 108)/59700$ ] in the whole community. As vaccination was targeted mainly at students, the vaccination campaign only increased seroconversion in the student group, and hepatitis A virus could still circulate among the susceptible individuals in preschool children and adults. We believe that incomplete interruption of HAV is the consequence of a lack of vaccination coverage in preschool children and adults.

A corroborative survey was conducted to investigate the anti-HAV positive rate when the outbreak occurred in the community (Hai-Tao He, Xiuzhou District Center for Disease Control and Prevention, personal communication, 2006) (Table 2). The anti-HAV positive rate was low in those at the age of 0-4 and 20-39 years, suggesting that a large number of susceptible individuals are the preschool children and adults. These data provide evidence that a low anti-HAV seropositive rate and the lack of a vaccination campaign in preschool children and adults might be the cause of the aforementioned second wave of hepatitis A epidemic.

It was reported that inactivated hepatitis A vaccine was used in prevention of community-wide outbreaks of hepatitis A<sup>[13]</sup>. Studies showed that an epidemic of hepatitis A could end eight weeks after 80% of susceptible children and adults received one dose of hepatitis A vaccine<sup>[14]</sup>; the epidemic may persist for up to 30 wk in another location where less than 50% of the susceptible individuals were vaccinated<sup>[15]</sup>. It is still unknown what level of vaccination coverage is required to curtail a community-wide outbreak of hepatitis A.

It was difficult to promote vaccination in the local

community, as vulnerable individuals besides the students refused to receive the vaccination in spite of our recommendation. Since inactivated hepatitis A vaccine is at expense of the recipients and whole-family vaccination is still unaffordable for many households, school children enjoy the priority in receiving the vaccination. We hope that the government and public health authorities can allocate a larger budget to support emergency vaccination programs to control outbreaks of hepatitis A more rapidly and effectively, especially in areas with a high incidence of HAV infection.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Hepatitis A is an acute, usually self-limited disease of the liver caused by hepatitis A virus. Although the use of inactivated hepatitis A vaccine as a prophylactic treatment has been reported in Europe and USA, little is known about the application of such a vaccine in control of hepatitis A in China.

### Research frontiers

Although hepatitis A is regarded as a benign infection, it may cause serious symptoms. The disease can be prevented by vaccination and hepatitis A vaccine has been proved effective in controlling its outbreak worldwide.

### Innovations and breakthroughs

Our study showed that inactivated hepatitis A vaccine demonstrating a good protective effect against outbreak of hepatitis A could be used for emergency vaccination. The study reports the first clinical study evaluating inactivated hepatitis A vaccine in an emergency vaccination campaign during an outbreak of hepatitis A in China.

### Applications

Hepatitis A vaccine has several advantages over immune globulin, including long-term protection effect, ease of administration, and widespread availability. Substitution of immunoglobulin by inactivated hepatitis A vaccine and its application in both routine and emergency vaccination during an outbreak of hepatitis are proposed.

### Terminology

Hepatitis A: an acute infectious disease of the liver caused by hepatitis A virus. Emergency vaccination: vaccination used during an outbreak of hepatitis A.

### Peer review

The present study demonstrated the protective effect of inactivated hepatitis A vaccine against hepatitis A during the outbreak in an open rural community. It provides an interesting insight into the control and prevention of hepatitis in a rural community of China.

## REFERENCES

- Fiore AE, Wasley A, Bell BP. Prevention of hepatitis A through active or passive immunization: recommendations of the Advisory Committee on Immunization Practices (ACIP). *MMWR Recomm Rep* 2006; **55**: 1-23
- Chinese center for disease control and prevention. National incidence and death cases of notifiable class A or class B infectious diseases. Available from: URL: <http://www.chinacdc.net.cn/n272442/n272530/n272757/index.html>

- 3 **Mao JS.** Development of live, attenuated hepatitis A vaccine (H2-strain). *Vaccine* 1990; **8**: 523-524
- 4 **Ren A,** Feng F, Ma J, Xu Y, Liu C. Immunogenicity and safety of a new inactivated hepatitis A vaccine in young adults: a comparative study. *Chin Med J (Engl)* 2002; **115**: 1483-1485
- 5 **Sui HT,** Liang XF, Yin DP, Cui FQ, Wang HQ. Epidemic characteristics on hepatitis A in China during 1990-2006. *Zhongguo Jihua Mianyi* 2007; **13**: 466-469
- 6 **Wang X,** Ma J, Xu Z, Liu H, Zhang Y, Han C. [Effectiveness of post-exposure prophylaxis using live attenuated hepatitis Alpha vaccine (H(2) strain) among schoolchildren]. *Zhonghua Yixue Zazhi* 2002; **82**: 955-957
- 7 **Ma JC,** Han CQ, Ding YX, Liu HB, Zhang Y, Zhang YL, Wang XY, Zhang YW, Xing ZC, Zhao H, Meng ZD, Xu ZY. Effect of booster immunization of live attenuated hepatitis A vaccine. *Zhongguo Shengwu Zhipinxue Zazhi* 2003; **16**: 56-58
- 8 **Ren YH,** Wu WT, Zhang YC, Xue WH, Kang WX, Ren YF, Han LJ, Li SP, Gao SJ, Cui LY, Liu CB. The study on immunoreaction of low-dose inactivated Chinese hepatitis A vaccine. *Zhongguo Jihua Mianyi* 2003; **9**: 114-116
- 9 **Andre FE,** D'Hondt E, Delem A, Safary A. Clinical assessment of the safety and efficacy of an inactivated hepatitis A vaccine: rationale and summary of findings. *Vaccine* 1992; **10** Suppl 1: S160-S168
- 10 **D'Argenio P,** Adamo B, Cirrincione R, Gallo G. The role of vaccine in controlling hepatitis A epidemics. *Vaccine* 2003; **21**: 2246-2249
- 11 **Victor JC,** Monto AS, Surdina TY, Suleimenova SZ, Vaughan G, Nainan OV, Favorov MO, Margolis HS, Bell BP. Hepatitis A vaccine versus immune globulin for postexposure prophylaxis. *N Engl J Med* 2007; **357**: 1685-1694
- 12 **Shen YG,** Jiang XL, Gu XJ. Surveillance of Human Immune Level for Hepatitis A in Xiuzhou District of Jiashan City. *Zhejiang Yufang Yixue* 2004; **16**(10): 13
- 13 **Werzberger A,** Mensch B, Kuter B, Brown L, Lewis J, Sitrin R, Miller W, Shouval D, Wiens B, Calandra G. A controlled trial of a formalin-inactivated hepatitis A vaccine in healthy children. *N Engl J Med* 1992; **327**: 453-457
- 14 **McMahon BJ,** Beller M, Williams J, Schloss M, Tanttala H, Bulkow L. A program to control an outbreak of hepatitis A in Alaska by using an inactivated hepatitis A vaccine. *Arch Pediatr Adolesc Med* 1996; **150**: 733-739
- 15 **Craig AS,** Sockwell DC, Schaffner W, Moore WL Jr, Skinner JT, Williams IT, Shaw FE, Shapiro CN, Bell BP. Use of hepatitis A vaccine in a community-wide outbreak of hepatitis A. *Clin Infect Dis* 1998; **27**: 531-535

**S- Editor** Liu JN **L- Editor** Wang XL **E- Editor** Ma WH



## CASE REPORT

# Hemosuccus pancreaticus: Problems and pitfalls in diagnosis and treatment

Yoshikazu Toyoki, Kenichi Hakamada, Shunji Narumi, Masaki Nara, Keinosuke Ishido, Mutsuo Sasaki

Yoshikazu Toyoki, Kenichi Hakamada, Shunji Narumi, Masaki Nara, Keinosuke Ishido, Mutsuo Sasaki, Department of Gastroenterological Surgery, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8652, Japan  
Correspondence to: Dr. Yoshikazu Toyoki, Department of Gastroenterological Surgery, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 036-8652, Japan. [ytoyoki@cc.hirosaki-u.ac.jp](mailto:ytoyoki@cc.hirosaki-u.ac.jp)  
Telephone: +81-172-395079 Fax: +81-172-395080  
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## Abstract

Hemosuccus pancreaticus is a rare cause of intermittent upper gastrointestinal bleeding. We report two cases of hemosuccus pancreaticus with multiple episodes of upper gastrointestinal bleeding. The causes of hemorrhage were rupture of pseudoaneurysm of the splenic artery and bleeding from the wall of pancreatic pseudocyst. Interventional radiology is the first modality for early diagnosis and possible treatment of hemosuccus pancreaticus. When angiography shows no abnormal findings or interventional radiological therapy can not be successful, surgery should be considered without delay. Our patients herein underwent surgery without recurrence or sequelae. Intraoperative ultrasonography and pancreatoscopy were helpful modalities for confirming the source of hemorrhage and determining the cutting line of the pancreas. When we encounter intermittent upper gastrointestinal bleeding with an obscure source, hemosuccus pancreaticus should be included in differential diagnoses especially in patients with chronic pancreatitis, which would lead to a prompt and proper treatment.

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**Key words:** Hemosuccus pancreaticus; Gastrointestinal bleeding; Interventional radiology; Intraoperative sonography; Intraoperative pancreatoscopy

**Peer reviewer:** Yoshiharu Motoo, MD, PhD, FACP, FACG, Professor and Chairman, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

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## INTRODUCTION

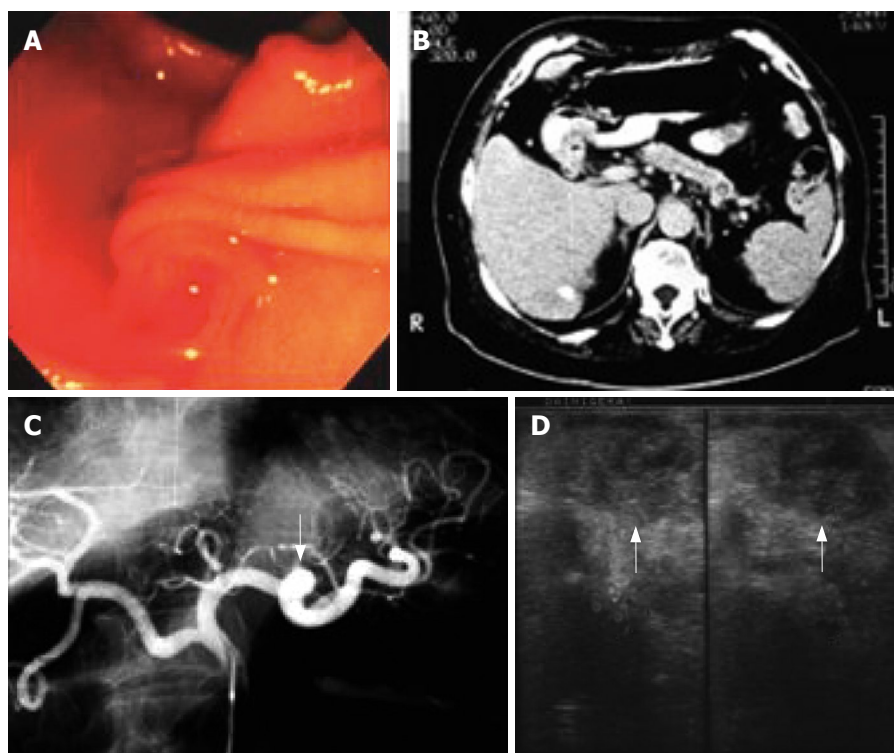
Hemorrhage from the papilla of Vater *via* the pancreatic duct, known as hemosuccus pancreaticus, is a rare cause of intermittent upper gastrointestinal bleeding. This condition was first reported in 1931 by Lower and Farrell who mentioned bleeding from an aneurysm of the splenic artery<sup>[1]</sup>. The expression "hemosuccus pancreaticus" was named by Sandblom in 1970<sup>[2]</sup>. Until now, reports on hemosuccus pancreaticus have been quite limited. Difficulties in determining the location of bleeding sometimes cause delay of treatment and critical condition of patients.

We herein report two cases of hemosuccus pancreaticus and discuss problems and pitfalls for managing this disease.

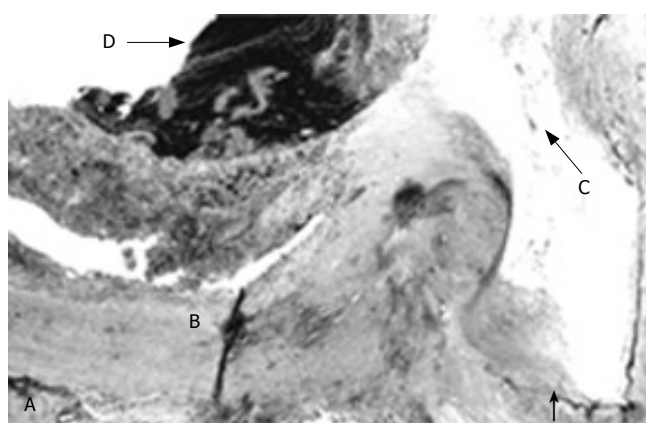
## CASE REPORTS

### Case 1

A 75-year-old woman had been followed up for epigastric discomfort and anemia for 3 years, but no abnormality had been elucidated by either upper gastrointestinal series or endoscopic examinations. She developed sudden hematoemesis and was emergently admitted to a referral hospital. Upper gastrointestinal endoscopy revealed fresh bleeding from the papilla of Vater (Figure 1A), and she was transferred to our institute. A CT scan showed a 2.0 cm × 1.8 cm cystic mass at the tail of the pancreas without remarkable findings of chronic pancreatitis (Figure 1B). Angiography identified aneurysms at the distal portion of the splenic artery and the right hepatic artery (Figure 1C). The patient came down with a pre-shock condition with continuous bleeding, and a diagnosis of the rupture of aneurysm of the splenic artery and/or the right hepatic artery was confirmed emergently. Intraoperative ultrasonography revealed a 2.0 cm × 2.0 cm low echoic mass at the body of the pancreas suspected as a hematoma (Figure 1D). Distal pancreatectomy and splenectomy were performed for the rupture of aneurysm of the splenic artery. The pancreas was diffusely hard and compatible with chronic pancreatitis. It seemed that this might be caused by obstruction of main pancreatic duct due to blood from hematoma. Pathological examination confirmed peripancreatic hematoma and pseudoaneurysm



**Figure 1** Case 1. Pseudoaneurysm of splenic artery communicating with pancreatic duct. **A:** Endoscopy reveals bleeding from papilla of Vater; **B:** CT scan shows a cystic mass at tail of pancreas; **C:** Angiography identifies aneurysms at distal portion of splenic artery and right hepatic artery (arrow); **D:** Operative ultrasonography demonstrates a low echoic mass at body of pancreas suspected as a hematoma (arrow).



**Figure 2** Histology of Case 1. **A:** Pancreatic parenchyma; **B:** Wall of pseudoaneurysm of splenic artery; **C:** Orifice of pseudoaneurysm into pancreatic duct; **D:** Hematoma.

of the splenic artery communicated with the pancreatic duct: hemosuccus pancreaticus (Figure 2). Postoperative course was uneventful without recurrence.

## Case 2

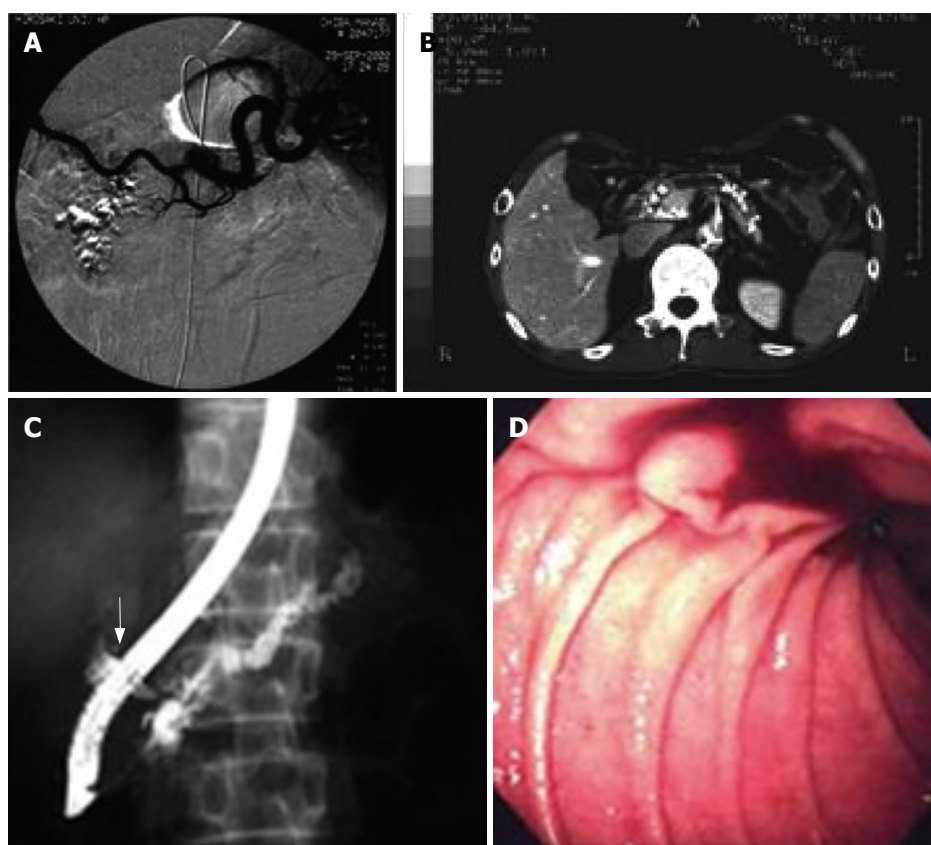
A 44-year-old man was hospitalized at a referral hospital developing tarry stool with severe anemia. Upon the first upper gastrointestinal endoscopy, a small amount of fresh blood was observed in the duodenum, but the source of bleeding was not identified. Colorectal endoscopy, angiography (Figure 3A) and scintigraphy failed to detect the bleeding source. A CT scan revealed multiple calcifications at the whole pancreas and dilatation of main pancreatic duct compatible with chronic pancreatitis and pancreatolithiasis (Figure 3B). Endoscopic retrograde pancreatography and magnetic resonance cholangio-

pancreatography revealed a dilated branch of pancreatic duct at the head of the pancreas (Figure 3C). Finally, bleeding from the papilla of Vater was seen by the upper gastrointestinal endoscopy (Figure 3D). He was transferred to our hospital and was electively explored with a diagnosis of hemosuccus pancreaticus and alcoholic chronic pancreatitis. The pancreas was macroscopically compatible with chronic pancreatitis and the bleeding source remained unclear because the stricture of the main pancreatic duct at the head of the pancreas prevented the endoscopic examination *via* the papilla of Vater. The pancreas was then divided right above the portal vein, and the bleeding source was identified at the tail of the pancreas by endoscopic examination (Figure 4A and B). Distal pancreatectomy and splenectomy were performed. Pathological examination demonstrated a pseudocyst filled with hematoma at the tail of the pancreas (Figure 4C).

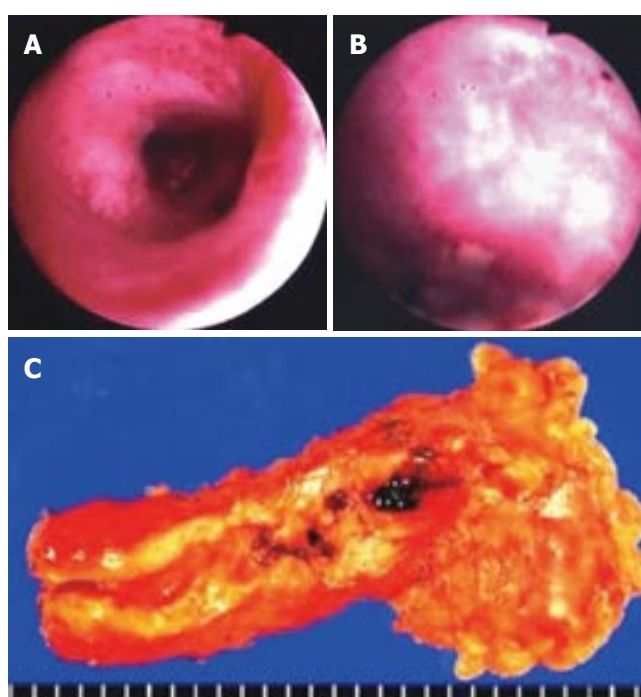
## DISCUSSION

Hemosuccus pancreaticus (HP), a rare cause of upper gastrointestinal bleeding from the papilla of Vater *via* the pancreatic duct, is most commonly caused by the rupture of aneurysm of the splenic artery associated with acute or chronic pancreatitis. Pseudoaneurysm of the hepatic, gastroduodenal or pancreaticoduodenal artery have also been reported as sources of bleeding<sup>[3-5]</sup>. Other uncommon causes are pancreatolithiasis and pseudocyst of the pancreas<sup>[6,7]</sup>. Our two patients demonstrated different pathogenetic mechanisms of HP: (1) a rupture of splenic arterial pseudoaneurysm communicating to the pancreatic duct (case 1), and (2) a communication between the peripancreatic artery and pancreatic pseudocyst (case 2).

It is difficult to make HP diagnosis because of intermittent hemorrhage from a source that is not



**Figure 3** Case 2. Pseudoaneurysm in pancreatic pseudocyst. **A:** Angiography failed to detect a bleeding point; **B:** CT scan shows multiple calcifications at the whole pancreas and dilatation of main pancreatic duct; **C:** Endoscopic retrograde pancreatography displays a dilated branch of pancreatic duct at head of pancreas (arrow); **D:** Endoscopy reveals bleeding from papilla of Vater.



**Figure 4** Case 2. Intraoperative pancreatoscopy and specimen. **A:** Pancreatic head by pancreatoscopy; **B:** Pancreatic tail by pancreatoscopy, and bleeding source was seen; **C:** Bleeding source at pancreatic tail.

readily accessible by endoscopy. Moreover, some patients underwent operations elsewhere of questionable benefit before establishment of the correct diagnosis<sup>[8]</sup>. Therefore, if patients present with obscure source of repeated upper gastrointestinal bleeding, especially underlying

chronic pancreatitis, repeated examinations and careful observations should be performed for the diagnosis of these conditions and HP should be included in differential diagnosis<sup>[9]</sup>. Koizumi *et al*<sup>[10]</sup> reported that MRI successfully identified the fistula and bleeding. However, MRI was not helpful for the diagnosis of HP in our cases, and reasons remain unknown.

The management for HP should be aimed to eradicate the source of bleeding completely. There are two choices for the treatment of HP: (1) surgery (e.g. resection of the pancreas head or tail), and (2) interventional radiological therapy<sup>[11-14]</sup>. Most HP cases can receive angiography. If the source of hemorrhage is found by arteriography, interventional radiological therapy should be done following this examination. Recently, Benz *et al*<sup>[13]</sup> reported the interventional radiological therapy of HP by implantation of an uncoated metal Palmaz stent across the aneurysmal segment of splenic artery. This interventional radiological treatment may be useful for the rupture of arterial pseudoaneurysm to the pancreatic duct so as to prevent emergency operation. However, surgical treatment is required when angiography shows no abnormal findings and interventional radiological therapy is not successful. For the patient with HP who has a pancreatic disease such as pancreatic pseudocyst, surgical treatment may be appropriate. However, it is very difficult to confirm the source of bleeding and determine the cutting line of pancreas. Therefore, intraoperative sonography and pancreatoscopy should be performed to confirm the origin of hemorrhage. They have also been frequently used during hepatobiliary and pancreas surgery. There has been no report about intraoperative ultrasonography



and pancreatoscopy in this disease. Case 1 in our report had two aneurysms of splenic artery and right hepatic artery. Preoperative angiography did not reveal fistula either between aneurysm and pancreatic duct or between aneurysm and bile duct. On intraoperative ultrasonography, no abnormality in hepatobiliary system was seen, but a hematoma could be seen in the pancreatic body. Finally, the diagnosis of a rupture of aneurysm of splenic artery was established. Case 2 in our study was diagnosed with bleeding from the pseudocyst at the pancreatic head by ERCP and MRCP. However, on intraoperative ultrasonography and pancreatoscopy, the bleeding point turned out to be a pseudocyst at the pancreatic tail. Intraoperative pancreatoscopy was also useful in finding the origin of bleeding.

In summary, we had experienced two cases of HP. Repeated examinations and careful observations should be performed to find the obscure source of repeated upper gastrointestinal bleeding and HP should be included in the differential diagnosis. Interventional radiological therapy should be tried at first for HP. Only when angiography shows no abnormal findings and interventional radiological therapy is not successful, surgical treatment is considered. Intraoperative ultrasonography and pancreatoscopy are often performed at surgery to confirm the origin of hemorrhage.

## REFERENCES

- 1 **Lower WE**, Farrell JI. Aneurysm of the splenic artery: Report of a case and review of the literature. *Arch Surg* 1931; **23**:182-190
- 2 **Sandblom P**. Gastrointestinal hemorrhage through the pancreatic duct. *Ann Surg* 1970; **171**: 61-66
- 3 **Stanley JC**, Frey CF, Miller TA, Lindenauer SM, Child CG 3rd. Major arterial hemorrhage: a complication of pancreatic pseudocysts and chronic pancreatitis. *Arch Surg* 1976; **111**: 435-440
- 4 **Rao RC**, Kumar A, Berry M. Pseudoaneurysm of anomalous right hepatic artery as a cause for hemosuccus pancreatitis. *Gastrointest Radiol* 1987; **12**: 313-314
- 5 **Fernandez-Cruz L**, Pera M, Vilella A, Llovera JM, Navasa M, Teres J. Hemosuccus pancreaticus from a pseudoaneurysm of the hepatic artery proper in a patient with a pancreatic pseudocyst. *Hepatogastroenterology* 1992; **39**: 149-151
- 6 **Risti B**, Marincek B, Jost R, Decurtins M, Ammann R. Hemosuccus pancreaticus as a source of obscure upper gastrointestinal bleeding: three cases and literature review. *Am J Gastroenterol* 1995; **90**: 1878-1880
- 7 **Jakobs R**, Riemann JF. [Hemosuccus pancreaticus due to a pressure ulcer in pancreatolithiasis] *Dtsch Med Wochenschr* 1992; **117**: 1956-1961
- 8 **Sakorafas GH**, Sarr MG, Farley DR, Que FG, Andrews JC, Farnell MB. Hemosuccus pancreaticus complicating chronic pancreatitis: an obscure cause of upper gastrointestinal bleeding. *Langenbecks Arch Surg* 2000; **385**: 124-128
- 9 **Cahow CE**, Gusberg RJ, Gottlieb LJ. Gastrointestinal hemorrhage from pseudoaneurysms in pancreatic pseudocysts. *Am J Surg* 1983; **145**: 534-541
- 10 **Koizumi J**, Inoue S, Yonekawa H, Kunieda T. Hemosuccus pancreaticus: diagnosis with CT and MRI and treatment with transcatheter embolization. *Abdom Imaging* 2002; **27**: 77-81
- 11 **Mandel SR**, Jaques PF, Sanofsky S, Mauro MA. Nonoperative management of peripancreatic arterial aneurysms. A 10-year experience. *Ann Surg* 1987; **205**: 126-128
- 12 **Baker KS**, Tisnado J, Cho SR, Beachley MC. Splanchnic artery aneurysms and pseudoaneurysms: transcatheter embolization. *Radiology* 1987; **163**: 135-139
- 13 **Benz CA**, Jakob P, Jakobs R, Riemann JF. Hemosuccus pancreaticus--a rare cause of gastrointestinal bleeding: diagnosis and interventional radiological therapy. *Endoscopy* 2000; **32**: 428-431
- 14 **Sugiki T**, Hatori T, Imaizumi T, Harada N, Fukuda A, Kamikozuru H, Yazawa T, Noguchi T, Takasaki K. Two cases of hemosuccus pancreaticus in which hemostasis was achieved by transcatheter arterial embolization. *J Hepatobiliary Pancreat Surg* 2003; **10**: 450-454

S- Editor Zhu WL L- Editor Ma JY E- Editor Lu W



## CASE REPORT

# Undifferentiated connective tissue diseases-related hepatic injury

Ying Zhang, Fu-Kui Zhang, Xiao-Ning Wu, Tai-Ling Wang, Ji-Dong Jia, Bao-En Wang

Ying Zhang, Fu-Kui Zhang, Xiao-Ning Wu, Ji-Dong Jia, Bao-En Wang, Liver research center, Beijing Friendship Hospital Affiliated to Capital Medical University, Beijing 100050, China  
Tai-Ling Wang, Department of Pathology, China-Japan Friendship Hospital, Beijing 100050, China  
Correspondence to: Bao-En Wang, Professor, Liver Research Center, Beijing Friendship Hospital Affiliated to Capital Medical University, Beijing 100050, China. [wangbbee@126.com](mailto:wangbbee@126.com)  
Telephone: +86-10-63164411 Fax: +86-10-63164411  
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## Abstract

Hepatic injury is rarely associated with undifferentiated connective tissue diseases (UCTD). We report, here, a case of a middle-aged woman with UCTD-related hepatic injury, including its case history, clinical manifestations, laboratory findings, treatment and its short-term effect. The patient was admitted to the hospital with symptoms of fatigue, anorexia, low-grade fever and skin rashes. She had a past history of left knee joint replacement. Laboratory tests showed elevated levels of serum transaminase, IgG and globulin, accelerated erythrocyte sedimentation rate, eosinophilia and a high titer of antinuclear antibodies (1:320). Imaging studies showed interstitial pneumonitis and hydropericardium. Liver biopsy showed the features which were consistent with those of connective tissue diseases-related polyangitis. After treatment with a low-dose of oral prednisone, both symptoms and laboratory findings were significantly improved. UCTD-related hepatic injury should be considered in the differential diagnosis of connective tissue diseases with abnormal liver function tests. Low-dose prednisone may effectively improve both symptoms and laboratory tests.

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**Key words:** Undifferentiated connective tissue diseases; Hepatic injury; Glucocorticoid

**Peer reviewer:** Francesco Perri, MD, Department of Gastroenterology, CSS Hospital, IRCCS, Via Cappuccini, San Giovanni Rotondo 71013, Italy

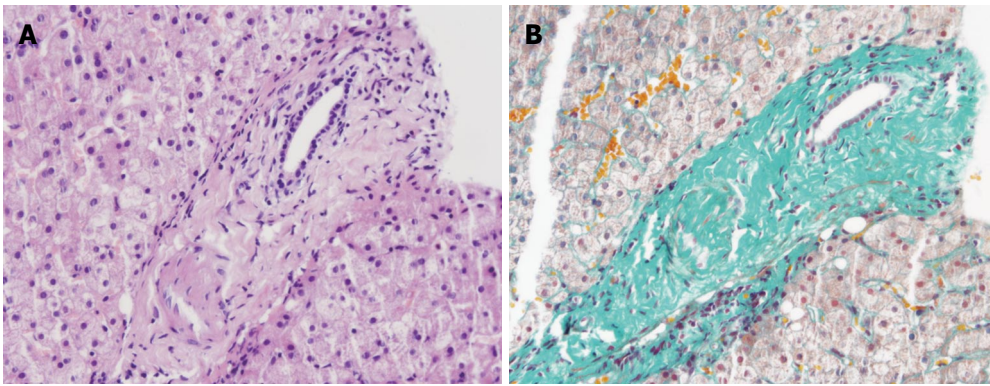
Zhang Y, Zhang FK, Wu XN, Wang TL, Jia JD, Wang BE. Undifferentiated connective tissue diseases-related hepatic injury. *World J Gastroenterol* 2008; 14(17): 2780-2782 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2780.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2780>

## INTRODUCTION

The term of undifferentiated connective tissue disease (UCTD) is used to define conditions characterized by the presence of signs and symptoms suggestive of a systemic autoimmune disease that do not satisfy the classification criteria for defined connective tissue diseases (CTD), such as systemic lupus erythematosus (SLE), Sjögren's syndrome (SS), rheumatoid arthritis (RA) and others. Patients may present with systemic symptoms, such as fatigue, fever, or weight loss, preceding any organ involvement. The most common symptoms include arthralgias, unexplained or undifferentiated polyarthritides, Raynaud syndrome, mucocutaneous manifestations, and sicca symptoms. However, it is unusual for a patient with UCTD to have major organ involvement<sup>[1]</sup>. Here, we report a case of UCTD-related hepatic injury.

## CASE REPORT

A 58-year-old woman was admitted to our hospital with chief complaints of fatigue, anorexia, low-grade fever and skin rashes for two months. At physical examination, she had a BP of 118/79 mmHg, a pulse of 82 beats/min, a respiratory rate of 16, and a temperature of 37.3°C. BMI was 25.2. Red maculae and desquamation were found on her face, upper limbs and buttocks. Family history was negative for rheumatic or inherited liver disease. She had a past medical history of psoriasis, hypertension, acute hepatitis A and blood transmission, but no history of ethanol consumption, no exposure to possible hepatotoxic drugs. She accepted left knee joint replacement three years ago because of osteoarthritis. Abdominal ultrasonography revealed echo enhancement of the liver and mild splenomegaly, no ascites was detected. Chest CT examination revealed interstitial pneumonia and hydropericardium. Laboratory analysis revealed a white blood cell count of  $9.86 \times 10^9/L$ , eosinophile of 10.1%, hemoglobin of 108 g/L, ESR of 90 mm/h, aspartate aminotransferase level of 157 U/L, alanine aminotransferase level of 141 U/L, albumin of 27.1 g/L, globulin of 34.7 g/L, pre-albumin of 69 mg/L, seropositivity for antinuclear antibodies with a titer of 1:320. Total bilirubin, prothrombin time, renal function and urine routine test were within normal range. Investigations for the underlying cause of her hepatic injury including alfa-fetoprotein, CEA, thyroid function, cytomegalovirus, EB virus, adenovirus antinephrotic cytoplasmic antibody, antibodies to liver/kidney microsome, smooth muscle antibodies, anti-neutrophil cytoplasmic antibody,



**Figure 1** Biopsy of the liver showing dense fibrosis of intrahepatic portal tracts with obliteration of portal vein channels, consistent with connective tissue diseases-related polyangitis with HE staining (A) and Masson trichrome staining (B).

immunoglobulin pattern, hepatitis B, C and E serology were all negative or within the normal range. Liver biopsy showed the features consistent with those of connective tissue diseases-related polyangitis (Figure 1). These findings suggested that she had UCTD-related hepatic injury. The patient was treated with a low dose of oral prednisone (40 mg/d) at the beginning. Three weeks later, the dose of oral prednisone was decreased to 30 mg/d. Her symptoms and laboratory tests improved markedly four weeks later. The levels of hemoglobin, albumin and pre-albumin were elevated to 107 g/L, 31.7 g/L and 158 mg/L, respectively. The levels of white blood cells, eosinophile, PLT, ESR, ALT and AST were decreased to  $8.72 \times 10^9$ /L, 6.4%,  $356 \times 10^9$ /L, 44 mm/h, 100 U/L, and 86 U/L, respectively. She was discharged on the 40th d. Twenty-eight days after discharge, follow-up laboratory tests showed that the levels of ESR and eosinophiles were decreased to their normal range. The levels of ALT and AST were also decreased to 60 U/L and 70 U/L, respectively.

## DISCUSSION

The most characteristic symptoms of UCTD are arthritis, arthralgias, Raynaud's phenomenon, and leukopenia. It is clear that the clinical profile of UCTD is characterized by the absence of major organ involvement. During follow-up, the symptoms may improve or mildly flare spontaneously. A positive ANA is common, with a positive rate ranging from 60%-100%, and a stable profile over time<sup>[2]</sup>. After reviewing the UCTD literature, Mosca *et al*<sup>[3]</sup> proposed that the preliminary classification criteria for UCTD include: (1) signs and symptoms suggestive of a connective tissue disease, but not fulfilling the criteria for any defined CTD, (2) positive antinuclear antibodies, and (3) a disease course of at least 3 years. As these are mild and benign conditions, only a small number of UCTD patients are treated. The most widely used drugs are NSAID (40%), corticosteroids (30%-50%) and antimalarials at a low dose (10%-30%)<sup>[4,5]</sup>. Our patient was treated with a low dose of prednisone. Four weeks later, both symptoms and laboratory findings were improved markedly. According to the above findings, the diagnosis of UCTD was established.

Our report was evaluated for hereditary (Wilson's disease,  $\alpha$ -1 antitrypsin deficiency, and genetic hemochromatosis), infections (hepatitis B, C and CMV,

EBV), drinking history and drug-induced liver injury, some of which may have autoimmune features. These possible causes for liver damage can be excluded. Likewise, although echogram of the liver showed fatty change, histological findings did not show steatosis in hepatocytes. However, even after careful exclusion of the above stated etiologies, the question remains whether the patient should be diagnosed having a primary liver disease associated with autoimmune or clinical and laboratory features and liver involvement should be considered manifestations of UCTD. The main pathogenetic dilemma is autoimmune hepatitis (AIH) and UCTD-related hepatic injury, although they have some common features of autoimmune syndrome. In this paper, we present a case of a patient with hepatic injury. UCTD is a systemic autoimmune condition characterized by a mild clinical profile and a simplified autoimmune repertoire. Although UCTD is generally benign, it may progress to CTD and changes in the disease course may occur. A small number of patients presenting with an undifferentiated profile will develop CTD during the first year follow-up. However, an average of 75% patients will maintain an undifferentiated clinical course. These patients may be defined having a stable UCTD. Bodolay *et al*<sup>[6]</sup> followed up 665 Hungarian patients with UCTD and found that most of the UCTD patients do not develop a definite CTD, but have new clinical and serological manifestations during the follow-up period and UCTD progresses to different types of specific CTD in one-third of them. Margaret *et al*<sup>[7]</sup> reviewed 11 patients meeting the diagnostic criteria for AIH. Of these 11 patients, three with a definitive diagnosis of AIH developed systemic CTD, one developed systemic lupus erythematosus (SLE) with vasculitis and peripheral neuropathy, 2 developed limited scleroderma, and 3 developed UCTD and interstitial lung disease. There appears to be a shared susceptibility of alleles to AIH and CTD in addition to the shared positive autoantibodies. Therefore, it is necessary to exclude the possibility of AIH if the definite diagnosis of UCTD-related hepatic injury is made. In the present case, the possibility of a diagnosis of AIH should be considered. The criteria for the diagnosis of AIH in adult patients have been established by the International Autoimmune Hepatitis Group (IAIHG)<sup>[8]</sup>. The diagnosis of UCTD-related hepatic injury is based on the presence of characteristic laboratory and liver histology features and the exclusion of conditions that

resemble AIH. Interface hepatitis is the hallmark of AIH<sup>[9]</sup>. However, the liver biopsy did not show interface hepatitis, which is essential for the diagnosis of AIH. Therefore, the diagnosis of AIH cannot be established. Instead, the diagnosis of UCTD-related hepatic injury can be established. Laing *et al*<sup>[10]</sup> reported that potential risk factors for UCTD are found in women implanted medical devices, of which, non-silicone-containing devices, non-silicone-containing artificial joints (OR = 5.01, 95% CI = 1.60-15.71) and orthopedic metallic fixation devices (OR = 1.95, 95% CI = 1.05-3.60) are associated with UCTD. Laing *et al*<sup>[10]</sup> have also reviewed the intervals between surgical implantation of artificial joints and diagnosis of UCTD (mean, 4.5 years; range, 3.0-6.9 years). Our patient received left knee joint replacement because of a 3-year history of osteoarthritis. Whether this plays a role in the pathogenesis of UCTD needs further study.

Just as most of other connective-tissue diseases, the pathogenesis of UCTD is unclear. Like most connective-tissue diseases, the theory and research have been concentrated on genetically susceptible hosts, T- and B-cell abnormalities, and environmental triggers, such as ultraviolet light or infection. In general, activities are not restricted unless specific functional limitations, such as interstitial lung disease, associated with UCTD are present<sup>[11]</sup>. Therefore, UCTD-related hepatic injury is an auto-reactive tissue injury which may arise *via* either the TH<sub>1</sub> pathway (leading to cell-mediated cytotoxic reactions) or the TH<sub>2</sub> pathway (resulting in antibody-mediated cell damage). These processes are under a certain form of immunoregulatory control which normally maintains self-tolerance by “switching off” the autoimmune response, although whether this involves discrete subsets of “suppressor” T lymphocytes is still controversial<sup>[12]</sup>. Whether the above factors induce UCTD-related hepatic injury is uncertain, in part because the pathogenetic mechanism underlying the development of these two conditions is poorly understood.

In conclusion, we report this case for its unusual hepatic injury after UCTD. UCTD-related hepatic injury should be considered in the differential diagnosis of connective tissue diseases with abnormal liver function tests. Low-dose prednisone can effectively improve both symptoms and laboratory findings of UCTD.

## REFERENCES

- 1 Mosca M, Tani C, Neri C, Baldini C, Bombardieri S. Undifferentiated connective tissue diseases (UCTD). *Autoimmun Rev* 2006; **6**: 1-4
- 2 Mosca M, Tavoni A, Neri R, Bencivelli W, Bombardieri S. Undifferentiated connective tissue diseases: the clinical and serological profiles of 91 patients followed for at least 1 year. *Lupus* 1998; **7**: 95-100
- 3 Mosca M, Neri R, Bencivelli W, Tavoni A, Bombardieri S. Undifferentiated connective tissue disease: analysis of 83 patients with a minimum followup of 5 years. *J Rheumatol* 2002; **29**: 2345-2349
- 4 Swaak AJ, van de Brink H, Smeenk RJ, Manger K, Kalden JR, Tosi S, Marchesoni A, Domljan Z, Rozman B, Logar D, Pokorny G, Kovacs L, Kovacs A, Vlachoyiannopoulos PG, Moutsopoulos HM, Chwalinska-Sadowska H, Dratwianka B, Kiss E, Cikes N, Anic B, Schneider M, Fischer R, Bombardieri S, Mosca M, Graninger W, Smolen JS. Incomplete lupus erythematosus: results of a multicentre study under the supervision of the EULAR Standing Committee on International Clinical Studies Including Therapeutic Trials (ESCISIT). *Rheumatology* (Oxford) 2001; **40**: 89-94
- 5 Greer JM, Panush RS. Incomplete lupus erythematosus. *Arch Intern Med* 1989; **149**: 2473-2476
- 6 Bodolay E, Csiki Z, Szekanecz Z, Ben T, Kiss E, Zeher M, Szucs G, Danko K, Szegedi G. Five-year follow-up of 665 Hungarian patients with undifferentiated connective tissue disease (UCTD). *Clin Exp Rheumatol* 2003; **21**: 313-320
- 7 West M, Jasin HE, Medhekar S. The development of connective tissue diseases in patients with autoimmune hepatitis: a case series. *Semin Arthritis Rheum* 2006; **35**: 344-348
- 8 Alvarez F, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, Chapman RW, Cooksley WG, Czaja AJ, Desmet VJ, Donaldson PT, Eddleston AL, Fainboim L, Heathcote J, Homberg JC, Hoofnagle JH, Kakumu S, Krawitt EL, Mackay IR, MacSween RN, Maddrey WC, Manns MP, McFarlane IG, Meyer zum Buschenfelde KH, Zeniya M. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; **31**: 929-938
- 9 Czaja AJ, Freese DK. Diagnosis and treatment of autoimmune hepatitis. *Hepatology* 2002; **36**: 479-497
- 10 Laing TJ, Schottenfeld D, Lacey JV Jr, Gillespie BW, Garabrant DH, Cooper BC, Heeringa SG, Alcsér KH, Mayes MD. Potential risk factors for undifferentiated connective tissue disease among women: implanted medical devices. *Am J Epidemiol* 2001; **154**: 610-617
- 11 Mosca M, Baldini C, Bombardieri S. Undifferentiated connective tissue diseases in 2004. *Clin Exp Rheumatol* 2004; **22**: S14-S18
- 12 McFarlane IG, Heneghan MA. Autoimmunity and the female liver. *Hepatol Res* 2004; **28**: 171-176

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## Eosinophilic cholecystitis caused by *Ascaris lumbricoides*

Alvaro Montiel-Jarquín

Alvaro Montiel-Jarquín, Hospital General de Zona No. 15, Tehuacan, Puebla 72550, México

Author contributions: Montiel-Jarquín A analyzed and wrote the paper.

Correspondence to: Alvaro Montiel-Jarquín, MD, Hospital General de Zona No. 15, Tehuacan, Instituto Mexicano del Seguro Social, Puebla 72550, México. [dralmoja@hotmail.com](mailto:dralmoja@hotmail.com)

Telephone: +52-222-2446781 Fax: +52-222-2444386

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### Abstract

Eosinophilic cholecystitis is caused by the accumulation of eosinophils in the gallbladder wall and diagnosis is usually made based on histopathologic studies. The purpose of this paper is to comment on a case report published in *World J Gastroenterol* 2007 July; 13 (27): 3760-3762, about eosinophilic cholecystitis along with pericarditis without histopathological studies, which are considered necessary for its diagnosis.

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**Key words:** Eosinophilic cholecystitis; *Ascaris lumbricoides*; Pericarditis

**Peer reviewers:** Xian-Ming Chen, MD, Associate Professor, Department of Medical Microbiology and Immunology, Creighton University, 2500 California Plaza, Omaha NE 68178, United States; Richard A Kozarek, MD, Department of Gastroenterology, Virginia Mason Medical Center, 1100 Ninth Avenue, PO Box 900, Seattle 98111-0900, United States

Montiel-Jarquín A. Eosinophilic cholecystitis caused by *Ascaris lumbricoides*. *World J Gastroenterol* 2008; 14(17): 2783 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2783.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2783>

### TO THE EDITOR

Regarding the article eosinophilic cholecystitis along with pericarditis caused by *Ascaris lumbricoides*: A case report<sup>[1]</sup>, a coproparasitoscopic test should have been conducted to demonstrate the presence of the parasite in the digestive tract of the patient, since the presence of the *A. lumbricoides*'s antigen in the blood is nonspecific and only refers to the contact of the patient with the parasite<sup>[2-4]</sup>. It is likely that the parasitosis is the cause both of the eosinophilia and the cholecystitis. Nevertheless, because the histopathological study to demonstrate an increase of eosinophils in the gallbladder wall was not conducted<sup>[1]</sup>, the following doubt remains: did eosinophilia cause the cholecystitis? Because of clinical improvement with medical treatment with albendazole, surgery was not performed and the acute cholecystitis that went into remission following administration of the drug may be a consequence of migration of the parasite from the biliary tract<sup>[2]</sup>.

### REFERENCES

- 1 Kaji K, Yoshiji H, Yoshikawa M, Yamazaki M, Ikenaka Y, Noguchi R, Sawai M, Ishikawa M, Mashitani T, Kitade M, Kawaratani H, Uemura M, Yamao J, Fujimoto M, Mitoro A, Toyohara M, Yoshida M, Fukui H. Eosinophilic cholecystitis along with pericarditis caused by *Ascaris lumbricoides*: a case report. *World J Gastroenterol* 2007; 13: 3760-3762
- 2 Montiel-Jarquín A, Carrillo-Ríos C, Flores-Flores J. [Gallbladder ascariasis with acute hepatitis. Conservative treatment]. *Cir Cir* 2003; 71: 314-318
- 3 Benítez García F, Pacahuala del Carmen M. Ascariasis en vías biliares. Presentación de dos casos. *Rev Med IMSS* 1999; 37 (1): 19-23
- 4 Sánchez-Pobre P, López-Ríos M, Colima F, Yela C, Manzano M, Rodríguez S, Martín A, Casis B, Garfía C, Castellano G, Solís-Herruzo JA. [Eosinophilic cholecystitis: an infrequent cause of cholecystectomy] *Gastroenterol Hepatol* 1997; 20: 21-23

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### **Rakesh Aggarwal, Additional Professor**

Department of Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, India

### **Takafumi Ando, MD, PhD**

Department of Gastroenterology, Nagoya University Graduate School of Medicine, Therapeutic Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

### **Marc Basson, MD, PhD, MBA, Chief of Surgery**

John D Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48301, United States

### **Carla W Brady, MD, MHS**

Duke University Medical Center, Division of Gastroenterology, DUMC Box 3913, Durham, NC 27705, United States

### **Jordi Camps, PhD**

Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan, C. Sant Joan s/n, 43201 Reus, Catalunya, Spain

### **Ravi S Chari, MD, Associate Professor**

Division of Hepatobiliary Surgery and Liver Transplantation, Departments of Surgery and Cancer Biology, 1313 21<sup>st</sup> Avenue South Suite 801 Oxford House, Vanderbilt University Medical Center, Nashville, TN 37232-4753, United States

### **Abdellah Essaid, Professor**

Hospital Ibn Sina, Rabat 10100, Morocco

### **Alessandro Fichera, MD, FACS, FASCRS, Assistant Professor**

Department of Surgery-University of Chicago, 5841 S Maryland Ave, MC 5031, Chicago, IL 60637, United States

### **Zvi Fireman, MD, Associate Professor of Medicine, Head**

Gastroenterology Department, Hillel Yaffe Med Ctr, POB 169, 38100, Hadera, Israel

### **Jean L Frossard, Dr**

Division of gastroenterology, Geneva University Hospital, Rue Micheli du Crest, 1211 Geneva 14, Switzerland

### **Mitsuhiro Fujishiro, Dr**

Department of Gastroenterology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

### **Diego Garcia-Compean, MD, Professor**

Faculty of Medicine, University Hospital, Department of Gastroenterology, Autonomous University of Nuevo Leon, Ave Madero y Gonzalitos, 64700 Monterrey, NL, México

### **Diego Garcia-Compean, MD, Professor**

Faculty of Medicine, University Hospital, Department of

Gastroenterology, Autonomous University of Nuevo Leon, Ave Madero y Gonzalitos, 64700 Monterrey, NL, México

### **Paul Y Kwo, Professor**

Gastroenterology and Hepatology Division, Indiana University School of Medicine, 975 West Walnut, IB 327, Indianapolis, Indiana 46202-5121, United States

### **Anders E Lehmann, PhD, Associate Professor**

Senior Principal Scientist, Bioscience, AstraZeneca R&D Mölndal, Mölndal, Sweden

### **Peter J Mannon, MD**

Mucosal Immunity Section, Laboratory of Host Defense, National Institute of Allergy, Laboratory of Clinical Investigation, Building 10/CRC, Room 6-3742, 9000 Rockville Pike, Bethesda, Maryland 20892, United States

### **Sri P Misra, Professor**

Gastroenterology, Moti Lal Nehru Medical College, Allahabad 211001, India

### **James M Scheiman, Professor**

Division of Gastroenterology, University of Michigan Medical Center, 3912 Taubman Center, Box 0362, Ann Arbor, Michigan 48109-0362, United States

### **Francis Seow-Choen, Professor**

Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, 3 Mt Elizabeth Medical Centre #09-10, 228510, Singapore

### **Wing-Kin Syn, MD**

Division of Gastroenterology, GSRB-1, Suite 1073, DUMC 3256, 595 LaSalle Street, Durham, NC 27710, United States

### **Simon D Taylor-Robinson, MD**

Department of Medicine A, Imperial College London, Hammersmith Hospital, Du Cane Road, London W12 0HS, United Kingdom

### **Michael Torbenson, MD, Associate Professor of Pathology**

Room B314, 1503 E Jefferson (Bond Street Building), The Johns Hopkins University School of Medicine, Baltimore, MD 21231, United States

### **Debbie Trinder, PhD**

School of Medicine and Pharmacology, University of Western Australia, Fremantle Hospital, PO Box 480, Fremantle 6959, Western Australia, Australia

### **Yvan Vandenplas, Professor**

Department of Pediatrics, AZ-VUB, Laarbeeklaan 101, Brussels 1090, Belgium

### **Jian Wu, Associate Professor of Medicine**

Internal Medicine/Transplant Research Program, University of California, Davis Medical Center, 4635 2nd Ave. Suite 1001, Sacramento CA 95817, United States

### **Eric M Yoshida, MD**

Department of Medicine, University of British Columbia, 100-2647 Willow Street, Vancouver V5Z 3P1, Canada

### **Hiroshi Yoshida, MD**

First Department of Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan

## Meetings

### Events Calendar 2008-2009

#### FALK SYMPOSIA 2008

January 24-25, Frankfurt, Germany  
 Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008  
 February 14-16, Paris, France  
 EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
 8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
 3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

March 10-13, Birmingham, UK  
 British Society of Gastroenterology Annual Meeting  
 E-mail: [BSG@mailbox.ulcc.ac.uk](mailto:BSG@mailbox.ulcc.ac.uk)

March 14-15, HangZhou, China  
 Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
 Asian Pacific Association for the Study of the Liver  
 18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
 Shanghai - Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
 OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation - Management of Adeno- carcinomas  
 Email: [robert.giuli@oeso.org](mailto:robert.giuli@oeso.org)

April 18-22, Buenos Aires, Argentina  
 9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
 Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
 43<sup>rd</sup> Annual Meeting of the European

Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary  
 Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA  
 Digestive Disease Week 2008  
 June 4-7, Helsinki, Finland

The 39<sup>th</sup> Nordic Meeting of Gastroenterology  
[www.congrex.com/ngc2008](http://www.congrex.com/ngc2008)  
 June 6-8, Prague, Czech Republic  
 3<sup>rd</sup> Annual European Meeting: Perspectives in Inflammatory Bowel Diseases  
 Email: [meetings@imedex.com](mailto:meetings@imedex.com)

June 13-14, Amsterdam, Netherlands  
 Falk Symposium 165: XX International Bile Acid Meeting, Bile Acid Biology and Therapeutic Actions

June 25-28, Barcelona, Spain  
 10<sup>th</sup> World Congress on Gastrointestinal Cancer  
 Imedex and ESMO  
 Email: [meetings@imedex.com](mailto:meetings@imedex.com)

June 25-28, Lodz, Poland  
 Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatology (IAP)  
 E-mail: [office@epc-iap2008.org](mailto:office@epc-iap2008.org)  
[www.e-p-c.org](http://www.e-p-c.org)  
[www.pancreatology.org](http://www.pancreatology.org)

June 26-28, Bratislava, Slovakia  
 5<sup>th</sup> Central European Gastroenterology Meeting  
[www.ceurgem2008.cz](http://www.ceurgem2008.cz)  
 September 10-13, Budapest, Hungary

11<sup>th</sup> World Congress of the International Society for Diseases of the Esophagus  
 Email: [isde@isde.net](mailto:isde@isde.net)

September 13-16, New Delhi, India  
 Asia Pacific Digestive Week  
 E-mail: [apdw@apdw2008.net](mailto:apdw@apdw2008.net)

III FALK GASTRO-CONFERENCE  
 September 17, Mainz, Germany  
 Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany  
 Falk Symposium 166:  
 GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic  
 Prague Hepatology Meeting 2008  
[www.czech-hepatology.cz/phm2008](http://www.czech-hepatology.cz/phm2008)

September 20-21, Mainz, Germany  
 Falk Symposium 167:  
 Liver Under Constant Attack - From

Fat to Viruses  
 September 24-27, Nantes, France  
 Third Annual Meeting  
 European Society of Coloproctology  
[www.escp.eu.com](http://www.escp.eu.com)



October 8-11, Istanbul, Turkey  
 18<sup>th</sup> World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists  
 E-mail: [orkun.sahin@serenas.com.tr](mailto:orkun.sahin@serenas.com.tr)

October 18-22, Vienna, Austria  
 16<sup>th</sup> United European Gastroenterology Week  
[www.negf.org](http://www.negf.org)  
[www.acv.at](http://www.acv.at)

October 22-25, Brisbane, Australia  
 Australian Gastroenterology Week 2008  
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October 31-November 4, Moscone West Convention Center, San Francisco, CA  
 59<sup>th</sup> AASLD Annual Meeting and Postgraduate Course  
 The Liver Meeting  
 Information: [www.aasld.org](http://www.aasld.org)

November 6-9, Lucerne, Switzerland  
 Neurogastroenterology & Motility Joint International Meeting 2008  
 Email: [ngm2008@mci-group.com](mailto:ngm2008@mci-group.com)  
[www.ngm2008.com](http://www.ngm2008.com)

November 12, Santiago de Chile, Chile  
 Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

December 7-9, Seoul, Korea  
 6<sup>th</sup> International Meeting Hepatocellular Carcinoma: Eastern and Western Experiences  
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International Gastroenterological

Congresses 2009  
 March 23-26, Glasgow, Scotland  
 Meeting of the British Society of Gastroenterology (BSG)  
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May 17-20, Denver, Colorado, USA  
 Digestive Disease Week 2009

November 21-25, London, UK  
 Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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WJG publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidermiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

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- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaobua Zazhi* 1999; **7**: 285-287

*In press*

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

*Organization as author*

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

*No author given*

- 6 21st century heart solution may have a sting in the tail. *BMJ*



2002; 325: 184 [PMID: 12142303]

#### Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; 42 Suppl 2: S93-99 [PMID: 12028325]

#### Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (401): 230-238 [PMID: 12151900]

#### No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

### Books

#### Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

#### Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

#### Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wicczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

#### Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

#### Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

#### Electronic journal (list all authors)

Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

#### Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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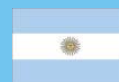
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<sup>[1]</sup>Passed away on October 20, 2007

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 Editorial Board of *World Journal of Gastroenterology*, 77 Shuangta Xijie, Taiyuan 030001, Shanxi Province, China  
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 E-mail: [wjg@wjgnet.com](mailto:wjg@wjgnet.com)

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# Occult persistence and lymphotropism of hepatitis C virus infection

Tram NQ Pham, Tomasz I Michalak

Tram NQ Pham, Tomasz I Michalak, Molecular Virology and Hepatology Research Group, Division of BioMedical Sciences, Faculty of Medicine, Health Sciences Centre, Memorial University, St. John's NL A1B 3V6, Canada

Author contributions: Pham TNQ and Michalak TI contributed equally to this paper.

Correspondence to: Tomasz I Michalak, MD, PhD, Molecular Virology and Hepatology Research Group, Faculty of Medicine, Health Sciences Centre, Memorial University, 300 Prince Philip Drive, St. John's NL A1B 3V6, Canada. [timich@mun.ca](mailto:timich@mun.ca)

Telephone: +1-709-7777301 Fax: +1-709-7778279

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## Abstract

Recent discovery of occult hepatitis C virus (HCV) infection persisting after spontaneous or antiviral therapy-induced resolution of hepatitis C was made possible by the introduction of nucleic acid amplification assays capable of detecting HCV RNA at sensitivities superseding those offered by clinical tests. Although individuals with this seemingly silent HCV infection are usually anti-HCV antibody reactive and have normal liver function tests, occult HCV infection has also been reported in anti-HCV-negative individuals with persistently elevated liver enzymes of unknown etiology. Studies have shown that HCV RNA can persist for years in serum, lymphomononuclear cells and liver in the absence of clinical symptoms, although histological evidence of a mild inflammatory liver injury can be occasionally encountered. Furthermore, while HCV RNA can be detected in circulating lymphoid cells in approximately 30% of cases, a short-term culture under stimulatory conditions augments HCV replication in these cells allowing detection of virus in otherwise HCV-negative cases. HCV infects different immune cell subsets, including CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, B cells and monocytes. Studies employing clonal sequencing and single-stranded conformational polymorphism analyses have revealed unique HCV variants residing in immune cells, further strengthening the notion of HCV lymphotropism. Overall, the data accumulated suggest that occult HCV infection is a common consequence of resolution of symptomatic hepatitis C and that examination of the cells of the immune system is an effective approach to diagnosis of HCV infection and its long-term persistence. Further work is required to fully realize pathogenic and epidemiological consequences of occult HCV persistence.

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**Key words:** Hepatitis C virus; Chronic hepatitis C; Occult viral persistence; HCV lymphotropism; Consequences of occult HCV persistence

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## INTRODUCTION

Hepatitis C virus (HCV) is an important human pathogen which infects over 170 million people world-wide and causes symptomatic chronic hepatitis in up to 85% of those inflicted. In a significant number of patients, chronic hepatitis C (CHC) eventually progresses to fibrosis, cirrhosis and hepatocellular carcinoma (HCC). In fact, it is estimated that cirrhosis can manifest in up to 35% of patients with CHC, of whom approximately 3% would develop HCC<sup>[1,2]</sup>. End-stage liver disease due to CHC is currently the number one reason for liver transplantation in many parts of the world. The global socioeconomic burden of HCV infection is further magnified by hundreds of thousands of infections identified each year.

HCV is a positive single-stranded RNA virus which belongs to the *Flaviviridae* family and replicates by making the so-called negative strand, which is also referred to as the anti-genomic strand. The virus genome of approximately 9600 base pairs in length contains an internal ribosomal entry site (IRES) located at the 5'-untranslated region (5'-UTR), which drives the translation of the viral RNA transcript. Subsequent processing of the polyprotein precursor gives rise to over ten proteins, including structural proteins which form the viral nucleocapsid and envelope, as well as several non-structural proteins which are essential to replication<sup>[3]</sup>.

The current standard treatment for CHC is a combination of pegylated-interferon alpha and Ribavirin (P-IFN $\alpha$ /RBV), which is administered for 24 wk to patients infected with HCV genotype 2 or 3 and for 48 wk

to those with genotype 1 or 4<sup>[4,5]</sup>. At present, patients who are serum HCV RNA non-reactive for at least 6 mo after completion of treatment, as determined by clinical laboratory assays, of which the sensitivities range between 9.6 and 615 IU or 30 and 1000 virus genome copies (also referred to as virus genome equivalents, vge) per mL, are considered to have achieved a sustained virological response (SVR) and would clinically be deemed “cured” of HCV. Thus, by this definition, SVR is attainable in approximately 40%-45% of patients infected with genotype 1<sup>[6,7]</sup> and in up to 69% of those carrying genotype 4<sup>[5]</sup>. HCV genotypes 2 and 3 are generally easiest to treat with up to 80% of patients afflicted with these strains achieving a SVR, as defined above<sup>[6,7]</sup>.

## IDENTIFICATION OF OCCULT HCV INFECTION

In the past four years, the identification of a new entity of HCV infection termed as occult HCV infection was made possible by the introduction of research assays which are capable of detecting HCV RNA at lower quantities ( $\leq 2$  IU or  $\leq 10$  virus genome copies per mL) than those used in clinical laboratories. One such research assay sequentially involves: (1) a reverse transcription (RT) of high quality intact total RNA extracted from serum or plasma, peripheral blood mononuclear cells (PBMC) or, when feasible, hepatic tissues; (2) a two-round (i.e., direct and nested) amplification of the resulting cDNA by polymerase chain reaction (PCR) using primers spanning different regions of the HCV genome; and (3) validation of the amplified products by nucleic acid hybridization (NAH) to recombinant HCV DNA probe<sup>[8]</sup>. By employing this assay or those with comparable sensitivities, low levels of HCV RNA can be detected in individuals for many years after clinical and biochemical recovery from hepatitis C<sup>[9-12]</sup>. In our studies, HCV RNA loads, as determined by the aforementioned method, in individuals who were followed for up to 7 years after SVR, were usually below 100 virus copies per mL of plasma or serum and, in most cases, ranged 100-1000 virus copies per  $10^7$  circulating lymphoid cells<sup>[9,13]</sup>. Comparable levels of HCV genomes were also observed by others<sup>[10]</sup>. Furthermore, longitudinal analyses of serum or plasma and PBMC samples obtained from the same patients at different time points of SVR duration or after spontaneous recovery from hepatitis C revealed that HCV RNA typically would not fluctuate by more than ten-fold between collections and that screening sequential samples enhanced identification of occult HCV persistence<sup>[8,13,14]</sup>. In this regard, when serum samples collected 12 mo after the first one were analyzed, the overall HCV RNA positivity was increased by as much as 15% of the cases examined<sup>[13]</sup>.

The discovery of occult HCV infection has, in essence, directly challenged the accepted paradigm that apparent complete resolution of hepatitis C, either spontaneously or therapeutically-induced, would be indicative of eradication of HCV<sup>[8]</sup>. It should be pointed out that although HCV persistence after resolution of CHC was first made evident from studies using the RT-PCR/NAH or equivalent

research assays, data from more recent studies suggested that this form of clinically unapparent, but molecularly evident HCV infection could also be identified when clinical assays of enhanced sensitivity are employed. On this note, it was reported that using the Roche Cobas-Amplicor assay (sensitivity: 50 virus copies/mL), HCV RNA was detected in freshly isolated blood mononuclear cells of approximately 20% of individuals with clinical SVR<sup>[12]</sup>. Furthermore, in another study conducted by another group, over 11% of CHC patients who initially failed IFN $\alpha$  monotherapy, but achieved clinical SVR after successful completion of P-IFN $\alpha$ /RBV were also found to carry residual HCV RNA when their sera were tested by the Cobas-Amplicor assay<sup>[15]</sup>.

In addition to the documentation of HCV RNA in serum or plasma, PBMC and hepatic tissue in patients with resolved hepatitis C in whom alanine aminotransferase (ALT) levels were deemed repeatedly normal, HCV genomes were also identified in the same three aforementioned compartments in patients with persistently elevated liver enzymes, who were consistently negative for serological markers typical of a *bona fide* HCV infection<sup>[11,16-18]</sup>.

## LYMPHOTROPISM OF HCV

Although hepatocytes are considered to be primary targets of HCV, clinical and experimental evidence strongly indicates that the virus also invades and replicates in cells of other organs, particularly the immune system<sup>[19-21]</sup>. In doing so, HCV may effectively equip itself with one of the most efficient mechanisms to establish long-term, if not life-long, persistence, as it has been documented for other viruses equally capable of inducing protracted infections<sup>[22-24]</sup>. The notion of HCV lymphotropism is further supported by a greater representation of disorders of the lymphatic system in patients with CHC than in those without, including type II mixed cryoglobulinemia<sup>[25]</sup> and non-Hodgkin's lymphoma<sup>[26]</sup>.

Recent findings from our studies with different cohorts of patients with either spontaneous or therapy-induced resolution of hepatitis C showed that HCV RNA, on average, is detectable in freshly isolated PBMC in about 30% of cases, at levels ranging 100-1000 virus copies per  $10^7$  cells. However, in approximately 10% of such individuals, HCV RNA can reach  $10^4$  virus copies per  $10^7$  cells or higher, a level which is typically observed in patients with CHC<sup>[13]</sup>. In individuals where PBMC were apparently negative for HCV RNA, the use of mitogen cocktails supplemented with interleukin-2 (IL-2) and IL-4 to stimulate T and B lymphocytes and monocytes in 72-h cultures, augments HCV replication in the respective cells leading to enhanced detection of the residing virus<sup>[9,13,27]</sup>. Using this approach, HCV RNA positive strand could be identified in approximately 75% of seemingly HCV-negative cases<sup>[9,13,14,27]</sup>. Of note, the presence of HCV RNA positive strand in mitogen-treated cells is nearly always accompanied by that of HCV RNA negative (replicative) strand, indicative of authentic viral replication. Moreover, non-structural HCV proteins, such as NS5A, are also

detectable in circulating immune cells, as our recent study clearly documented<sup>[14]</sup>. Interestingly, in many cases of occult infection, HCV RNA expression was found to be higher in cells treated with a cocktail of mitogens, which simultaneously stimulated different immune cell types, compared to those treated with single mitogens<sup>[13,27]</sup>. This implied that within a given individual, different immune cell subsets may be infected with HCV. Indeed, this observation was unequivocally confirmed in our most recent study<sup>[14]</sup> in which different affinity-purified immune cell types, e.g. CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes, B cells and monocytes, were found to be infected to a varying extent in different individuals. We have also established that HCV infection can be confined to a specific immune cell subtype, as evidenced by the presence of replicating HCV in affinity-purified cell types but not in total PBMC. This finding highlights the need to screen individual immune cell populations, in addition to PBMC, for possible HCV presence before occult infection can be irrefutably excluded.

The use of clonal sequencing and highly sensitive single-stranded conformational analysis (SSCP), which allows for identification of even single nucleotide polymorphisms, has enabled the identification of HCV variants which appeared unique to immune cells. For example, sequence polymorphisms located at the IRES of the 5'-UTR and the hypervariable region (HVR) of the second envelope glycoprotein (E2) were observed in lymphoid cells of individuals with occult persistent HCV infection<sup>[10,12-14,28]</sup>. The fact that these variants were different from those found in the serum or liver obtained in parallel further strengthened the notion of HCV lymphotropism<sup>[10,12-14]</sup>. Additional support for the inherent propensity of HCV to infect and propagate in cells of the immune system came from *in vitro* studies documenting that certain substitutions found in individuals with occult HCV infection<sup>[10,12,14]</sup> were identical to those that emerged from wild-type HCV passaged through untransformed T cell and lymphoblastoid cell cultures<sup>[29,30]</sup>.

## POTENTIAL CLINICAL CONSEQUENCES OF OCCULT HCV INFECTION

As of now, clinical data pertinent to the clinical significance of occult HCV persistence are still in its infancy. However, it is hypothesized that persistent HCV replication in hepatocytes and lymphoid cells would likely lead to continuous antigenic stimulation of the immune system in immunocompetent patients, which in turn, allows the host to keep this silent infection under relative control. This concept has been supported by demonstration of sustained HCV-specific T cell cytotoxic and proliferative responses in patients years after recovery from hepatitis C<sup>[31-33]</sup>. Similarly, T cell responses to HCV antigens were also evident in patients with persistently elevated liver enzymes of unknown etiology who were HCV RNA reactive in both lymphoid cells and the liver<sup>[34]</sup>. On the other hand, such prolonged HCV replication associated with the continuous presentation of HCV antigens by infected B cells and monocytes may contribute to the

immune tolerance of HCV, hence, favouring even further virus persistence. At present, it remains unknown whether and how infection of the immune cells by HCV may alter their functions, although impairment in the allostimulatory capacity of HCV-infected dendritic cells derived from patients with CHC has been reported<sup>[35]</sup>.

In certain scenarios, including immunosuppression, immunomodulatory therapy or co-infection, instead of eliciting desirable T cell responses in the host, persistent replicating HCV could represent a potential source for virus reactivation, as it has been shown in other viruses, including hepatitis B virus<sup>[8]</sup> and human herpesvirus 6<sup>[36]</sup>. In this regard, corticosteroid-induced immunosuppression has been shown to affect HCV reactivation years after spontaneous resolution of acute hepatitis C<sup>[37]</sup>. Along this line, recurrent HCV infection has been reported in liver transplant recipients who were deemed free of HCV RNA at the time of transplantation, as evidenced by negativity of HCV RNA in serum and the explanted liver tissue assessed by Cobas Amplicor assay with a sensitivity of 50 copies/mL<sup>[38]</sup>. Furthermore, HCV replication was found to be frequent among patients positive for antibodies to HCV (anti-HCV) who received HCV-negative kidney<sup>[39]</sup>. In this study, HCV became detectable around 30 d with the viremia peaking on d 62 post-transplantation. Similarly, approximately 18% of bone marrow recipients who were HCV seropositive prior to transplantation became reactive to HCV RNA after receiving a transplant from an apparently HCV-negative donor<sup>[40]</sup>.

## CONCLUDING REMARKS

The availability of research assays capable of detecting HCV RNA at sensitivities superior to those offered by clinical assays significantly contributed to the recent discovery of occult HCV infection in individuals years after having been clinically deemed to have cleared the virus. Interestingly, when HCV reactivity in both plasma/serum and peripheral lymphomononuclear cells is taken into consideration, nearly all individuals with apparent complete resolution of hepatitis C can be found to carry low levels of HCV RNA. The fact that the HCV RNA replicative strand and viral proteins are detectable in immune cells, as well as that certain HCV variants are unique to immune cells, lends strong support to the existence of an extrahepatic compartment of HCV replication. Overall, the data accumulated in recent years highlight not only the need for development and implementation of more sensitive HCV RNA diagnostic assays but also the importance of screening both serum and peripheral immune cells for HCV RNA<sup>[8]</sup>. At present, the data pertinent to pathogenic and epidemiological consequences of occult HCV infection remain very sparse. Further research in this area is of significant clinical relevance in which, as works from our and other groups have shown, an involvement of the infected immune system should not be neglected.

## REFERENCES

- 1 El-Serag HB, Rudolph KL. Hepatocellular carcinoma:

- epidemiology and molecular carcinogenesis. *Gastroenterology* 2007; **132**: 2557-2576
- 2 **Freeman AJ**, Dore GJ, Law MG, Thorpe M, Von Overbeck J, Lloyd AR, Marinos G, Kaldor JM. Estimating progression to cirrhosis in chronic hepatitis C virus infection. *Hepatology* 2001; **34**: 809-816
  - 3 **Brass V**, Moradpour D, Blum HE. Molecular virology of hepatitis C virus (HCV): 2006 update. *Int J Med Sci* 2006; **3**: 29-34
  - 4 **Strader DB**, Wright T, Thomas DL, Seeff LB. Diagnosis, management, and treatment of hepatitis C. *Hepatology* 2004; **39**: 1147-1171
  - 5 **Kamal SM**, El Tawil AA, Nakano T, He Q, Rasenack J, Hakam SA, Saleh WA, Ismail A, Aziz AA, Madwar MA. Peginterferon {alpha}-2b and ribavirin therapy in chronic hepatitis C genotype 4: impact of treatment duration and viral kinetics on sustained virological response. *Gut* 2005; **54**: 858-866
  - 6 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982
  - 7 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965
  - 8 **Michalak TI**, Pham TNQ, Mulrooney-Cousins PM. Molecular diagnosis of occult hepatitis C and hepatitis B virus infections. *Future Virol* 2007; **2**: 451-465
  - 9 **Pham TN**, MacParland SA, Mulrooney PM, Cooksley H, Naoumov NV, Michalak TI. Hepatitis C virus persistence after spontaneous or treatment-induced resolution of hepatitis C. *J Virol* 2004; **78**: 5867-5874
  - 10 **Radkowski M**, Gallegos-Orozco JF, Jablonska J, Colby TV, Walewska-Zielecka B, Kubicka J, Wilkinson J, Adair D, Rakela J, Laskus T. Persistence of hepatitis C virus in patients successfully treated for chronic hepatitis C. *Hepatology* 2005; **41**: 106-114
  - 11 **Castillo I**, Rodriguez-Inigo E, Lopez-Alcorocho JM, Pardo M, Bartolome J, Carreno V. Hepatitis C virus replicates in the liver of patients who have a sustained response to antiviral treatment. *Clin Infect Dis* 2006; **43**: 1277-1283
  - 12 **Di Liberto G**, Roque-Afonso AM, Kara R, Ducoulombier D, Fallot G, Samuel D, Feray C. Clinical and therapeutic implications of hepatitis C virus compartmentalization. *Gastroenterology* 2006; **131**: 76-84
  - 13 **Pham TN**, Mulrooney-Cousins PM, Mercer SE, MacParland SA, Inglot M, Zalewska M, Simon K, Michalak TI. Antagonistic expression of hepatitis C virus and alpha interferon in lymphoid cells during persistent occult infection. *J Viral Hepat* 2007; **14**: 537-548
  - 14 **Pham TN**, King D, Macparland SA, McGrath JS, Reddy SB, Bursey FR, Michalak TI. Hepatitis C virus replicates in the same immune cell subsets in chronic hepatitis C and occult infection. *Gastroenterology* 2008; **134**: 812-822
  - 15 **Ciancio A**, Smedile A, Giordanino C, Colletta C, Croce G, Pozzi M, Cariti G, Macor A, Biglino A, Di Napoli A, Tappero GF, Andreoni M, Manca A, Prandi G, Calleri G, Orsi PG, Ciccone G, Rizzetto M, Saracco G. Long-term follow-up of previous hepatitis C virus positive nonresponders to interferon monotherapy successfully retreated with combination therapy: are they really cured? *Am J Gastroenterol* 2006; **101**: 1811-1816
  - 16 **Castillo I**, Pardo M, Bartolome J, Ortiz-Movilla N, Rodriguez-Inigo E, de Lucas S, Salas C, Jimenez-Heffernan JA, Perez-Mota A, Graus J, Lopez-Alcorocho JM, Carreno V. Occult hepatitis C virus infection in patients in whom the etiology of persistently abnormal results of liver-function tests is unknown. *J Infect Dis* 2004; **189**: 7-14
  - 17 **Bartolome J**, Lopez-Alcorocho JM, Castillo I, Rodriguez-Inigo E, Quiroga JA, Palacios R, Carreno V. Ultracentrifugation of serum samples allows detection of hepatitis C virus RNA in patients with occult hepatitis C. *J Virol* 2007; **81**: 7710-7715
  - 18 **Carreno V**. Occult hepatitis C virus infection: a new form of hepatitis C. *World J Gastroenterol* 2006; **12**: 6922-6925
  - 19 **Blackard JT**, Kemmer N, Sherman KE. Extrahepatic replication of HCV: insights into clinical manifestations and biological consequences. *Hepatology* 2006; **44**: 15-22
  - 20 **Pham TN**, Michalak TI. Occult hepatitis C virus persistence: identification and characteristics. *MLO Med Lab Obs* 2006; **38**: 20-22
  - 21 **Radkowski M**, Wilkinson J, Nowicki M, Adair D, Vargas H, Ingui C, Rakela J, Laskus T. Search for hepatitis C virus negative-strand RNA sequences and analysis of viral sequences in the central nervous system: evidence of replication. *J Virol* 2002; **76**: 600-608
  - 22 **Oldstone MB**. Virus-lymphoid cell interactions. *Proc Natl Acad Sci USA* 1996; **93**: 12756-12758
  - 23 **Ciurea A**, Klenerman P, Hunziker L, Horvath E, Odermatt B, Ochsenbein AF, Hengartner H, Zinkernagel RM. Persistence of lymphocytic choriomeningitis virus at very low levels in immune mice. *Proc Natl Acad Sci USA* 1999; **96**: 11964-11969
  - 24 **Michalak TI**. Occult persistence and lymphotropism of hepadnaviral infection: insights from the woodchuck viral hepatitis model. *Immunol Rev* 2000; **174**: 98-111
  - 25 **Agnello V**, De Rosa FG. Extrahepatic disease manifestations of HCV infection: some current issues. *J Hepatol* 2004; **40**: 341-352
  - 26 **Matsuo K**, Kusano A, Sugumar A, Nakamura S, Tajima K, Mueller NE. Effect of hepatitis C virus infection on the risk of non-Hodgkin's lymphoma: a meta-analysis of epidemiological studies. *Cancer Sci* 2004; **95**: 745-752
  - 27 **Pham TN**, Macparland SA, Coffin CS, Lee SS, Bursey FR, Michalak TI. Mitogen-induced upregulation of hepatitis C virus expression in human lymphoid cells. *J Gen Virol* 2005; **86**: 657-666
  - 28 **Ducoulombier D**, Roque-Afonso AM, Di Liberto G, Penin F, Kara R, Richard Y, Dussaix E, Feray C. Frequent compartmentalization of hepatitis C virus variants in circulating B cells and monocytes. *Hepatology* 2004; **39**: 817-825
  - 29 **MacParland SA**, Pham TN, Gujar SA, Michalak TI. De novo infection and propagation of wild-type Hepatitis C virus in human T lymphocytes in vitro. *J Gen Virol* 2006; **87**: 3577-3586
  - 30 **Nakajima N**, Hijikata M, Yoshikura H, Shimizu YK. Characterization of long-term cultures of hepatitis C virus. *J Virol* 1996; **70**: 3325-3329
  - 31 **Cramp ME**, Carucci P, Rossol S, Chokshi S, Maertens G, Williams R, Naoumov NV. Hepatitis C virus (HCV) specific immune responses in anti-HCV positive patients without hepatitis C viraemia. *Gut* 1999; **44**: 424-429
  - 32 **Quiroga JA**, Llorente S, Castillo I, Rodriguez-Inigo E, Lopez-Alcorocho JM, Pardo M, Carreno V. Virus-specific T cell responses associated with hepatitis C virus (HCV) persistence in the liver after apparent recovery from HCV infection. *J Med Virol* 2006; **78**: 1190-1197
  - 33 **Takaki A**, Wiese M, Maertens G, Depla E, Seifert U, Liebetrau A, Miller JL, Manns MP, Rehermann B. Cellular immune responses persist and humoral responses decrease two decades after recovery from a single-source outbreak of hepatitis C. *Nat Med* 2000; **6**: 578-582
  - 34 **Quiroga JA**, Llorente S, Castillo I, Rodriguez-Inigo E, Pardo M, Carreno V. Cellular immune responses associated with occult hepatitis C virus infection of the liver. *J Virol* 2006; **80**: 10972-10979
  - 35 **Bain C**, Fatmi A, Zoulim F, Zarski JP, Trepo C, Inchauspe G. Impaired allostimulatory functions of dendritic cells in chronic hepatitis C patients. *Gastroenterology* 2001; **120**: 512-524
  - 36 **Caserta MT**, McDermott MP, Dewhurst S, Schnabel K, Carnahan JA, Gilbert L, Lathan G, Lofthus GK, Hall CB. Human herpesvirus 6 (HHV6) DNA persistence and reactivation in healthy children. *J Pediatr* 2004; **145**: 478-484
  - 37 **Lee WM**, Polson JE, Carney DS, Sahin B, Gale M Jr.



- Reemergence of hepatitis C virus after 8.5 years in a patient with hypogammaglobulinemia: evidence for an occult viral reservoir. *J Infect Dis* 2005; **192**: 1088-1092
- 38 **Forns X**, Garcia-Retortillo M, Serrano T, FeliuSuarez F, de la Mata M, Garcia-Valdecasas JC, Navasa M, Rimola A, Rodes J. Antiviral therapy of patients with decompensated cirrhosis to prevent recurrence of hepatitis C after liver transplantation. *J Hepatol* 2003; **39**: 389-396
- 39 **Melon S**, Galarraga MC, Villar M, Laures A, Boga JA, de Ona M, Gomez E. Hepatitis C virus reactivation in anti-hepatitis C virus positive renal transplant recipients. *Transplant Proc* 2005; **37**: 2083-2085
- 40 **Zekri AR**, Mohamed WS, Samra MA, Sherif GM, El-Shehaby AM, El-Sayed MH. Risk factors for cytomegalovirus, hepatitis B and C virus reactivation after bone marrow transplantation. *Transpl Immunol* 2004; **13**: 305-311

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OBSERVER

Hugh James Freeman, MD, Professor, Series Editor

## Recent developments on the role of *Clostridium difficile* in inflammatory bowel disease

Hugh James Freeman

Hugh James Freeman, Department of Medicine (Gastroenterology), University of British Columbia, Vancouver V6T 1W5, Canada

Author contributions: Freeman HJ contributed all to this paper.  
Correspondence to: Dr. Hugh James Freeman, MD, FRCPC, FACP, Department of Medicine Gastroenterology, University of British Columbia, 2211 Wesbrook Mall, Vancouver V6T 1W5, Canada. [hugfree@shaw.ca](mailto:hugfree@shaw.ca)

Telephone: +1-604-8227216 Fax: +1-604-8227236

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### Abstract

*Clostridium difficile* (CD), specifically its toxins, have been implicated as a risk factor for exacerbation of the inflammatory process in up to 5% of patients with ulcerative colitis or Crohn's disease. Typical evidence of colonic changes with CD infection, including pseudomembranous exudate, are often not present; however, a severe clinical course may result, including precipitation of toxic colitis and toxic megacolon. Recently, hypervirulent CD strains have been reported raising concern for a more severe disease process in patients with underlying inflammatory bowel disease. Moreover, small bowel involvement or CD enteritis has been increasingly described, usually in those with a history of a prior colectomy or total proctocolectomy for prior severe and extensive inflammatory bowel disease. Finally, refractory or treatment-resistant pouchitis may occur with CD infection.

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**Key words:** Crohn's disease; Ulcerative colitis; Antibiotic-associated colitis; Cytotoxin; Enterotoxin; Pseudomembranous colitis; *Clostridium difficile* colitis

**Peer reviewer:** Andrew Ukleja, MD, Assistant Professor, Clinical Assistant Professor of Medicine, Director of Nutrition Support Team, Director of Esophageal Motility Laboratory, Cleveland Clinic Florida, Department of Gastroenterology, 2950 Cleveland Clinic Blvd., Weston, FL 33331, United States

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### INTRODUCTION

Considerable information has emerged on the intriguing relationship between the intestinal luminal microflora and the pathogenesis of inflammatory bowel disease<sup>[1]</sup>. While not believed to play an etiologic role, one particular organism, *Clostridium difficile* (CD) has become increasingly recognized as a risk factor for exacerbation of the inflammatory process in ulcerative colitis or Crohn's colitis<sup>[2]</sup>. In recent years, there has also been a marked increase in the apparent severity of disease associated with CD per se, especially with a hypervirulent strain (e.g. B1/NAP1/027) that exhibits fluoroquinolone resistance and has been detected in spite of metronidazole treatment. There have also been reports showing increased mortality and more complex CD disease with this hypervirulent strain, initially in Quebec, an eastern province of Canada, and later from other centers in North America and Europe<sup>[3-5]</sup>.

### CD TOXINS AND CD DISEASE

After 1977, evidence rapidly accumulated to show that toxins produced by the microbial agent, CD, rather than the organism, were responsible for significant and sometimes severe inflammatory changes in the colon, particularly pseudomembranous colitis. This usually occurred after antibiotic use that was thought to alter the normal intestinal microflora so that CD could colonize the intestine. Larson *et al*<sup>[6]</sup> made the initial observation during attempts to isolate a virus from stool of a 12-year-old female with penicillin-associated pseudomembranous colitis. Diluted fecal ultrafiltrates were toxic to tissue-cultured cells; however, this effect was not due to a viral agent. In addition, toxin concentration decreased with improved clinical status. Others examined clindamycin-induced cecitis in a hamster model and showed that vancomycin was protective, further implicating a bacterial cause<sup>[7]</sup>. Rifkin *et al*<sup>[8]</sup> showed that stool toxin from patients with the disease could be specifically neutralized in tissue culture by antitoxin. Later, toxigenic CD was cultured from fecal material of patients with antibiotic-associated pseudomembranous colitis and CD toxin was also neutralized by antitoxin.

CD causes diarrhea, often watery, rather than bloody, developing within 48 to 72 h after infection. In some, symptoms may be delayed for 2 to 3 mo, usually after an

antimicrobial agent had been administered. In some, only a single antibiotic tablet may lead to severe disease. Over time, the clinical spectrum has become better appreciated with illness severity noted to be broad ranging from an asymptomatic carrier state (without detectable toxin) to severe and life-threatening pseudomembranous colitis with toxic megacolon<sup>[2]</sup>. In others, persistent symptoms or recurrent bouts of disease develop, in part, likely reflecting the capability of the CD organism to form spores.

CD produces at least two distinct toxins<sup>[9]</sup>. These have been labeled toxin A and toxin B. Although initially thought to have distinctive actions, both now appear to be cytotoxic and enteropathic. These disrupt the actin cytoskeleton of intestinal epithelial cells by uridine diphosphate-glucose dependent glycosylation of Rho and Ras proteins<sup>[10]</sup>. Other toxins have been described, but their significance is not clear<sup>[11,12]</sup>. The most widely used laboratory assays for CD involve toxin A and/or toxin B detection and both are usually detected if diarrhea is present. Atypical toxin variant strains that may cause symptoms have also been described from Asia<sup>[13]</sup>. So far, there is no widely available clinical detection method for hypervirulent strains. Treatment for hypervirulent CD strains, however, appears to be no different from other CD infections, including oral vancomycin<sup>[14]</sup>. Recent evidence suggests that PCR (rather than the widely used ELISA assays) may not only permit detection of toxins, but also identify virulent strains, including epidemic strains<sup>[14]</sup>.

## CD AND INFLAMMATORY BOWEL DISEASE

CD toxin was later detected in patients with inflammatory bowel disease, especially with symptomatic relapse<sup>[15-23]</sup>. In some, no prior antibiotic administration was recorded and symptoms responded to vancomycin. Previously, some “relapses” may have been assumed to be due to “disease activity” of the underlying inflammatory bowel disease. Some thought that drugs used in medical treatment (e.g. sulfasalazine) might alter the intestinal flora and promote CD colonization<sup>[20]</sup>. Others theorized that altered immune status, possibly related to therapeutic agents, or nutritional status might be important. Pseudomembranous exudates were not always present with underlying colitis<sup>[15,17]</sup>. Also, CD toxin was detected in ileostomy fluid with symptomatically increased ileostomy output; this resolved with vancomycin. This finding suggested that CD, under special circumstances, might be able to cause small bowel as well as colonic disease<sup>[15]</sup>. Another report described toxic megacolon with CD toxin in two patients that resolved with metronidazole<sup>[23]</sup>. In both, underlying inflammatory bowel disease was noted, including Crohn’s colitis and ulcerative colitis. Thus, early recognition of CD in those with known colitis might permit antibiotic treatment and reversal of toxic megacolon.

More recent investigations have confirmed and extended these early reports<sup>[24,25]</sup>. CD was the most common infecting organism in hospitalized patients with inflammatory bowel disease, recently estimated to occur in up to 5% of patients<sup>[25]</sup>. Their numbers also appear to be increasing and account for a large proportion of all

patients in hospital with CD infections<sup>[24,25]</sup>. This may be due to several factors<sup>[2]</sup>: first, increased awareness of the need to test for CD toxins, particularly soon after hospital admission as many CD infections in colitis patients are community acquired; second, increased detection, due to the sensitivity of modern toxin tests; third, many hospitalized patients (including those with Crohn’s or ulcerative colitis) may have other co-morbidities or reduced resistance to infection; and finally, increased use of proton pump inhibitors, antibiotics and immune modulators may also alter the normal intestinal microbial flora.

Reports have also noted the occurrence of CD enteritis usually with colitis, but very rarely as an independent small intestinal infection in the absence of colitis<sup>[26-32]</sup>. In the latter, this usually occurs after substantive colon resections, often for underlying severe and extensive colitis<sup>[28]</sup>. This is not entirely surprising since prior autopsy studies and cultures of jejunal aspirates have suggested that the small bowel per se may be a reservoir for CD<sup>[33,34]</sup>. Most often, these have been identified in elderly patients and pseudomembranous enterocolitis was found<sup>[32]</sup>. Most patients had prior gastrointestinal surgery, especially colonic resections, and usually these were patients that had a colectomy or total proctocolectomy for severe ulcerative colitis. Often, the CD infection occurred soon after colectomy, but in some, the colon resection was done even years earlier<sup>[32]</sup>. In most, a severe, often fatal, clinical course was initially noted<sup>[32]</sup>, although this may now be reduced<sup>[35]</sup>.

The pathogenesis has not been precisely defined. Most had prior use of broad spectrum antibiotics. As CD usually affects the colon, the factors that predispose to small bowel disease are not known. Changes in the small intestinal flora after colectomy may lead to development of a small intestinal environment similar to the colon, susceptible to CD overgrowth following antibiotic usage. Some have shown the colonic-type bacteria grow rapidly in the distal small bowel after ileocolonic resection<sup>[36]</sup>. Others have reported that the phenotypic histological changes develop in distal ileum so that colonic epithelial characteristics are seen<sup>[37]</sup>. CD toxin has also been detected in patients that have undergone pelvic pouch reconstruction<sup>[38-40]</sup>. In these, pouchitis or refractory pouchitis may be present.

## CONCLUSION

The diagnosis of CD-related disease with a positive toxin assay as a cause for new or worsening symptoms in patients with underlying inflammatory bowel disease is significant as it may lead to antibiotic treatment that entirely reverses the exacerbation of clinical symptoms. Usually, disease affects the colon, but, interestingly, in patients with underlying Crohn’s colitis or ulcerative colitis, pseudomembranous changes may not occur. In addition, the ileal mucosa may be at increased risk for inflammatory disease in a specific subset of patients that have undergone a prior colectomy. As a result, CD enteritis may result, possibly because the residual ileum has developed phenotypic features of the colonic luminal environment or the colonic mucosa per se. Similarly, chronic or refractory pouchitis may result from CD toxin after colectomy due

to CD colonization of the proximal small bowel, pouch mucosa or the residual rectal cuff mucosa. In these pouch patients, CD treatment may resolve the pouchitis.

## REFERENCES

- 1 Sartor RB. Microbial influences in inflammatory bowel diseases. *Gastroenterology* 2008; **134**: 577-594
- 2 Tremaine WJ. Inflammatory Bowel Disease and Clostridium difficile-associated diarrhea: a growing problem. *Clin Gastroenterol Hepatol* 2007; **5**: 310-311
- 3 Cookson B. Hypervirulent strains of Clostridium difficile. *Postgrad Med J* 2007; **83**: 291-295
- 4 Pepin J, Valiquette L, Alary ME, Villemure P, Pelletier A, Forget K, Pepin K, Chouinard D. Clostridium difficile-associated diarrhea in a region of Quebec from 1991 to 2003: a changing pattern of disease severity. *CMAJ* 2004; **171**: 466-472
- 5 Pepin J, Valiquette L, Cossette B. Mortality attributable to nosocomial Clostridium difficile-associated disease during an epidemic caused by a hypervirulent strain in Quebec. *CMAJ* 2005; **173**: 1037-1042
- 6 Larson HE, Parry JV, Price AB, Davies DR, Dolby J, Tyrrell DA. Undescribed toxin in pseudomembranous colitis. *Br Med J* 1977; **1**: 1246-1248
- 7 Bartlett JG, Onderdonk AB, Cisneros RL, Kasper DL. Clindamycin-associated colitis due to a toxin-producing species of Clostridium in hamsters. *J Infect Dis* 1977; **136**: 701-705
- 8 Rifkin GD, Fekety FR, Silva J Jr. Antibiotic-induced colitis: implication of a toxin neutralised by Clostridium sordellii antitoxin. *Lancet* 1977; **2**: 1103-1106
- 9 McFarland LV. Update on the changing epidemiology of Clostridium difficile-associated disease. *Nat Clin Pract Gastroenterol Hepatol* 2008; **5**: 40-48
- 10 Rupnik M, Dupuy B, Fairweather NF, Gerding DN, Johnson S, Just I, Lysterly DM, Popoff MR, Rood JL, Sonenshein AL, Thelestam M, Wren BW, Wilkins TD, von Eichel-Streiber C. Revised nomenclature of Clostridium difficile toxins and associated genes. *J Med Microbiol* 2005; **54**: 113-117
- 11 Geric B, Rupnik M, Gerding DN, Grabnar M, Johnson S. Distribution of Clostridium difficile variant toxinotypes and strains with binary toxin genes among clinical isolates in an American hospital. *J Med Microbiol* 2004; **53**: 887-894
- 12 Goncalves C, Decre D, Barbut F, Burghoffer B, Petit JC. Prevalence and characterization of a binary toxin (actin-specific ADP-ribosyltransferase) from Clostridium difficile. *J Clin Microbiol* 2004; **42**: 1933-1939
- 13 Rupnik M, Kato N, Grabnar M, Kato H. New types of toxin A-negative, toxin B-positive strains among Clostridium difficile isolates from Asia. *J Clin Microbiol* 2003; **41**: 1118-1125
- 14 Bartlett JG. Narrative review: the new epidemic of Clostridium difficile-associated enteric disease. *Ann Intern Med* 2006; **145**: 758-764
- 15 LaMont JT, Trnka YM. Therapeutic implications of Clostridium difficile toxin during relapse of chronic inflammatory bowel disease. *Lancet* 1980; **1**: 381-383
- 16 Bolton RP, Sherriff RJ, Read AE. Clostridium difficile associated diarrhoea: a role in inflammatory bowel disease? *Lancet* 1980; **1**: 383-384
- 17 Trnka YM, LaMont JT. Association of Clostridium difficile toxin with symptomatic relapse of chronic inflammatory bowel disease. *Gastroenterology* 1981; **80**: 693-696
- 18 Meyers S, Mayer L, Bottone E, Desmond E, Janowitz HD. Occurrence of Clostridium difficile toxin during the course of inflammatory bowel disease. *Gastroenterology* 1981; **80**: 687-690
- 19 Keighley MR, Youngs D, Johnson M, Allan RN, Burdon DW. Clostridium difficile toxin in acute diarrhoea complicating inflammatory bowel disease. *Gut* 1982; **23**: 410-414
- 20 Pokorney BH, Nichols TW Jr. Pseudomembranous colitis. A complication of sulfasalazine therapy in a patient with Crohn's colitis. *Am J Gastroenterol* 1981; **76**: 374-376
- 21 Rolny P, Jarnerot G, Mollby R. Occurrence of Clostridium difficile toxin in inflammatory bowel disease. *Scand J Gastroenterol* 1983; **18**: 61-64
- 22 Dorman SA, Liggoria E, Winn WC Jr, Beeken WL. Isolation of Clostridium difficile from patients with inactive Crohn's disease. *Gastroenterology* 1982; **82**: 1348-1351
- 23 Bolton RP, Read AE. Clostridium difficile in toxic megacolon complicating acute inflammatory bowel disease. *Br Med J (Clin Res Ed)* 1982; **285**: 475-476
- 24 Rodemann JF, Dubberke ER, Reske KA, Seo da H, Stone CD. Incidence of Clostridium difficile infection in inflammatory bowel disease. *Clin Gastroenterol Hepatol* 2007; **5**: 339-344
- 25 Issa M, Vijayapal A, Graham MB, Beaulieu DB, Otterson MF, Lundeen S, Skaros S, Weber LR, Komorowski RA, Knox JF, Emmons J, Bajaj JS, Binion DG. Impact of Clostridium difficile on inflammatory bowel disease. *Clin Gastroenterol Hepatol* 2007; **5**: 345-351
- 26 Kuntz DP, Shortsleeve MJ, Kantrowitz PA, Gauvin GP. Clostridium difficile enteritis. A cause of intramural gas. *Dig Dis Sci* 1993; **38**: 1942-1944
- 27 Tsutaoka B, Hansen J, Johnson D, Holodniy M. Antibiotic-associated pseudomembranous enteritis due to Clostridium difficile. *Clin Infect Dis* 1994; **18**: 982-984
- 28 Yee HF Jr, Brown RS Jr, Ostroff JW. Fatal Clostridium difficile enteritis after total abdominal colectomy. *J Clin Gastroenterol* 1996; **22**: 45-47
- 29 Vesoulis Z, Williams G, Matthews B. Pseudomembranous enteritis after proctocolectomy: report of a case. *Dis Colon Rectum* 2000; **43**: 551-554
- 30 Freiler JF, Durning SJ, Ender PT. Clostridium difficile small bowel enteritis occurring after total colectomy. *Clin Infect Dis* 2001; **33**: 1429-1431; discussion 1432
- 31 Kim KA, Wry P, Hughes E Jr, Butcher J, Barbot D. Clostridium difficile small-bowel enteritis after total proctocolectomy: a rare but fatal, easily missed diagnosis. Report of a case. *Dis Colon Rectum* 2007; **50**: 920-923
- 32 Hayetian FD, Read TE, Brozovich M, Garvin RP, Caushaj PF. Ileal perforation secondary to Clostridium difficile enteritis: report of 2 cases. *Arch Surg* 2006; **141**: 97-99
- 33 Taylor RH, Borriello SP, Taylor AJ. Isolation of Clostridium difficile from the small bowel. *Br Med J (Clin Res Ed)* 1981; **283**: 412
- 34 Testore GP, Nardi F, Babudieri S, Giuliano M, Di Rosa R, Panichi G. Isolation of Clostridium difficile from human jejunum: identification of a reservoir for disease? *J Clin Pathol* 1986; **39**: 861-862
- 35 Lunde SJ, Otterson MF, Binion DG, Carman ET, Peppard WJ. Clostridium difficile enteritis: an early postoperative complication in inflammatory bowel disease patients after colectomy. *J Gastrointest Surg* 2007; **11**: 138-142
- 36 Apel R, Cohen Z, Andrews CW Jr, McLeod R, Steinhart H, Odze RD. Prospective evaluation of early morphological changes in pelvic ileal pouches. *Gastroenterology* 1994; **107**: 435-443
- 37 Shepherd NA, Healey CJ, Warren BF, Richman PI, Thomson WH, Wilkinson SP. Distribution of mucosal pathology and an assessment of colonic phenotypic change in the pelvic ileal reservoir. *Gut* 1993; **34**: 101-105
- 38 Mann SD, Pitt J, Springall RG, Thillainayagam AV. Clostridium difficile infection—an unusual cause of refractory pouchitis: report of a case. *Dis Colon Rectum* 2003; **46**: 267-270
- 39 Shen B, Goldblum JR, Hull TL, Remzi FH, Bennett AE, Fazio VW. Clostridium difficile-associated pouchitis. *Dig Dis Sci* 2006; **51**: 2361-2364
- 40 Wood MJ, Hyman N, Hebert JC, Blaszyk H. Catastrophic Clostridium difficile Enteritis in a Pelvic Pouch Patient: Report of a Case. *J Gastrointest Surg* 2008; **12**: 350-352





# Chemotherapy with enteric-coated tegafur/uracil for advanced hepatocellular carcinoma

Toru Ishikawa

Toru Ishikawa, Department of Gastroenterology and Hepatology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata 950-1104, Japan

Correspondence to: Toru Ishikawa, MD, Department of Gastroenterology and Hepatology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata 950-1104,

Japan. [toruishi@ngt.saiseikai.or.jp](mailto:toruishi@ngt.saiseikai.or.jp)

Telephone: +81-25-2336161 Fax: +81-25-2338880

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## Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide, including Japan. Although the development of imaging modalities has made the early diagnosis of HCC possible, surgically resectable cases are relatively uncommon because of hepatic function reserve and/or an advanced stage at presentation. Several modalities, such as transcatheter arterial chemoembolization, percutaneous ethanol injection, microwave coagulation therapy and radiofrequency ablation are reportedly useful in treating patients with non-resectable disease. However, unfortunately, many HCC patients have tumor recurrence. The overall prognosis of patients with HCC is very poor, and treatment of the advanced form is still problematic. In this article, we review the clinical efficacy and toxicity of enteric-coated tegafur/uracil in the treatment of patients with advanced non-resectable HCC.

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**Key words:** Advanced hepatocellular carcinoma; Tumor dormancy; Enteric-coated tegafur/uracil; Chemotherapy; Portal vein tumor thrombus; Lung metastasis

**Peer reviewer:** Susumu Ohwada, Associate Professor, Department of Surgery, Gunma University Graduate School of Medicine, 3-39-15 Shoma-Machi, Maebashi 371-8511, Japan

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## INTRODUCTION

Chronic liver disease predisposes patients to hepatocellular

carcinoma (HCC), therefore, a high-risk group can be identified<sup>[1]</sup>. Progress in diagnostic imaging studies has facilitated the relatively early diagnosis of HCC. In many patients, however, the disease is already advanced at the time of detection. Patients with recurrence after local treatment and those with far-advanced HCC should receive effective systemic chemotherapy. In this review, we outline the mechanism of action and outcomes of enteric-coated tegafur/uracil (UFT-E Taiho Pharmaceutical, Co. Ltd., Tokyo, Japan) used as systemic chemotherapy in patients with HCC.

## DEVELOPMENT OF UFT

Heidelberger *et al*<sup>[2]</sup> synthesized 5-fluorouracil in 1957. Since that time, fluoropyrimidine antimetabolites have been used as broad-spectrum anticancer drugs to treat various types of tumors. However, the serum half-life of 5-fluorouracil is very short (8-12 min)<sup>[3,4]</sup>. Therefore, many derivatives have been developed to maintain high serum concentrations of 5-fluorouracil, enhance response, and reduce toxicity. In 1968, Hiller *et al*<sup>[5]</sup> synthesized tegafur, a prodrug of 5-fluorouracil, which is gradually converted to 5-fluorouracil by cytochrome P450 2A6. Subsequently, UFT was developed to enhance the tumor specificity and effectiveness of 5-fluorouracil. In 1978, Fujii *et al*<sup>[6]</sup> conducted a series of experiments on combination therapy with fluoropyrimidines and pyrimidines and reported that the antitumor activity of tegafur was most strongly enhanced by uracil. They also found that combinations of tegafur and uracil in certain ratios enhanced the antitumor activity of tegafur, without increasing toxicity. A combination of tegafur and uracil in a molar ratio of 1:4 was experimentally shown to be optimal.

These findings led to the development of UFT. This preparation produces and maintains high concentrations of 5-fluorouracil and its active metabolites in tumors, and has specific characteristics not obtained with tegafur or 5-fluorouracil alone. In some patients, however, the conventional formulation of UFT capsules is associated with adverse upper gastrointestinal effects such as loss of appetite, nausea and vomiting, and requires dose reduction or treatment withdrawal. To reduce such adverse effects, UFT-E was developed from 1987 to 1988. Subsequently, the therapeutic usefulness of UFT-E has been confirmed in clinical studies<sup>[7,8]</sup>. In 1990, a new drug application was submitted. UFT-E was approved in 1992 and is now widely used.

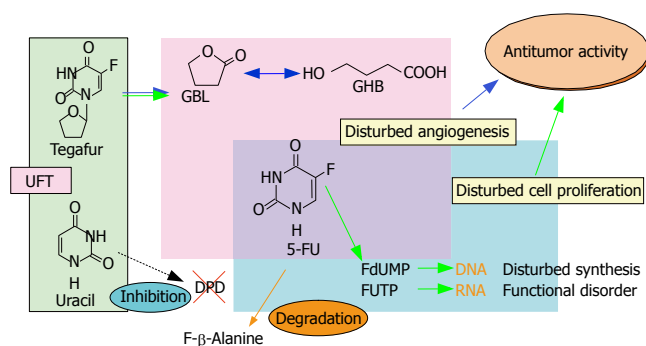


Figure 1 Mechanism of action of UFT.

## MECHANISM OF ACTION OF UFT

The mechanism of action of UFT is shown in Figures 1 and 2. The anticancer activity of UFT is derived from 5-fluorouracil, to which tegafur is gradually converted. As for the mechanism of action of 5-fluorouracil, 5-fluoro-2'-deoxyuridine 5'-monophosphate (FdUMP), the active metabolite of 5-fluorouracil, competes with 2'-deoxyuridine 5'-monophosphate (dUMP) and inhibits thymidylate synthase, thereby blocking DNA synthesis. 5-fluorouridine 5'-triphosphate (FUTP) is incorporated into RNA, which disrupts RNA function (*in vitro*)<sup>[9,10]</sup>. The antitumor activity of 5-fluorouracil depends mainly on inhibition of DNA synthesis. 5-fluorouracil is a time-dependent anticancer drug; therefore, prolonged exposure of cancer cells to even low concentrations of 5-fluorouracil results in antitumor activity. Dihydropyrimidine dehydrogenase (DPD), the main metabolizing enzyme of 5-fluorouracil, is present throughout the body, including the liver, epithelium of the gastrointestinal tract, and peripheral leukocytes, but is most abundant in the liver<sup>[11]</sup>. After intravenous administration, > 90% of the dose of 5-fluorouracil is metabolized during the first pass through the liver<sup>[12]</sup>. Tumors contain DPD, which metabolizes 70% of the 5-fluorouracil that enters tumor tissue. Therefore, outcomes of 5-fluorouracil monotherapy in patients with HCC are poor. On the other hand, uracil is a pyrimidine-based analogue, a component of nucleic acids. Uracil alone has no appreciable antitumor activity or toxicity, but has a high affinity for DPD in the liver and tumors, thereby inhibiting DPD and preventing the metabolism of 5-fluorouracil. This mechanism provides the theoretical basis for UFT, a combination of uracil and tegafur that produces prolonged concentrations of 5-fluorouracil in serum, thereby enhancing antitumor activity against HCC and reducing toxicity<sup>[13]</sup>. Previous studies have reported that  $\gamma$ -hydroxy butyrate (GHB), the metabolite of the protecting group of tegafur,  $\gamma$ -butyrolactone (GBL), and high-dose tegafur inhibit neovascularization<sup>[14]</sup>. UFT-E is thus expected to be effective against HCC.

## REGIMENS OF UFT

Recent controlled studies have reported the usefulness of postoperative adjuvant chemotherapy with UFT, given continuously in a high daily dose (400 mg/m<sup>2</sup>) for 1-2 years.

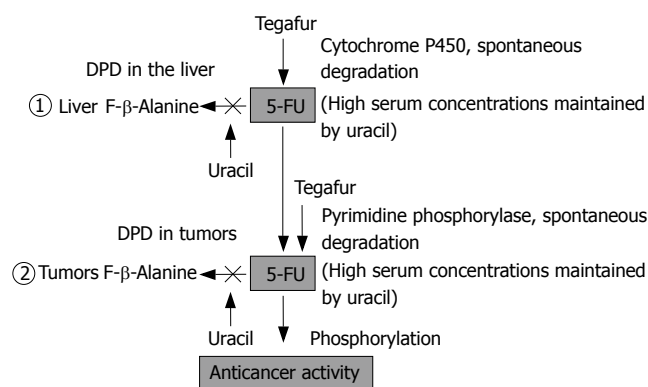


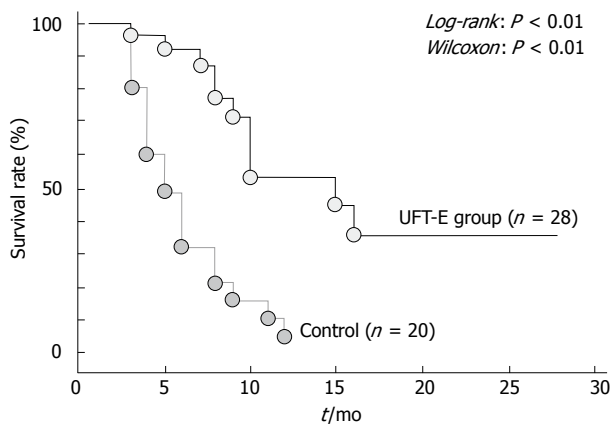
Figure 2 Biochemical modulation of UFT.

In a representative study of patients with resected rectal cancer, the 3-year recurrence-free survival rate was 78% in patients who received postoperative adjuvant chemotherapy with UFT (400 mg/m<sup>2</sup> per day) for 1 year, and 60% in those who underwent surgery alone. The 3-year survival rate was 91% in the UFT group and 81% in the surgery alone group. The rates of recurrence-free and overall survival were significantly improved by postoperative adjuvant chemotherapy with UFT<sup>[15]</sup>. In this study, because UFT was given at a high dose of 400 mg/m<sup>2</sup> per day (in two divided doses), adequate concentrations of 5-fluorouracil in serum might have been maintained, which inhibited the growth of small amounts of residual tumor cells. Severe myelosuppression and gastrointestinal disorders associated with 5-fluorouracil were rare. This was attributed to the fact bone marrow cells and gastrointestinal mucosal cells were rescued by treatment with UFT, given for five consecutive days followed by two days rest. However, in patients with HCC, long-term treatment with oral anticancer drugs may negatively affect liver function as a side effect because of underlying chronic liver disease. Oral anticancer drugs should thus be administered cautiously. The next section describes the use of a reduced dose of UFT-E, equivalent to two-thirds to less than half of the usual dose, in patients with HCC.

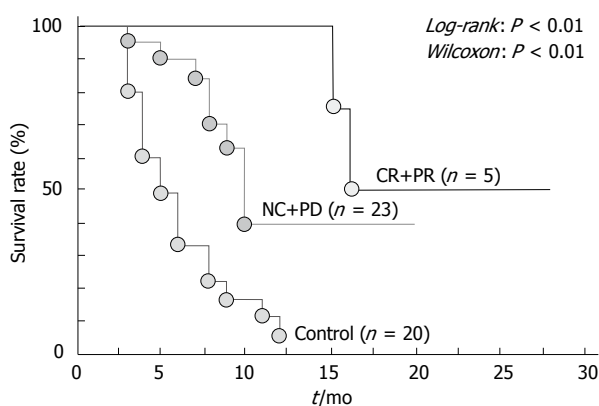
## TREATMENT OF HCC WITH UFT-E

### Effectiveness of UFT-E for stage IVA HCC

We have previously described our own experience with a patient who had stage IVA HCC, in whom all tumors disappeared after monotherapy with UFT-E, with no evidence of recurrence<sup>[16]</sup>. Subsequently, we assessed the usefulness of monotherapy with UFT-E in 28 patients with stage IVA HCC who could not undergo hepatectomy, interventional radiology, or intra-arterial infusion chemotherapy<sup>[17]</sup>. In a study that compared patients who received UFT-E with those who did not, the survival rate was significantly higher in the UFT-E group (Figure 3). Moreover, survival was significantly longer in the UFT-E group than in the untreated group among patients who had a complete or partial response, as well as among those with no change or progressive disease (Figure 4)<sup>[17]</sup>.



**Figure 3** Cumulative survival rates in patients with stage IVA HCC who received UFT-E and those who did not.



**Figure 4** Cumulative survival rates in patients with stage IVA hepatocellular carcinoma according to response to treatment. CR: Complete response; PR: Partial response; NC: No change; PD: Progressive disease.

Although patients with clinical disease progression tended to have poor outcome, multivariate analysis showed that treatment with UFT-E contributes to improved survival, which indicates UFT-E is effective (Table 1)<sup>[17]</sup>. These results indicate administration of UFT-E improves the survival time by inducing a cytostatic phase, rather than by tumor reduction<sup>[18]</sup>.

### Effectiveness of UFT-E for far-advanced HCC

**HCC with portal vein tumor thrombosis (PVTT):** We have previously reported that UFT-E is therapeutically useful in patients with stage IVA HCC<sup>[16,17]</sup>. However, in patients with advanced HCC accompanied by PVTT, outcome is extremely poor, and UFT-E monotherapy is apparently not beneficial. Combination therapy with UFT-E has, therefore, been tried. Recently, Kusunoki *et al*<sup>[19,20]</sup> have reported that pharmacokinetic modulating chemotherapy, based on the concept that the benefit of continuous venous 5-fluorouracil infusion can be potentiated by low-dose oral UFT, is useful for a variety of cancers.

We have previously reported on patients with various types of cancer who had a complete response to pharmacokinetic modulating chemotherapy with UFT-E and continuous intravenous infusion of 5-fluorouracil<sup>[21-27]</sup>.

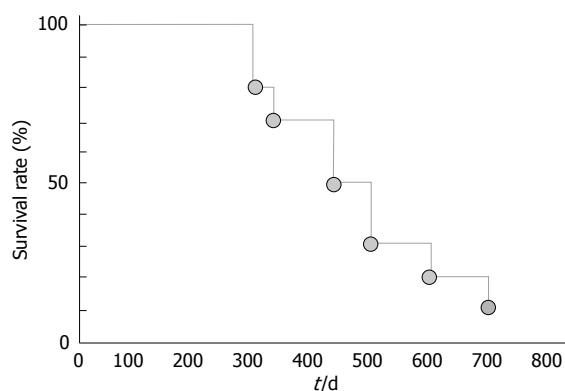
**Table 1** Multivariate analysis of prognostic factors in patients with stage IVA hepatocellular carcinoma

Variable	No. of patients	Risk ratio	95% CI	P value
Treatment				
Tegafur/uracil	28	0.170	0.069-0.441	< 0.001
Control	20	1		
Cirrhosis				
Yes	43	2.592	0.541-12.423	NS (P = 0.235)
No	5	1		
Sex				
Men	35	0.751	0.289-1.955	NS (P = 0.557)
Women	13	1		
Age				
≥ 65	22	0.678	0.307-1.499	NS (P = 0.337)
< 65	26	1		
AFP				
≥ 100	23	1.741	0.724-4.188	NS (P = 0.216)
< 100	25	1		
Serum bilirubin				
≥ 2.0	18	0.573	0.211-1.557	NS (P = 0.275)
< 2.0	30	1		
Serum albumin				
≥ 3.0	28	1.290	0.525-3.171	NS (P = 0.579)
< 3.0	20	1		
Tumor thrombus				
Yes	20	3.516	0.927-8.661	NS (P = 0.062)
No	28	1		
Okuda staging				
I / II	42	0.368	0.099-1.370	NS (P = 0.136)
III	6	1		

NS: Not significant.

Such regimens have recently been used to increase tissue 5-fluorouracil concentrations in patients with advanced gastric cancer or colorectal cancer. We, therefore, modified the regimen for intra-arterial infusion chemotherapy with epirubicin, etoposide, and cisplatin (EEP therapy) described by Takayasu *et al*<sup>[28]</sup> to treat HCC patients with PVTT. Our modified EEP regimen consisted of intra-arterial infusion chemotherapy with epirubicin (30 mg), carboplatin (200 mg), and etoposide (60 mg), given once weekly, followed by a 24-h infusion of 5-fluorouracil (500 mg) as standard therapy, plus continuous treatment with UFT-E (200 mg/d). We have previously described a patient with UFT-E who had a complete response to this regimen. We then studied outcomes in several similar patients. Despite PVTT, all patients could receive chemotherapy on an outpatient basis. The mean survival from the beginning of intra-arterial infusion chemotherapy, excluding the period of previous treatment, was 457.2 d (Figure 5). Further studies are needed to determine the optimal dosage and treatment intervals. However, preliminary evidence indicates modified EEP therapy, including oral anticancer treatment with UFT-E, might be a useful treatment strategy for advanced HCC accompanied by PVTT (Vp3, Vp4)<sup>[29]</sup>.

**Effectiveness of UFT-E against stage IVB HCC with extrahepatic metastasis:** Systemic chemotherapy is usually given to patients who have HCC with extrahepatic metastasis. However, drug recommendations based on



**Figure 5** Cumulative survival rates in patients with tumor occlusion of the portal vein who received modified EEP therapy.

scientific evidence are currently not available. Although the effectiveness of UFT-E has been confirmed in patients with stage IVA HCC, monotherapy with UFT-E does not improve outcome in patients who have stage IVB disease with distant metastasis, usually an indication for systemic chemotherapy. We have previously reported a case of HCC with multiple lung metastases that had a complete response to combination therapy with UFT-E plus docetaxel/cisplatin<sup>[30]</sup>. At present, additional patients with distant metastasis are being studied. Long-term studies of larger numbers of patients are needed to assess the clinical significance of combination therapy including oral anticancer drugs.

## TOXIC EFFECTS OF UFT

Toxic effects of UFT include severe liver dysfunction and diarrhea. Leukoencephalopathy has been reported as a neurological effect, and pigmentation as a skin symptom. Loss of appetite, nausea and vomiting commonly occur in clinical practice. These adverse signs and symptoms may require a reduction in the dose of UFT or even the cessation of treatment in some patients<sup>[31]</sup>. In a study that compared the incidence of toxicity between UFT capsules and UFT-E granules, which were developed to reduce upper gastrointestinal symptoms and thereby improve compliance, patients who received UFT-E granules had a lower incidence of adverse upper gastrointestinal symptoms<sup>[31]</sup>. Grade 1 stomatitis developed in three patients with stage IVA HCC who received monotherapy with UFT-E. However, hepatic function reserve was undisturbed. Monotherapy with UFT-E thus contributed to an improved quality of life. Ikeda *et al*<sup>[32]</sup> conducted a controlled study to compare outcome between patients who received UFT-E adjuvant chemotherapy before transcatheter arterial embolization and those who did not. Outcomes did not significantly differ between the groups, but ascites and hepatic encephalopathy developed in the UFT-E group. However, in our studies, no patient had UFT-E-induced liver failure. In patients with tumor occlusion of the portal vein, who received combination chemotherapy, toxic effects such as stomatitis and leukopenia developed, but treatment could be continued after a rest period.

## CONCLUSION

The effectiveness of UFT-E should be evaluated at different stages of HCC. At the same time, different dosages and treatment with UFT-E regimens, including rest periods, should be assessed, rather than administering anticancer drugs continuously. Unlike other types of cancer, HCC is usually associated with cirrhosis. Long-term treatment with anticancer drugs may therefore adversely affect liver function. Further studies are needed to determine optimal regimens, doses, and treatment periods for anticancer drugs. Other clinical questions, such as whether systemic chemotherapy with UFT-E is an effective adjuvant therapy after liver transplantation, remain to be answered.

## REFERENCES

- 1 **Ishikawa T**, Ichida T, Yamagiwa S, Sugahara S, Uehara K, Okoshi S, Asakura H. High viral loads, serum alanine aminotransferase and gender are predictive factors for the development of hepatocellular carcinoma from viral compensated liver cirrhosis. *J Gastroenterol Hepatol* 2001; **16**: 1274-1281
- 2 **Heidelberger C**, Chaudhuri NK, Dannerberg P, Mooren D, Griesbach L, Duschinsky R, Schnitzer RJ, Plevin E, Scheiner J. Fluorinated pyrimidines, a new class of tumour-inhibitory compounds. *Nature* 1957; **179**: 663-666
- 3 **Diasio RB**, Harris BE. Clinical pharmacology of 5-fluorouracil. *Clin Pharmacokinet* 1989; **16**: 215-237
- 4 **Baker SD**, Khor SP, Adjei AA, Doucette M, Spector T, Donehower RC, Grochow LB, Sartorius SE, Noe DA, Hohnaker JA, Rowinsky EK. Pharmacokinetic, oral bioavailability, and safety study of fluorouracil in patients treated with 776C85, an inactivator of dihydropyrimidine dehydrogenase. *J Clin Oncol* 1996; **14**: 3085-3096
- 5 **Hiller SA**, Zhuk RA, Lidak MJ, Zidermane AA. *Br Patent* 1968; **1**: 391-395
- 6 **Fujii S**, Ikenaka K, Fukushima M, Shirasaka T. Effect of uracil and its derivatives on antitumor activity of 5-fluorouracil and 1-(2-tetrahydrofuryl)-5-fluorouracil. *Gann* 1978; **69**: 763-772
- 7 **Takahashi H**, Kamano T. [Clinical results of UFT enteric-coated granule therapy under cooperative study (phase II study). Tokyo Cancer Chemotherapy Cooperative Study Group] *Gan To Kagaku Ryoho* 1990; **17**: 2043-2049
- 8 **Kikuchi K**, Wakui A. [Cooperative research of UFT E phase II study. Cooperative Study Group of UFT E in Tohoku Area] *Gan To Kagaku Ryoho* 1990; **17**: 2183-2190
- 9 **Heidelberger C**, Kaldor G, Mukherjee KL, Danneberg PB. Studies on fluorinated pyrimidines. XI. In vitro studies on tumor resistance. *Cancer Res* 1960; **20**: 903-909
- 10 **Hartmann KU**, Heidelberger C. Studies on fluorinated pyrimidines. XIII. Inhibition of thymidylate synthetase. *J Biol Chem* 1961; **236**: 3006-3013
- 11 **Naguib FN**, el Kouni MH, Cha S. Enzymes of uracil catabolism in normal and neoplastic human tissues. *Cancer Res* 1985; **45**: 5405-5412
- 12 **Diasio RB**, Harris BE. Clinical pharmacology of 5-fluorouracil. *Clin Pharmacokinet* 1989; **16**: 215-237
- 13 **Yonekura K**, Basaki Y, Chikahisa L, Okabe S, Hashimoto A, Miyadera K, Wierzbica K, Yamada Y. UFT and its metabolites inhibit the angiogenesis induced by murine renal cell carcinoma, as determined by a dorsal air sac assay in mice. *Clin Cancer Res* 1999; **5**: 2185-2191
- 14 **Basaki Y**, Yonekura K, Chikahisa L, Okabe S, Hashimoto A, Miyadera K, Aoyagi K, Yamada Y. [Anti-angiogenic activities of UFT and its metabolites, GHB and GBL, in the dorsal air sac (DAS) model in mice] *Gan To Kagaku Ryoho* 2000; **27**: 93-98
- 15 **Akasu T**, Moriya Y, Ohashi Y, Yoshida S, Shirao K, Kodaira S.



- Adjuvant chemotherapy with uracil-tegafur for pathological stage III rectal cancer after mesorectal excision with selective lateral pelvic lymphadenectomy: a multicenter randomized controlled trial. *Jpn J Clin Oncol* 2006; **36**: 237-244
- 16 **Ishikawa T**, Ichida T, Ishimoto Y, Yokoyama J, Nomoto M, Ebe Y, Usuda H, Naito M, Asakura H. Complete remission of multiple hepatocellular carcinomas associated with hepatitis C virus-related, decompensated liver cirrhosis by oral administration of enteric-coated tegafur/uracil. *Am J Gastroenterol* 1999; **94**: 1682-1685
- 17 **Ishikawa T**, Ichida T, Sugitani S, Tsuboi Y, Genda T, Sugahara S, Uehara K, Inayoshi J, Yokoyama J, Ishimoto Y, Asakura H. Improved survival with oral administration of enteric-coated tegafur/uracil for advanced stage IV-A hepatocellular carcinoma. *J Gastroenterol Hepatol* 2001; **16**: 452-459
- 18 **Takahashi Y**, Nishioka K. Survival without tumor shrinkage: re-evaluation of survival gain by cytostatic effect of chemotherapy. *J Natl Cancer Inst* 1995; **87**: 1262-1263
- 19 **Kusunoki M**, Yanagi H, Kotera H, Noda M, Yamamura T. Effects of pharmacokinetic modulating chemotherapy using oral UFT and continuous venous 5FU infusion on the prognosis of irradiated rectal carcinomas with p53 overexpression. *Int J Oncol* 1998; **13**: 653-657
- 20 **Kusunoki M**, Yanagi H, Noda M, Yamamura T. The usefulness of pharmacokinetic modulating chemotherapy (UFT plus 5FU) in the treatment of unresectable colorectal carcinomas. *Oncol Rep* 1999; **6**: 547-552
- 21 **Ishikawa T**, Sato S, Matsuzawa J, Mita Y, Matsui S, Tashiro K, Tashiro S, Matsuki H. [A case of successful management of nonresectable pancreas cancer with liver metastasis by intra-arterial infusion chemotherapy with angiotensin-II and administration of tegafur/uracil] *Gan To Kagaku Ryoho* 2001; **28**: 521-525
- 22 **Ishikawa T**, Mita Y, Kobayashi M, Tashiro K, Tashiro S, Matsuki H. [A case of nonresectable scirrhous type gastric cancer treated by hypertensive subselective chemotherapy with pharmacokinetic modulating chemotherapy] *Gan To Kagaku Ryoho* 2001; **28**: 1137-1140
- 23 **Ishikawa T**, Nomura K, Baba Y, Hayashi S, Oota H, Yoshida T, Kamimura T. [A case of advanced gastric cancer with liver and intra-abdominal lymph node metastasis treated by hypertensive selective chemotherapy with pharmacokinetic modulating chemotherapy] *Gan To Kagaku Ryoho* 2003; **30**: 1151-1155
- 24 **Ishikawa T**, Mizuno K, Togashi T, Watanabe K, Seki K, Ohta H, Yoshida T, Kamimura T. [A case of advanced gastric cancer with bone metastasis and severe DIC responding to hypertensive subselective chemotherapy with pharmacokinetic modulating chemotherapy] *Gan To Kagaku Ryoho* 2005; **32**: 523-527
- 25 **Ishikawa T**, Mizuno K, Togashi T, Watanabe K, Seki K, Ohta H, Yoshida T, Kamimura T. [Modified pharmacokinetic modulating chemotherapy for progressive gastric cancer accompanied by peritoneal dissemination] *Gan To Kagaku Ryoho* 2005; **32**: 469-472
- 26 **Ishikawa T**, Mizuno K, Watanabe K, Baba Y, Oota H, Yoshida T, Kamimura T. [A case of successful management of nonresectable pancreas cancer with liver metastasis by intra-arterial infusion chemotherapy with gemcitabine hydrochloride, 5-FU, CDDP and administration of tegafur/uracil] *Gan To Kagaku Ryoho* 2004; **31**: 1555-1558
- 27 **Ishikawa T**, Ishikawa N, Oota H, Yoshida T, Honma A, Kamimura T, Takeda K, Ishikawa N, Ozaki T. [A case of common bile duct cancer responding to MMC leucovorin, 5-FU, and UFT combination chemotherapy and radiation] *Gan To Kagaku Ryoho* 1996; **23**: 773-777
- 28 **Takayasu Y**. [Hepatic arterial infusion chemotherapy for hepatocellular carcinoma by EEP regimen] *Nippon Rinsho* 2001; **59** Suppl 6: 624-628
- 29 **Ishikawa T**, Imai M, Kamimura H, Tsuchiya A, Togashi T, Watanabe K, Seki K, Ohta H, Yoshida T, Kamimura T. Improved survival for hepatocellular carcinoma with portal vein tumor thrombosis treated by intra-arterial chemotherapy combining etoposide, carboplatin, epirubicin and pharmacokinetic modulating chemotherapy by 5-FU and enteric-coated tegafur/uracil: a pilot study. *World J Gastroenterol* 2007; **13**: 5465-5470
- 30 **Ishikawa T**, Ichida T, Yokoyama J, Matsuda Y, Watanabe T, Asakura H. Complete disappearance of pulmonary metastases in a case of hepatocellular carcinoma treated with docetaxel-based systemic chemotherapy. *J Gastroenterol Hepatol* 2004; **19**: 1423-1426
- 31 **Ohyama M**, Matsumura M, Katsuta K, Nobori T, Matsuyama H, Fukami K, Kiyota R, Yano H, Shima T, Ogawa K. [A comparative study of UFT enteric-coated granules with UFT capsules on the occurrence of side effects in patients with head and neck cancers--a special attention to the upper gastrointestinal tract disorders] *Gan To Kagaku Ryoho* 1990; **17**: 1211-1216
- 32 **Ikeda K**, Saitoh S, Koida I, Tsubota A, Arase Y, Chayama K, Kumada H. A prospective randomized evaluation of a compound of tegafur and uracil as an adjuvant chemotherapy for hepatocellular carcinoma treated with transcatheter arterial chemoembolization. *Am J Clin Oncol* 1995; **18**: 204-210

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COLORRECTAL CANCER

## Relationship between expression of gastrin, somatostatin, Fas/FasL and caspases in large intestinal carcinoma

Jia-Ding Mao, Pei Wu, Ying-Lin Yang, Jian Wu, He Huang

Jia-Ding Mao, Pei Wu, Ying-Lin Yang, Jian Wu, He Huang, Department of General Surgery, the First Affiliated Yijishan Hospital of Wannan Medical College, Wuhu 241001, Anhui Province, China

Author contributions: Mao JD, Wu P, and Yang YL designed and performed the research; Yang YL provided new reagents/analytic tools; Mao JD analyzed data and wrote the paper; and Wu J and Huang H contributed equally to this work.

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Correspondence to: Professor Pei Wu, Department of General Surgery, The First Affiliated Yijishan Hospital of Wannan Medical College, Wuhu 241001, Anhui Province, China. [maojiading0205@sina.com](mailto:maojiading0205@sina.com)

Telephone: +86-553-5739343

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### Abstract

**AIM:** To explore the correlation between the mRNAs and protein expression of gastrin (GAS), somatostatin (SS) and apoptosis index (AI), apoptosis regulation gene Fas/FasL and caspases in large intestinal carcinoma (LIC).

**METHODS:** Expression of GAS and SS mRNAs were detected by nested RT-PCR in 79 cases of LIC. Cell apoptosis was detected by molecular biology *in situ* apoptosis detecting methods (TUNEL). Immunohistochemical staining for GAS, SS, Fas/FasL, caspase-3 and caspase-8 was performed according to the standard streptavidin-biotin-peroxidase (S-P) method.

**RESULTS:** There was a significant positive correlation between mRNA and protein expression of GAS and SS ( $r_s^{\text{GAS}}=0.99, P < 0.01$ ;  $r_s^{\text{SS}}=0.98, P < 0.01$ ). There was significant difference in positive expression rates of GAS, SS mRNAs and protein among different histological differentiation, histological types and Dukes' stage of LIC. The AI in GAS high and moderate expression groups was significantly lower than that in low expression groups ( $3.75 \pm 2.38$  vs  $7.82 \pm 2.38, P < 0.01$ ;  $5.51 \pm 2.66$  vs  $7.82 \pm 2.38, P < 0.01$ ), and the AI in SS high and moderate expression groups was significantly higher than that in low expression groups ( $9.03 \pm 1.76$  vs  $5.35 \pm 3.00, P < 0.01$ ;  $7.44 \pm 2.67$  vs  $5.35 \pm 3.00, P < 0.01$ ). There was a significant negative correlation between the integral ratio of GAS to SS and the AI ( $r_s = -0.41, P < 0.01$ ). The positive expression rate of FasL in GAS high and moderate expression groups was higher than that

in low expression group (90.9% and 81.0% vs 53.2%,  $P < 0.05$ ). The positive expression rates of Fas, caspase-8 and caspase-3 in SS high (90.0%, 90.0% and 100%) and moderate (80.0%, 70.0%, 75.0%) expression groups were higher than that in low expression group (53.1%, 42.9%, 49.0%) (90.0% and 80.0% vs 53.1%,  $P < 0.05$ ; 90.0% and 70.0% vs 42.9%,  $P < 0.05$ ; 100.0% and 75.0% vs 49.0%,  $P < 0.05$ ). There was a significant positive correlation between the integral ratio of GAS to SS and the semiquantitative integral of FasL ( $r_s = 0.32, P < 0.01$ ).

**CONCLUSION:** GAS and SS play important roles in the regulation and control of cell apoptosis in LIC, and the mechanism may be directly related to the aberrant expression of Fas/FasL. The GAS and SS will be valuable targets of the biological behavior of LIC.

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**Key words:** Large intestinal carcinoma; Gastrin; Somatostatin; Apoptosis index; Fas; FasL; Caspase

**Peer reviewers:** Rene Lambert, Professor, International Agency for Research on Cancer, 150 Cours Albert Thomas, Lyon 69372 cedex 8, France; Takayuki Yamamoto, MD, Inflammatory Bowel Disease Center, Yokkaichi Social Insurance Hospital, 10-8 Hazuyamacho, Yokkaichi 510-0016, Japan

Mao JD, Wu P, Yang YL, Wu J, Huang H. Relationship between expression of gastrin, somatostatin, Fas/FasL and caspases in large intestinal carcinoma. *World J Gastroenterol* 2008; 14(18): 2802-2809 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2802.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2802>

### INTRODUCTION

Large intestinal cancer (LIC) is one of the most common digestive tract malignant tumors in the world, with a high incidence rate in North America, Western Europe, Australia, New Zealand and France, and is the second leading cause of gastrointestinal cancer-related mortality worldwide<sup>[1-4]</sup>. In China, it now ranks fourth<sup>[5]</sup>. Although great progress in understanding the molecular aspects of LIC has been made and several therapeutic agents have been developed, it is still difficult to cure the tumor. It is posing a serious threat to public health and remains a major killer among Chinese patients. The general survival rate

of LIC patients does not exceed 40%. Thereby, it is very important to know what kind of cell factor can influence cell apoptosis, which will enrich the etiology theory of tumor. In addition, while studying the mechanism of loss of control of tumor cell apoptosis from signal transduction pathway, we may find a new way to treat malignant tumors. Previous studies have demonstrated that the occurrence of LIC is directly related to the abnormal expression of gastrointestinal hormones such as gastrin (GAS), somatostatin (SS), *etc*<sup>[6,7]</sup>. Some studies found that GAS was able to promote growth and inhibit apoptosis of LIC cells, but the role of SS was opposite<sup>[8-10]</sup>. However, the detailed molecule mechanism by which GAS and SS mediate cell apoptosis of LIC is not fully known. We used nested RT-PCR method to detect the expression of GAS and SS mRNAs in LIC tissues, and TUNEL method to detect cell apoptosis, and immunohistochemical staining S-P method to detect the protein expression of GAS, SS, Fas/FasL, caspase-3 and caspase-8. The aim of this study was to explore the correlation between the mRNAs and protein expression of GAS and SS, and between the protein expression of GAS, SS and apoptosis index (AI), and apoptosis regulation gene Fas/FasL and caspases in LIC, and to further confirm whether GAS and SS could mediate LIC cell apoptosis mainly via affecting the expression of Fas/FasL.

## MATERIALS AND METHODS

### Clinical data

Seventy-nine cases of cancer tissue samples were randomly and retrospectively selected from patients with LIC hospitalized in our hospital from January 2004 to October 2006. All patients were confirmed as having LIC by clinical pathology. Among them, 36 were cases of rectum cancer, and 43 were cases of colorectal carcinoma. Thirty-seven were females and 42 were males. The median age was  $50.8 \pm 11.2$  years, with a range of 21-79 years. There were 27 patients with well differentiated carcinoma, 28 patients with moderately differentiated carcinoma, and 24 patients with poorly differentiated carcinoma (inclusive of undifferentiated carcinoma). Among them, there were 40 cases of ulcerative type, 32 protruded type, 7 infiltrating type, 19 papillary adenocarcinoma, 35 glandular adenocarcinoma, 10 mucoid carcinoma, 6 signet-ring cell carcinoma and 9 undifferentiated carcinoma. The clinical stage was determined according to the Dukes' stage. Dukes' stages A and B were found in 36 patients, and stages C and D in 43 patients.

### Main reagents

The polyclonal rabbit antibodies against human SS protein and human GAS, monoclonal rabbit antibodies against human Fas and FasL, caspase-3, caspase-8, and immunohistochemical staining kit and *in situ* cell apoptosis detection kit II were all purchased from Beijing Zhongshan Biological Technology Co, Ltd. Trizol liquid, M-MLV reverse transcriptase, SK312, Taq DNA polymerase, RNasin, oligo (dT) 15 and dNTPs primers were purchased from Shanghai Sangon Biological Engineering Technology

and Service Co. Ltd and PCR primers were synthesized by the same company.

### Total RNA extraction and cDNA synthesis

Total RNA was extracted from frozen tissue specimens (50-100 mg) using TRIzol reagent according to the protocol provided by the manufacturer. Total RNA was primed with an Oligo (dT) 15 oligonucleotide and reverse-transcribed following the manufacturer's instructions.

### PCR amplification

The primers of GAS, SS and  $\beta$ -actin were synthesized according to the primer design principles, all primers spanned an intron to control against amplification of genomic DNA sequences. Two point five  $\mu$ L first strand cDNA was amplified in 25  $\mu$ L total volume. The first PCR amplifications were performed in a UNO II thermocycler (Perkin-Elmer, USA) under the following conditions. After an initial denaturation for 5 min at 94°C, samples were subjected to 30 cycles, each amplification consisting of denaturation at 94°C for 30 s, primer annealing at 60°C for 45 s, extension at 72°C for 45 s, followed by a final extension of 6 min 72°C. Conditions and parameters of the second PCR amplifications were the same with the first PCR amplifications except for primers. The length of final PCR products was 174 base pair (bp) (GAS) and 231 bp (SS), respectively. At last, 6  $\mu$ L of reaction product was analyzed by electrophoresis on a 1.5% agarose gel followed by ethidium bromide staining and digital camera photography (Table 1, Figure 1).

### Detection of cell apoptosis

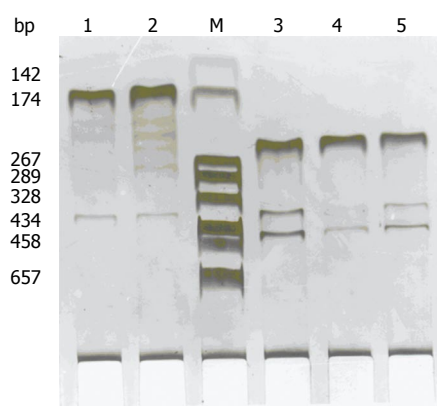
Cell apoptosis was detected using molecular biology *in situ* apoptosis detecting methods (TUNEL). The sections were fixed and embedded conventionally. Dnase (1 mg/L) was used to digest the positive control specimen for 10 min before put into TUNEL response mixture. Only label liquor was added into the negative control response mixture. The detailed manipulation was conducted according to the introductions for users. Cells with brown or yellow nuclei were assumed as apoptotic cells. The number of apoptotic cells and total cancer cells was counted under light microscope at  $400 \times$  magnification in 10 fields of vision and the average values were obtained for the calculation of apoptosis index (AI) according to the formula:  $AI = (\text{apoptotic cells} / \text{total cancer cells}) \times 100\%$ .

### Immunohistochemical staining

Specimens obtained at surgery were routinely fixed in 10% neutral formalin and embedded in paraffin. Serial 4  $\mu$ m thick sections were cut. Immunohistochemical staining for Fas, FasL, caspase-3, caspase-8, GAS and SS was performed by the standard streptavidin-biotin-peroxidase (S-P) method. Positive pancreatic tissue and stomach antrum mucous membrane were used as a positive control for SS and GAS. Positive controls for Fas, FasL, caspase-3 and caspase-8 were purchased from Shanghai Sangon Biological Engineering Technology and Service Co. Ltd. PBS 0.01 mol/L as a negative control replaced the primary antibody. The detailed

Table 1 Primers used for nested RT-PCR amplification of GAS and SS

Name	Primer sequence	PCR conditions	Size (bp)
GAS	1: 5'-TATGTGCTGATCTTTGCACTGGCT-3' (sense: 6307-6330) 2: 5'-CTCATCTCAGCACTGCGCGGCC-3' (antisense: 6718-6695)	94°C, 30 s	282
		60°C, 45 s	
		72°C, 45 s	
GAS	3: 5'-GAGCTACCTGGCTGGAGCAGCAG-3' (sense: 6415-6438) 2: 5'-CTCATCTCAGCACTGCGCGGCC-3' (antisense: 6718-6695)	94°C, 30 s	174
		60°C, 45 s	
		72°C, 45 s	
SS	1: 5'-ATGCTGTCTCTGCCGCTCCAG-3' (sense: 106-126) 2: 5'-ACAGGATGTGAAAGTCTTCCA-3' (antisense: 1330-1310)	94°C, 30 s	348
		60°C, 45 s	
		72°C, 45 s	
SS	3: 5'-GCTGTGCGCGGGGAAGCAG-3' (sense: 223-243) 2: 5'-ACAGGATGTGAAAGTCTTCCA-3' (antisense: 1330-1310)	94°C, 30 s	231
		60°C, 45 s	
		72°C, 45 s	



**Figure 1** Expression of GAS, SS mRNA by nested RT-PCR analysis in LIC tissue. Lane M: DNA marker DL 2000; Lane 1-2: Positive expression of GAS; Lane 3-5: Positive expression of SS.

manipulation of S-P immunohistochemical method was used according to its manual.

### Evaluation of scores

Brown-yellow staining mainly in cell plasma, partly in cell membranes was considered positive SS and GAS protein expression. When SS and GAS protein expression was scored, both the extent and intensity of immunopositivity were considered. The intensity of staining was scored as follows: 0, no staining; 1, weak-yellow; 2, brown-yellow; and 3, brown-black. The extent of positive cells was scored as follows (100 cells were counted by two independent observers, who did not know the clinicopathological features of these LIC): 1, positive staining cells < 5%; 2, positive staining cells in 5%-10%; 3, positive staining cells in 10%-20%; and 4, positive staining cells > 20%. The final score was determined by multiplying the intensity and extent of positivity scores, yielding a range from 1 to 12. According to the semi-quantitative integral evaluation, SS and GAS were divided into three groups: Scores 1-3 was defined as the low expression group, 4-6 as the moderate expression group, and 7-12 as the high expression group (Figure 2A and B).

The positive Fas and FasL protein expression was defined with brown-yellow staining mainly in cell membranes, or cell plasma, while positive caspase-3 and caspase-8 protein expression was defined with brown-yellow staining mainly in cell plasma. The degree of Fas, FasL, caspase-3 and caspase-8 staining was estimated by semi-quantitative evaluation and

categorized by the extent and intensity of staining<sup>[11]</sup>. The intensity of staining was scored as: 0, negative; 1, weak-yellow; 2, brown-yellow; and 3, brown-black. The extent of positive cells was scored as: 0 = positive staining cells in 0%-5%, 1 = positive staining cells in 6%-25%, 2 = positive staining cells in 26%-50%, 3 = positive staining cells in 51%-75%, and 4 = positive staining cells > 75%. Combined staining score was used to evaluate the results of Fas, FasL, caspase-3 and caspase-8 staining. The final score was determined by adding the intensity and extent of staining scores, yielding a range from 0 to 7. Scores 1-2 were defined as negative expression (-), 3 as weak staining (+), 4 as moderately staining (++), and  $\geq 5$  as strong staining (+++) (Figure 2C-F).

### Statistical analysis

Statistical evaluation was performed using Chi-square test and  $q$  test to differentiate the positive rates of different groups, and using Spearman test to analyze the correlation between the ratio of GAS to SS and the integral of Fas, FasL, caspase-3 and caspase-8. All data were analyzed with statistical package for social science (SPSS) version 10.0.  $P < 0.05$  was considered statistically significant.

## RESULTS

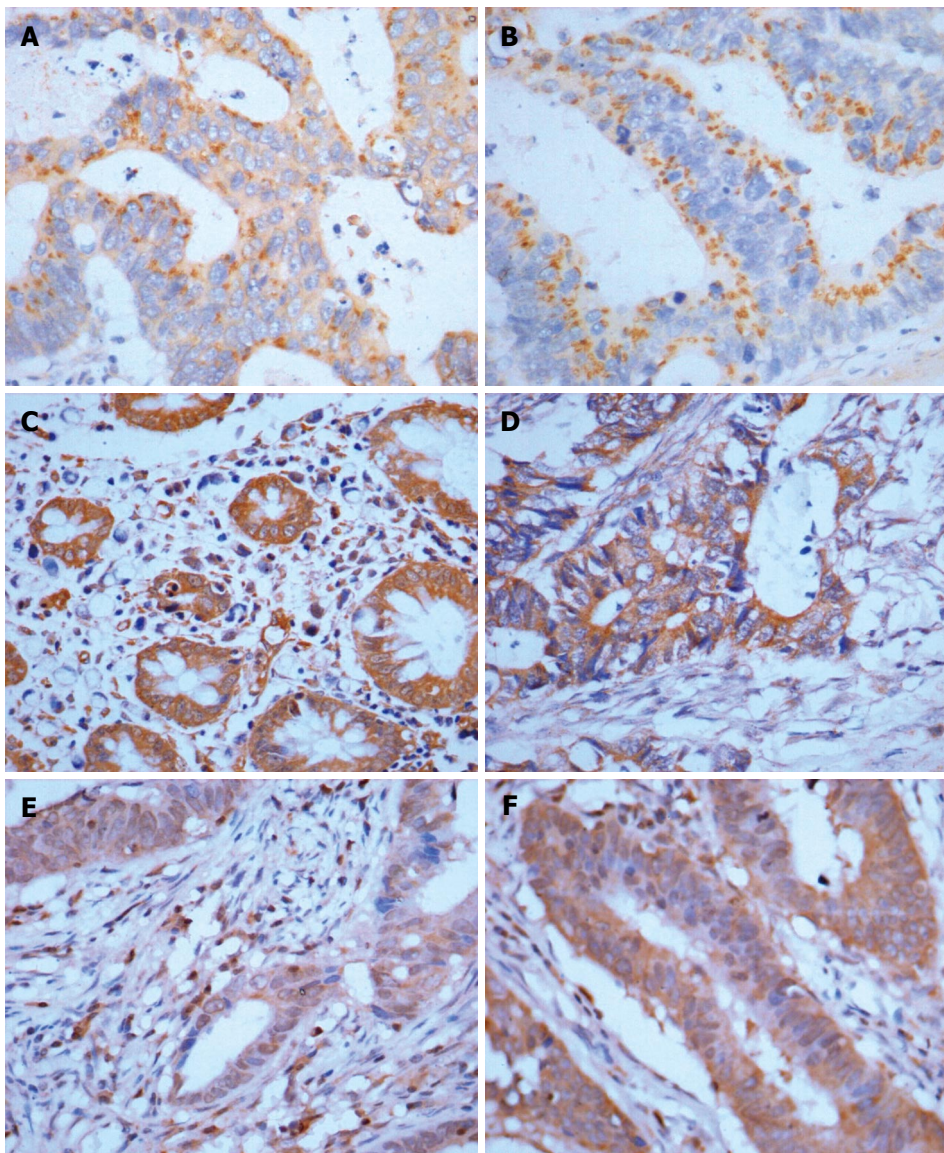
### Expression of GAS, SS mRNA and protein

The positive expression rates of GAS and SS mRNA were 46.8% (36/79), 41.8% (33/79), respectively. The positive expression rates of GAS and SS protein were 40.5% (32/79) and 38.0% (30/79), respectively. There was a significant positive correlation between mRNA and protein expression of GAS and SS ( $P < 0.01$ ,  $r_{\text{GAS}} = 0.99$ ;  $P < 0.01$ ,  $r_{\text{SS}} = 0.98$ ). There was significant difference in positive expression rates of GAS, SS mRNAs and protein among different histological differentiation, histological types and Dukes' stage of LIC, as shown in Table 2.

### Apoptosis index

The AI in GAS high and moderate expression groups was significantly lower than in low expression groups ( $q_{\text{high vs low}} = 6.71$ ,  $P < 0.01$ ;  $q_{\text{middle vs low}} = 4.60$ ,  $P < 0.01$ ), and the AI in SS high and moderate expression groups was significantly higher than in low expression groups ( $q_{\text{high vs low}} = 5.66$ ,  $P < 0.01$ ;  $q_{\text{middle vs low}} = 4.21$ ,  $P < 0.01$ ). There was a significant negative correlation between the integral ratio of GAS to SS and the AI ( $r_s = -0.41$ ,  $P < 0.01$ ) (Table 3).





**Figure 2** Strong expressions of GAS, SS, Fas, FasL, caspase-3 and caspase-8 in LIC tissues. **A:** Strong GAS expression in LIC. S-P  $\times$  400; **B:** Strong SS expression in LIC. S-P  $\times$  400; **C:** Strong Fas expression in SS high expression group. S-P  $\times$  400; **D:** Strong FasL expression in GAS high expression group of colorectal carcinoma. S-P  $\times$  400; **E:** Strong caspase-3 expression in SS high expression group. S-P  $\times$  400; **F:** Strong caspase-8 expression in SS high expression group. S-P  $\times$  400.

**Table 2** Correlation between clinicopathological factors and GAS and SS mRNA and protein expression in LIC

Groups	n	mRNA						Protein					
		GAS (+)	$\chi^2$	P	SS (+)	$\chi^2$	P	GAS (+)	$\chi^2$	P	SS (+)	$\chi^2$	P
Differentiation													
Well	27	8 <sup>b</sup>	10.47	< 0.01	18			6 <sup>b</sup>	10.23	< 0.01	17	5.24	< 0.05
Moderate	28	11 <sup>a</sup>	6.68	< 0.05	10 <sup>c</sup>		< 0.05	10 <sup>a</sup>	4.95	< 0.05	9 <sup>c</sup>	11.24	< 0.01
Poor	24	18			5 <sup>d</sup>	10.78	< 0.01	16			4 <sup>d</sup>		
Histological types													
Papillary	19	6 <sup>e</sup>	4.80	< 0.05	13			4 <sup>e</sup>	6.22	< 0.05	12		
Tubular	35	13 <sup>e</sup>	4.40	< 0.05	14			11 <sup>e</sup>	4.38	< 0.05	14		
Mucinous and signet-ring	16	11			4 <sup>g</sup>	6.56	< 0.05	10			3 <sup>g</sup>	6.99	< 0.05
Undifferentiated	9	7			2 <sup>g</sup>	5.44	< 0.05	7			1 <sup>g</sup>	7.33	< 0.05
Dukes' stage													
A and B	36	12 <sup>i</sup>	4.84	< 0.05	20 <sup>i</sup>	5.17	< 0.05	10 <sup>i</sup>	4.44	< 0.05	18 <sup>i</sup>	4.06	< 0.05
C and D	43	25			13			22			12		

<sup>a</sup>P < 0.05, <sup>b</sup>P < 0.01 vs poor differentiation; <sup>c</sup>P < 0.05, <sup>d</sup>P < 0.01 vs well differentiation; <sup>e</sup>P < 0.05 vs mucinous and signet-ring and undifferentiated; <sup>g</sup>P < 0.05 vs papillary; <sup>i</sup>P < 0.05 vs Dukes' stages C and D.

#### **Fas, FasL, caspase-3 and caspase-8 expression in GAS and SS high, moderate and low expression groups of LIC**

The positive expression rate of FasL in GAS high (10/11) and moderate (17/21) expression groups was higher than

that in low expression group (26/47) ( $\chi^2_{\text{high vs low}} = 6.24$ ,  $P < 0.05$ ;  $\chi^2_{\text{moderate vs low}} = 4.74$ ,  $P < 0.05$ ). There were no significant differences in positive expression rates of Fas, caspase-3 and caspase-8 among GAS high, moderate and

**Table 3** Comparison of AI in GAS and SS high, moderate and low expression groups of LIC (mean  $\pm$  SD)

Groups	<i>n</i>	AI (mean $\pm$ SD)	<i>F</i>	<i>P</i>
SS				
Low	10	5.35 $\pm$ 3.00	17.63	< 0.01
Moderate	20	7.44 $\pm$ 2.67 <sup>b</sup>		
High	49	9.03 $\pm$ 1.76 <sup>b</sup>		
GAS				
Low	47	7.82 $\pm$ 2.38	16.08	< 0.01
Moderate	21	5.51 $\pm$ 2.66 <sup>d</sup>		
High	11	3.75 $\pm$ 2.38 <sup>d</sup>		

<sup>b</sup>*P* < 0.01 vs low SS group; <sup>d</sup>*P* < 0.01 vs low GAS group.

low expression groups (*P* > 0.05). The positive expression rates of Fas, caspase-8 and caspase-3 in SS high (9/10, 9/10 and 10/10) and moderate (16/20, 14/20 and 15/20) expression groups was higher than that in low expression group (26/49, 21/49 and 24/49) ( $\chi^2_{\text{high}}$  vs low = 5.48, 5.62 and 6.89, *P* < 0.05;  $\chi^2_{\text{middle}}$  vs low = 4.32, 4.19 and 3.91, *P* < 0.05). There was no significant difference in positive expression rate of FasL among SS high, moderate and low expression groups (*P* > 0.05) (Table 4). There was a significant positive correlation between the integral ratio of GAS to SS and the semi-quantitative integral of FasL ( $r_s = 0.32$ , *P* < 0.01). But, there was no correlation between the integral ratio of GAS to SS and Fas, caspase-3 and caspase-8 (*P* > 0.05).

## DISCUSSION

LIC arises mainly from mutations in somatic cells. However, conversion of normal to cancer cells is not the result of a single mutation. It is achieved through a multi-step process that is closely associated with the accumulation of multiple gene changes including both oncogenes and tumor suppressor genes<sup>[12-16]</sup>. Uncontrolled cell proliferation and apoptosis are the main hallmarks of LIC. Apoptosis is a unique physiological mechanism that it can not only eliminate discrete cells in normal development, host defense and maintain the body in well stable condition, also play an important role in regulating and controlling tumor occurrence, development and treatment<sup>[17,18]</sup>. Impaired apoptosis has been implicated in the development of many human diseases, including cancer. It has been proved that occurrence of cancers is due to the loss of control of normal apoptosis and the disturbance of balance between cell proliferation and apoptosis<sup>[19-21]</sup>. Two main signaling pathways initiate the apoptotic program in mammalian cells<sup>[22]</sup>. The cell-extrinsic pathway, namely, death receptor signaling pathway triggers apoptosis in response to activation by their respective ligand of the tumor necrosis factor (TNF) family of death receptors, including TNF-RI for TNF $\alpha$ , Fas or CD95 for FasL, and death receptor 4 (DR4) or 5 (DR5) for TNF-related apoptosis-inducing ligand (TRAIL). On the other hand, the cell-intrinsic pathway, namely, mitochondria signaling pathway triggers apoptosis in response to DNA damage, loss of survival factors, or other types of cell distress. Both pathways involve the activation of cysteine proteases called caspases, which

**Table 4** Comparison of positive rates of Fas, FasL, caspase-3 and caspase-8 protein in GAS and SS high, moderate and low expression groups of LIC

Groups	<i>n</i>	Fas		FasL		Caspase-3		Caspase-8		
		+	(%)	-	+	(%)	-	+	(%)	-
GAS										
High	11	7	(63.6)	4	10	(90.9) <sup>a</sup>	1	9	(81.8)	2
Moderate	21	16	(76.2)	5	17	(81.0) <sup>a</sup>	4	14	(66.7)	7
Low	47	37	(78.7)	10	26	(53.2)	21	29	(61.7)	18
SS										
High	10	9	(90.0) <sup>c</sup>	1	8	(80.0)	2	10	(100.0) <sup>c</sup>	0
Moderate	20	16	(80.0) <sup>c</sup>	4	14	(70.0)	6	15	(75.0) <sup>c</sup>	5
Low	49	26	(53.1)	23	32	(65.3)	17	24	(49.0)	25

<sup>a</sup>*P* < 0.05 vs GAS low expression group; <sup>c</sup>*P* < 0.05 vs SS low expression group.

are constitutively expressed in the cytosol as proenzymes, and are activated to mature proteases by cleavage, which triggers the activation of the caspase-8 and caspase-9, followed by the activation of caspase-3. Caspase-3 is one of the key executioners in the apoptosis pathway. Caspase-3 activation is thought to be a major step in the apoptotic signal transduction cascade that commits cells to suicide. Caspase-3 cleavage during apoptosis leads to the proteolysis of several cytosolic and nuclear proteins and precedes DNA fragmentation. However, some recent studies have shown that activation of Fas/FasL signaling pathway is regulated by many kinds of cell factors, including GAS and SS<sup>[23-25]</sup>.

Many studies have pointed out that some tissue growths are regulated by hormones, and these tissues turned into tumors are still controlled by hormones<sup>[26-28]</sup>. Gastrointestinal hormones such as GAS and SS regulate the secretion, motility, absorption, blood flow and cell nutrition of the digestive tract. Abnormality of their secretion often affected the normal functions of digestive tract, even produced clinical symptoms or syndromes<sup>[29]</sup>. Xie *et al*<sup>[30]</sup> found that the disordered gastrointestinal hormones play a crucial role in the pediatric chronic gastritis and duodenal ulcer. There seems to be an increasing tendency in the expressions of GAS and SS in children with chronic gastritis and duodenal ulcer. In recent years, some studies have demonstrated that there is a high correlation between the aberrant expressions of GAS, SS and the occurrence and development of LIC<sup>[31-33]</sup>. D'Onghia *et al*<sup>[34]</sup> found that GAS serum levels were slightly higher in patients with colorectal cancer than in healthy controls. GAS levels were higher in patients carrying left colorectal cancer than the others, and higher in early stage than in late stage. Cao *et al*<sup>[35]</sup> found that GAS 17 can increase colorectal cancer cells' invasion, the mechanism of which is probably that GAS 17 makes FAK-Tyr397 to be phosphorylated and localized to lamellipodia, causing the formation of FAK-Src-p130 (Cas)-Dock180 signaling complex when it is bound to its receptor CCK-2 and activation of Rac. In this study, we found that there was a significant positive correlation between mRNA and protein expression of GAS and SS. The positive expression rate of GAS and SS mRNA and protein varied greatly in different histological type, differentiation degree, and Dukes' stage. The higher mRNA and protein expression of GAS was, the poorer of tissue differentiation degree and clinical stages.

For example, the expression GAS in poorly differentiated LIC, in particular, in mucinous adenocarcinomas and signet-ring cell carcinoma was prominently higher than the others, with poor prognosis. However, the action of SS was opposite. The results indicate that the expressions of GAS and SS were closely related to the biological behavior of LIC. It is obvious that the GAS and SS will be valuable targets of the biological behavior of LIC.

Tumor escape mechanisms in LIC result from dysregulation of the cell apoptosis signaling pathways, which are mediated by death ligands. Recently, great progress has been made in understanding the regulated signaling pathway mechanisms of GAS and SS. Some studies showed that the abnormal expressions of GAS and SS were closely related to cell apoptosis of LIC. GAS could promote cell proliferation and inhibit cell apoptosis. However, the action of SS was opposite in LIC<sup>[36,37]</sup>. Our studies showed that the expression of GAS protein in LIC was higher and the AI was lower, and the higher expression of SS protein, the higher AI. Despite abundant evidence that GAS may play an integral role in promoting tumor growth in the stomach and malignancies in the GI tract, the precise mechanisms about gastrin-restrained, somatostatin-induced apoptosis are still largely unknown. To elucidate the mechanisms by which GAS and SS might influence apoptotic signaling pathway, we analyzed their effects on the expression of Fas, FasL, caspase-3 and caspase-8 protein in human LIC tissue.

GAS is a gastrointestinal (GI) peptide that possesses potent trophic effects on most of the normal and neoplastic mucosa of the GI tract. GAS is mainly secreted from GAS secreting cells (G cells) in the antrum mucosa or upper small intestine and large intestine. Medulla oblongata and dorsal nuclei of vagus nerves in central nervous system also secrete GAS<sup>[38,39]</sup>. Recent reports indicate that chronic hypergastrinaemia increases the risk of colorectal cancer and cancer growth, and that interruption of the effects of GAS could be a potential target in the treatment of colorectal cancer<sup>[40]</sup>. Recent data have shown that GAS is not only able to induce cell proliferation, but also inhibit LIC cell apoptosis. However, the underlying mechanisms that GAS inhibits cell apoptosis, is still worthy of further elucidation. Wu *et al.*<sup>[41]</sup> recent studies found that down-regulation of GAS gene expression results in the activation of caspase 9 and 3 in gastrin-dependent human colon cancer cells inducing cell apoptosis. Cui *et al.*<sup>[42]</sup> found that GAS can induce apoptosis in gastric epithelial cells via GAS/CCK-2 receptor and synergized with FasL stimulation and contribute to the development of gastric carcinogenesis. In this study, we found that the level of GAS protein expression was higher and the positive expression rate of FasL was higher in LIC tissues, and expression of GAS protein was not related to expression of Fas, caspase-3 and caspase-8. The results indicate that the mechanisms of GAS inhibiting LIC cell apoptosis might be *via* up-regulation of expression of FasL inducing apoptosis with Fas-expressing T cells, making LIC cells escape from immune surveillance and immunocyte attack.

SS is a multifunctional hormone, which is secreted from SS secreting cells (D cells). D cells are distributed mainly in human central and peripheral nervous system, and in the gastrointestinal tract, including the large intestine. SS

inhibits multiple functions, including exocrine and endocrine secretions, inflammation, and angiogenesis, as well as cell proliferation and tumorigenesis, as shown in normal or tumoral cell models<sup>[43-46]</sup>. SS acts as an inhibitory peptide of various secretory and proliferative responses. The diverse biological effects of SS are mediated through a family of five SS receptors (sst1-sst5) that belong to the family of G-protein-coupled receptors and that regulate diverse signal transduction pathways including adenylate cyclase, phospholipase C- $\beta$ , phospholipase A2, guanylate cyclase, ionic conductance channels, and tyrosine phosphatase<sup>[47,48]</sup>. The mechanisms of the inhibition are the combined interaction of SS and its analogs with SST1-5R in tumor tissues, either directly inhibiting division and proliferation of tumor cells or inhibiting the activities of growth factors such as vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF), *etc.*<sup>[49-51]</sup>, thus counteracting tumorigenesis and tumor cell proliferation. The ability of SS and its stable analogues to promote tumor cell apoptosis has been demonstrated in various cell types including mammary, prostatic, gastric, pancreatic, colorectal, and small cell lung cancer cells. However, the underlying mechanisms of SS induced cell apoptosis are still poorly understood. Guillermet *et al.*<sup>[52]</sup> showed that sst2-dependent activation and cell sensitization to death ligand-induced apoptosis involved activation of the executioner caspases are key factors in both death ligand-or mitochondria-mediated apoptosis. Sst2 affected pathways by up-regulating expression of TRAIL and TNF $\alpha$  receptors, DR4 and TNFRI, respectively, and sensitizing the cells to death ligand-induced initiator caspase-8 activation; by down-regulating expression of the antiapoptotic mitochondrial Bcl-2 protein; and by enhancing TNF $\alpha$ -mediated activation of NF- $\kappa$ B, resulting in JNK inhibition and subsequent executioner caspase activation and cell death. Kang *et al.*<sup>[53]</sup> found that SS was able to induce peritoneal macrophages apoptosis by a Bax- and NO-independent p53 accumulation, and through Fas and caspase-8 activation pathways. Recent reports have demonstrated that SS analogs octreotide was able to induce human somatotroph tumor cell apoptosis by activating SST2 and also induce an increase in caspase-3 levels<sup>[54,55]</sup>. In this study, we found that the higher the integral of SS was, the higher the positive expression rate of Fas, caspase-3, caspase-8, and expression of SS protein was foreign to expression of FasL. Our results indicated that SS was able to induce over-expression of Fas, caspase-3 and caspase-8 protein, and the mechanisms of SS promoting LIC cell apoptosis can be *via* up-regulation of expression of Fas or through increasing caspase-3 levels and sensitivity of death receptor signaling pathway.

In this study, we found that the ratio of GAS to SS had an effect on biological characteristics such as malignant type, tissue differentiation and clinical stages of LIC. The increased ratio of GAS to SS is an event of significance in LIC occurrence and development<sup>[56,57]</sup>. Our results indicated that there was a positive correlation between the ratio of GAS to SS and the semi-quantitative integral of FasL, and negative correlation between the integral ratio of GAS to SS and the AI. Furthermore, the expression of GAS and SS proteins was directly related to the expression of Fas, FasL, caspase-3, 8 in LIC.



In conclusion, the regulation and control of GAS and SS in LIC cell apoptosis may be directly related to the aberrant expression of Fas/FasL. The GAS and SS will be valuable targets of the biological behavior of LIC.

## COMMENTS

### Background

Although great progress in understanding the molecular aspects of large intestinal cancer (LIC) has been made and several therapeutic agents have been developed, it is still difficult to cure the tumor. The general survival rate of LIC patients does not exceed 40%. Thereby it is highly important to know that what kind of cell factor can influence cell apoptosis, which will enrich the etiology theory of tumor. Despite abundant evidence that gastrin may play an integral role in promoting tumor growth in the large intestine, the precise mechanisms about gastrin-restrained and somatostatin-induced apoptosis are still largely unknown.

### Research frontiers

Recent some studies have shown that activation of Fas/FasL signaling pathway is regulated by many kinds of cell factors, including gastrin (GAS) and somatostatin (SS).

### Innovations and breakthroughs

Some studies found that GAS was able to inhibit apoptosis in LIC cell, but the role of SS was opposite. However, the detailed molecule mechanism by which GAS and SS mediate cell apoptosis of LIC is not fully known. In this study, the authors analyzed their effects on the expression of Fas, FasL, caspase-3 and caspase-8 protein. To further confirm whether GAS and SS could mediate LIC cell apoptosis mainly via affecting the expression of Fas/FasL, will help us find a new way to treat malignant tumors.

### Applications

The GAS and SS will be valuable targets of the biological behavior of LIC.

### Terminology

GAS: It is mainly secreted from GAS secreting cells (G cells) in antrum mucosa or upper small intestine and large intestine. Medulla oblongata and dorsal nuclei of vagus nerves in central nervous system also secrete GAS. SS: It is secreted from SS secreting cells (D cells). D cells are distributed mainly in human central and peripheral nervous system, and in the gastrointestinal tract. Fas/FasL: It belongs to the ligand of the tumor necrosis factor (TNF) family of death receptors. Caspase: Mammalian cysteine aspartate proteases (caspase) can be divided into initiator (e.g. caspases 2, 8, 9, 10) and effector (caspases 3, 4, 5, 6, 7, 11, 12, and 13) enzymes.

### Peer review

This paper demonstrates that GAS and SS play important roles in the regulation and control of cell apoptosis in LIC, and the mechanism may be directly related to the aberrant expression of Fas/FasL, which will enrich the etiology theory of LIC. In addition, it also provides a way to evaluate targets of the biological behavior of LIC.

## REFERENCES

- 1 **Parkin DM**, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74-108
- 2 **Peto J**. Cancer epidemiology in the last century and the next decade. *Nature* 2001; **411**: 390-395
- 3 **Tsukuma H**, Ajiki W. [Descriptive epidemiology of colorectal cancer--international comparison] *Nippon Rinsho* 2003; **61** Suppl 7: 25-30
- 4 **Jemal A**, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ, Thun MJ. Cancer statistics, 2005. *CA Cancer J Clin* 2005; **55**: 10-30
- 5 **Zhang MG**, Li JT, Yang HY, Zhao HC. Clinical analysis on 1143 cases of large intestine carcinoma. *Zhonghua Yixue Zazhi* 2003; **83**: 2087-2088
- 6 **Saga T**, Tamaki N, Itoi K, Yamazaki T, Endo K, Watanabe G, Maruno H, Machinami R, Koizumi K, Ichikawa T, Takami H, Ishibashi M, Kubo A, Kusakabe K, Hirata Y, Murata Y, Miyachi

- Y, Tsubuku M, Sakahara H, Katada K, Tonami N, Yamamoto K, Konishi J, Imamura M, Doi R, Shimatsu A, Noguchi S, Hasegawa Y, Ishikawa O, Watanabe Y, Nakajo M. [Phase III additional clinical study of <sup>111</sup>In-pentetreotide (MP-1727): diagnosis of gastrointestinal hormone producing tumors based on the presence of somatostatin receptors] *Kaku Igaku* 2003; **40**: 185-203
- 7 **Cobb S**, Wood T, Ceci J, Varro A, Velasco M, Singh P. Intestinal expression of mutant and wild-type progastrin significantly increases colon carcinogenesis in response to azoxymethane in transgenic mice. *Cancer* 2004; **100**: 1311-1323
- 8 **Beales IL**, Ogunwobi O. Glycine-extended gastrin inhibits apoptosis in colon cancer cells via separate activation of Akt and JNK pathways. *Mol Cell Endocrinol* 2006; **247**: 140-149
- 9 **Ogunwobi OO**, Beales IL. Glycine-extended gastrin stimulates proliferation and inhibits apoptosis in colon cancer cells via cyclooxygenase-independent pathways. *Regul Pept* 2006; **134**: 1-8
- 10 **Mao JD**, Wu P, Xia XH, Hu JQ, Huang WB, Xu GQ. Correlation between expression of gastrin, somatostatin and cell apoptosis regulation gene bcl-2/bax in large intestine carcinoma. *World J Gastroenterol* 2005; **11**: 721-725
- 11 **Fromowitz FB**, Viola MV, Chao S, Oravez S, Mishriki Y, Finkel G, Grimson R, Lundy J. ras p21 expression in the progression of breast cancer. *Hum Pathol* 1987; **18**: 1268-1275
- 12 **Joyce T**, Pintzas A. Microarray analysis to reveal genes involved in colon carcinogenesis. *Expert Opin Pharmacother* 2007; **8**: 895-900
- 13 **John R**, El-Rouby NM, Tomasetto C, Rio MC, Karam SM. Expression of TFF3 during multistep colon carcinogenesis. *Histol Histopathol* 2007; **22**: 743-751
- 14 **Zhang B**, Pan X, Cobb GP, Anderson TA. microRNAs as oncogenes and tumor suppressors. *Dev Biol* 2007; **302**: 1-12
- 15 **Michor F**, Iwasa Y, Lengauer C, Nowak MA. Dynamics of colorectal cancer. *Semin Cancer Biol* 2005; **15**: 484-493
- 16 **Lièvre A**, Laurent-Puig P. [Colorectal carcinogenesis: update] *Rev Prat* 2004; **54**: 143-150
- 17 **Kim JS**, Lee YC, Nam HT, Li G, Yun EJ, Song KS, Seo KS, Park JH, Ahn JW, Zee O, Park JI, Yoon WH, Lim K, Hwang BD. Apiculate A induces cell death through Fas ligand up-regulation and microtubule disruption by tubulin down-regulation in HM7 human colon cancer cells. *Clin Cancer Res* 2007; **13**: 6509-6517
- 18 **Fernández-Cebrián JM**, Nevado Santos M, Vorwald Kuborn P, Pardo de Lama M, Martín-Cavanna J, Pacheco Martínez P, Fernández Escudero B, Ramos Fernández M. Can the clinical outcome in stage II colon carcinomas be predicted by determination of molecular marker expression? *Clin Transl Oncol* 2007; **9**: 663-670
- 19 **Søreide K**. [Genetics and molecular classification of colorectal cancer] *Tidsskr Nor Lægeforen* 2007; **127**: 2818-2823
- 20 **He L**, Li X, Luo HS, Rong H, Cai J. Possible mechanism for the regulation of glucose on proliferation, inhibition and apoptosis of colon cancer cells induced by sodium butyrate. *World J Gastroenterol* 2007; **13**: 4015-4018
- 21 **Lev-Ari S**, Kazanov D, Liberman E, Ben-Yosef R, Arber N. Down-regulation of PGE2 by physiologic levels of celecoxib is not sufficient to induce apoptosis or inhibit cell proliferation in human colon carcinoma cell lines. *Dig Dis Sci* 2007; **52**: 1128-1133
- 22 **Zimmermann KC**, Bonzon C, Green DR. The machinery of programmed cell death. *Pharmacol Ther* 2001; **92**: 57-70
- 23 **Cui G**, Takaishi S, Ai W, Betz KS, Florholmen J, Koh TJ, Houghton J, Pritchard DM, Wang TC. Gastrin-induced apoptosis contributes to carcinogenesis in the stomach. *Lab Invest* 2006; **86**: 1037-1051
- 24 **Xie XZ**, Wang ZM, Zhang HY, Wang L, Gao BH, Li XM, Hu WG. [Expression of gastrin, somatostatin, PCNA and Fas-L in the mucosa of gastric antrum of children with chronic gastritis and duodenal ulcer] *Zhonghua Erke Zazhi* 2006; **44**: 774-777
- 25 **Guillemet J**, Saint-Laurent N, Rochaix P, Cuvillier O, Levade T, Schally AV, Pradayrol L, Buscail L, Susini C, Bousquet C. Somatostatin receptor subtype 2 sensitizes human pancreatic cancer cells to death ligand-induced apoptosis. *Proc Natl Acad Sci USA* 2003; **100**: 155-160
- 26 **Schmidt M**, Fink D, Lang U, Kimmig R. [Hormone replacement therapy: curse or blessing?] *Gynakol Geburtshilfliche Rundsch*



- 2006; **46**: 165
- 27 **Lam PM**, Chung TK, Haines C. Where are we with postmenopausal hormone therapy in 2005? *Gynecol Endocrinol* 2005; **21**: 248-256
  - 28 **Slattery ML**, Sweeney C, Murtaugh M, Ma KN, Wolff RK, Potter JD, Caan BJ, Samowitz W. Associations between ERalpha, ERbeta, and AR genotypes and colon and rectal cancer. *Cancer Epidemiol Biomarkers Prev* 2005; **14**: 2936-2942
  - 29 **Cho KH**, Lee HS, Ku SK. Changes in gastric endocrine cells in Balb/c mice bearing CT-26 carcinoma cells: an immunohistochemical study. *Eur J Histochem* 2006; **50**: 293-300
  - 30 **Xie XZ**, Wang ZM, Zhang HY, Wang L, Gao BH, Li XM, Hu WG. [Expression of gastrin, somatostatin, PCNA and Fas-L in the mucosa of gastric antrum of children with chronic gastritis and duodenal ulcer] *Zhonghua Erke Zazhi* 2006; **44**: 774-777
  - 31 **Tejeda M**, Gaál D, Hullán L, Hegymegi-Barakonyi B, Kéri G. Evaluation of the antitumor efficacy of the somatostatin structural derivative TT-232 on different tumor models. *Anticancer Res* 2006; **26**: 3477-3483
  - 32 **Singh P**. Role of Annexin-II in GI cancers: interaction with gastrins/progastrins. *Cancer Lett* 2007; **252**: 19-35
  - 33 **Schally AV**, Szepeshazi K, Nagy A, Comaru-Schally AM, Halmos G. New approaches to therapy of cancers of the stomach, colon and pancreas based on peptide analogs. *Cell Mol Life Sci* 2004; **61**: 1042-1068
  - 34 **D'Onghia V**, Leoncini R, Carli R, Santoro A, Giglioni S, Sorbellini F, Marzocca G, Bernini A, Campagna S, Marinello E, Vannoni D. Circulating gastrin and ghrelin levels in patients with colorectal cancer: correlation with tumour stage, Helicobacter pylori infection and BMI. *Biomed Pharmacother* 2007; **61**: 137-141
  - 35 **Cao J**, Yu JP, Zhou L, Song WC, Luo HS, Yu HG. [Molecular mechanism of gastrin increasing colon cancer cells' invasion] *Zhonghua Yixue Zazhi* 2007; **87**: 1704-1708
  - 36 **Beales IL**, Ogunwobi O. Glycine-extended gastrin inhibits apoptosis in colon cancer cells via separate activation of Akt and JNK pathways. *Mol Cell Endocrinol* 2006; **247**: 140-149
  - 37 **Qiu CZ**, Wang C, Huang ZX, Zhu SZ, Wu YY, Qiu JL. Relationship between somatostatin receptor subtype expression and clinicopathology, Ki-67, Bcl-2 and p53 in colorectal cancer. *World J Gastroenterol* 2006; **12**: 2011-2015
  - 38 **Ali MA**, Nyberg F, Chandranath SI, Dhanasekaran S, Tariq S, Petroianu G, Hasan MY, Adeghate EA, Adem A. Distribution of neuroendocrine cells in the small and large intestines of the one-humped camel (*Camelus dromedarius*). *Neuropeptides* 2007; **41**: 293-299
  - 39 **Rindi G**, Solcia E. Endocrine hyperplasia and dysplasia in the pathogenesis of gastrointestinal and pancreatic endocrine tumors. *Gastroenterol Clin North Am* 2007; **36**: 851-865, vi
  - 40 **Niv Y**, Delpre G, Sperber AD, Sandbank J, Zirklin H. Hyperplastic gastric polyposis, hypergastrinaemia and colorectal neoplasia: a description of four cases. *Eur J Gastroenterol Hepatol* 2003; **15**: 1361-1366
  - 41 **Wu H**, Owlia A, Singh P. Precursor peptide progastrin(1-80) reduces apoptosis of intestinal epithelial cells and upregulates cytochrome c oxidase Vb levels and synthesis of ATP. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**: G1097-G1110
  - 42 **Cui G**, Takaishi S, Ai W, Betz KS, Florholmen J, Koh TJ, Houghton J, Pritchard DM, Wang TC. Gastrin-induced apoptosis contributes to carcinogenesis in the stomach. *Lab Invest* 2006; **86**: 1037-1051
  - 43 **Williams GT**. Endocrine tumours of the gastrointestinal tract-selected topics. *Histopathology* 2007; **50**: 30-41
  - 44 **Badway AC**, Blake AD. Somatostatin: a hormone for the heart? *Curr Vasc Pharmacol* 2005; **3**: 125-131
  - 45 **Ueberberg B**, Tourne H, Redman A, Walz MK, Schmid KW, Mann K, Petersenn S. Differential expression of the human somatostatin receptor subtypes sst1 to sst5 in various adrenal tumors and normal adrenal gland. *Horm Metab Res* 2005; **37**: 722-728
  - 46 **Pichler R**, Pichler J, Mustafa H, Nussbaumer K, Zaunmüller T, Topakian R. Somatostatin-receptor positive brain stem glioma visualized by octreoscan. *Neuro Endocrinol Lett* 2007; **28**: 250-251
  - 47 **Guillemet-Guibert J**, Lahlou H, Cordelier P, Bousquet C, Pyronnet S, Susini C. Physiology of somatostatin receptors. *J Endocrinol Invest* 2005; **28**: 5-9
  - 48 **Arena S**, Pattarozzi A, Massa A, Esteve JP, Iuliano R, Fusco A, Susini C, Florio T. An intracellular multi-effector complex mediates somatostatin receptor 1 activation of phosphotyrosine phosphatase eta. *Mol Endocrinol* 2007; **21**: 229-246
  - 49 **Wilkinson-Berka JL**, Wraight C, Werther G. The role of growth hormone, insulin-like growth factor and somatostatin in diabetic retinopathy. *Curr Med Chem* 2006; **13**: 3307-3317
  - 50 **Sall JW**, Klisovic DD, O'Dorisio MS, Katz SE. Somatostatin inhibits IGF-1 mediated induction of VEGF in human retinal pigment epithelial cells. *Exp Eye Res* 2004; **79**: 465-476
  - 51 **Zatelli MC**, Piccin D, Vignali C, Tagliati F, Ambrosio MR, Bondanelli M, Cimino V, Bianchi A, Schmid HA, Scanarini M, Pontecorvi A, De Marinis L, Maira G, degli Uberti EC. Pasireotide, a multiple somatostatin receptor subtypes ligand, reduces cell viability in non-functioning pituitary adenomas by inhibiting vascular endothelial growth factor secretion. *Endocr Relat Cancer* 2007; **14**: 91-102
  - 52 **Guillemet J**, Saint-Laurent N, Rochaix P, Cuvillier O, Levade T, Schally AV, Pradayrol L, Buscail L, Susini C, Bousquet C. Somatostatin receptor subtype 2 sensitizes human pancreatic cancer cells to death ligand-induced apoptosis. *Proc Natl Acad Sci USA* 2003; **100**: 155-160
  - 53 **Kang BN**, Jeong KS, Park SJ, Kim SJ, Kim TH, Kim HJ, Ryu SY. Regulation of apoptosis by somatostatin and substance P in peritoneal macrophages. *Regul Pept* 2001; **101**: 43-49
  - 54 **Capello A**, Krenning EP, Bernard BF, Breeman WA, van Hagen MP, de Jong M. Increased cell death after therapy with an Arg-Gly-Asp-linked somatostatin analog. *J Nucl Med* 2004; **45**: 1716-1720
  - 55 **Ferrante E**, Pellegrini C, Bondioni S, Peverelli E, Locatelli M, Gelmini P, Luciani P, Peri A, Mantovani G, Bosari S, Beck-Peccoz P, Spada A, Lania A. Octreotide promotes apoptosis in human somatotroph tumor cells by activating somatostatin receptor type 2. *Endocr Relat Cancer* 2006; **13**: 955-962
  - 56 **Wu P**, Tu JS, Riu J, Hang H, Hang WB, Yuan P. To study the correlation between expression of gastrin, somatostatin and cell proliferation, apoptosis in colorectal carcinoma. *Zhonghua Shiyan Waike Zazhi* 2003; **20**: 947
  - 57 **Wu P**, Mao JD, Yan JY, Rui J, Zhao YC, Li XH, Xu GQ. Correlation between the expressions of gastrin, somatostatin and cyclin and cyclin-depend kinase in colorectal cancer. *World J Gastroenterol* 2005; **11**: 7211-7217

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## VIRAL HEPATITIS

# Pseudomonas exotoxin antisense RNA selectively kills hepatitis B virus infected cells

Peter Hafkemeyer, Ulrich Brinkmann, Elizabeth Brinkmann, Ira Pastan, Hubert E Blum, Thomas F Baumert

Peter Hafkemeyer, Hubert E Blum, Department of Medicine II, University Hospital Freiburg, Hugstetterstrasse 55, Freiburg D-79106, Germany

Ulrich Brinkmann, Elizabeth Brinkmann, Roche Diagnostics, Nonnenwald 2, Penzberg D-82377, Germany

Ira Pastan, National Cancer Institute, National Institutes of Health, Bethesda, United States

Thomas F Baumert, Inserm unit 748, Université Louis Pasteur, 3 Rue Koeberle, Strasbourg F-67000, France

**Author contributions:** Hafkemeyer P designed research/performed experiments/wrote paper; Brinkmann E, Brinkmann U performed experiments; Pastan I, Blum HE designed research; Baumert TF cloned plasmids/performed assays.

**Correspondence to:** Peter Hafkemeyer, MD, Department of Medicine II, University Hospital Freiburg, Hugstetterstrasse 55, Freiburg D-79106, Germany. [phafkemeyer@gmx.net](mailto:phafkemeyer@gmx.net)

Telephone: +49-761-2703403 Fax: +49-761-2703610

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## Abstract

**AIM:** To present an approach for selectively killing retrovirus-infected cells that combines the toxicity of Pseudomonas exotoxin (PE) and the presence of reverse transcriptase (RT) in infected cells.

**METHODS:** PE antisense toxin RNA has palindromic stem loops at its 5' and 3' ends enabling self-primed generation of cDNA in the presence of RT. The RT activity expressed in retrovirus-infected cells converts "antisense-toxin-RNA" into a lethal toxin gene exclusively in these cells.

**RESULTS:** Using cotransfection studies with PE-expressing RNAs and  $\beta$ -gal expressing reporter plasmids, we show that, in HepG2 and HepG2.2.15 hepatoma cells as well as in duck hepatitis B virus (DHBV) infected cells, HBV or DHBV-polymerase reverse transcribe a lethal cDNA copy of an antisense toxin RNA, which is composed of sequences complementary to a PE gene and eukaryotic transcription and translation signals.

**CONCLUSION:** This finding may have important implications as a novel therapeutic strategy aimed at the elimination of HBV infection.

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**Key words:** Gene therapy; Pseudomonas exotoxin; Retrovirus; Reverse transcription

## INTRODUCTION

Hepatitis B virus (HBV) infection results in a broad spectrum of clinical manifestations, ranging from asymptomatic course to acute self-limited or fulminant hepatitis or chronic hepatitis that may progress to liver cirrhosis and hepatocellular carcinoma (HCC). The individual host immune response as well as viral mutations largely determines the natural course of HBV infection.

Infection with retroviruses causes among others leukemias (HTLV-1) or AIDS (HIV)<sup>[1,2]</sup>. Because retroviruses encode a reverse transcriptase (RT), one therapeutic strategy is inhibition of RT. RT inhibitors such as lamivudine, adefovir, entecavir and other nucleotide analogues are presently being used to treat HBV infection<sup>[3-6]</sup>.

RT inhibitors are of limited efficacy, however, due to the development of viral resistance<sup>[7,8]</sup>. An alternative therapeutic approach for retroviral infection is to target infected cells by recombinant toxins or immunotoxins that bind to surface antigens<sup>[9-13]</sup> and kill the cells by the toxin moiety, as shown for HIV-infected cells<sup>[9,10]</sup>. One example is the CD4-Pseudomonas exotoxin (PE), composed of a CD4 binding domain fused to a truncated derivative of PE<sup>[14,15]</sup>, which is an extremely potent toxin. Because only few PE molecules are required to kill a cell, the full-length or truncated PE results in the death of eukaryotic cells<sup>[16]</sup>. The fusion of the HIV-binding domain CD4 to a truncated PE derivative proved extremely effective in HIV infection<sup>[17,18]</sup>.

Here, we describe a strategy for the selective killing of retrovirus-infected cells that combines the extreme toxicity of PE and the presence of RT in infected cells. The RT activity of HBV is recruited to selectively convert PE antisense toxin RNA into a lethal toxin gene exclusively in HBV infected cells. This experimental strategy was first evaluated in uninfected COS and MCF7 cells and

in RT positive MM5MT (murine mammary tumor) and HUT102 (human T-cell leukemia) cells (data not shown). Transfection of sense toxin RNA kills cells independently of RT, whereas antisense toxin RNA requires reverse transcription for toxin expression. In COS monkey kidney or MCF7 human breast carcinoma cells in which RT cannot be detected, antisense toxin RNA was not toxic, because it does not express a sense toxin. However, it reduces cell viability when transfected into RT positive HUT102 or MM5MT cells, infected with a human (HTLV1) and a murine (M-MLV) retrovirus, respectively. Here, we demonstrate that HBV RT converts PE antisense RNA into a cDNA expressing PE that selectively eliminates HBV infected cells. The antisense toxin RNA is complementary to the PE gene, flanked by eukaryotic transcription and translation signals. In addition, antisense toxin RNA has palindromic stem loops at its 5' and 3' ends enabling self-primed generation of cDNA in the presence of RT. Antisense toxin RNA does not affect uninfected cells but is lethal for RT containing cells and may therefore represent a novel antiviral strategy.

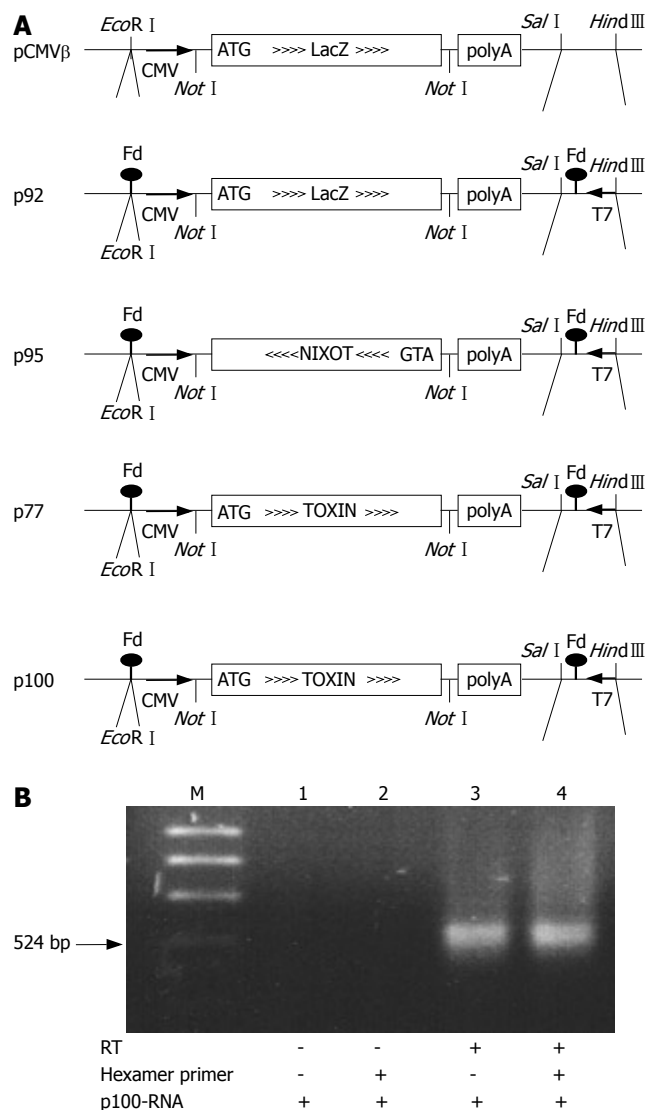
## MATERIALS AND METHODS

### Cell lines

HepG2 and HepG2.2.15 cells (American Type Culture Collection, ATCC, Manassas, VA, USA) were maintained as described<sup>[19]</sup>. HepG2.2.15 is a hepatoblastoma cell line based on Hep G2 cells transfected with a plasmid carrying the gene that confers resistance to G418 and four 5'-3' tandem copies of the HBV genome positioned such that two dimers of the genomic DNA are 3'-3' with respect to one another<sup>[19]</sup>.

### Plasmids for toxin expression and RNA production

The plasmids for PE expression and production of RNA are derived from pCMV $\beta$  (Clontech, Palo Alto, CA, USA)<sup>[17]</sup> which contain the *E. coli* LacZ gene under the control of the CMV promoter for expression of  $\beta$ -galactosidase ( $\beta$ -Gal) in eukaryotic cells, and SV40 splice and polyadenylation signals (Figure 1A). We introduced the T7 phage gene 10 promoter, the and 5'-stem loop sequence<sup>[20]</sup> and the Fd-phage transcription terminator<sup>[21]</sup> in opposite direction to the CMV expression cassette behind the polyadenylation signal and in front of the CMV promoter of pCMV $\beta$ , respectively. This generates a T7-transcription cassette that contains, and is complementary to, the CMV- $\beta$ Gal expression cassette (p92). p92 has the promoter and stem loop sequence of the T7 phage gene 10, obtained by PCR with the primers 5'-GTTCTTTCCAAAGCTTGTGAATTGAT-3' and 5'-GGGCGGAGTCGACCCCGCTAGAGGGAAA-3' inserted between the *Sal*I and *Hind*III restriction sites of pCMV $\beta$  and, in addition, the Fd phage transcription terminator inserted in the *Eco*R I site. The annealed terminator oligos, 5'-AATTACAAAATTAAGGCTCTTTTGAGCCTLTITG-3' and 5'-AATTCAAAAAAGGCTCCAAAAGGAGCCTTTAATTTTGT-3' are *Eco*R I compatible at both ends but reconstitute the site after ligation in the plasmid only at the 3'-end of the terminator.



**Figure 1** A: p92 has the promoter and stem loop sequence of the T7 phage gene 10 and the Fd phage transcription terminator. In p95, p100 and p77, the LacZ-gene *Not*I -fragment of p92 is replaced by fragments containing truncated mutated forms of *Pseudomonas* exotoxin (PE38). p92 represents the control  $\beta$ -Gal expressing plasmid. p95 contains the gene for PE38 in reverse orientation to the CMV promoter. p77 expresses a non-functional frameshift mutant of PE38. p100 expresses the fully active PE38. B: Reverse transcription of toxin antisense RNA *in vitro*. Ethidium bromide stained nondenaturing 2% agarose gel. PCR fragments indicate the presence of toxin cDNA; templates were p100 RNA incubated with (1) no M-MLV RT, no random hexamer primers, (2) no M-MLV RT, random hexamer primers, (3) M-MLV RT, no random hexamer primers, (4) M-MLV RT, random hexamer primers.

The T7 transcription cassette generated by these insertions is in opposite orientation to the CMV promoter. Toxin sense or antisense RNA coding plasmids were generated by replacing LacZ of the modified pCMV $\beta$  by a truncated but enzymatically fully active PE (PE35KDEL)<sup>[22]</sup>. We also constructed a PE frameshift mutation (p77) in which the active site of the toxin (Glu553) is inactivated<sup>[23]</sup>. In p95, p100 and p77, the LacZ gene *Not*I -fragment of p92 is replaced by fragments containing a translation initiation signal (Kozak) and ATG start codon at the 5'-end of truncated mutated forms of PE obtained by PCR with 5'-ACCCGTCATCGCGGCCGCCACCATGGGCTGGGAACAACCTGGA-3' and 5'-TCGGGCTTTTGCGGCC

GCCGAATTCCTTAGAGCTCG-3'; an *EcoR* I site in the latter primer was later filled in by polymerase, religated and thereby removed. p9 (active toxin)<sup>[23]</sup> was used as template for PE38KDEL; the introduction of an additional (fill-in) frameshift mutation in the *Bam*H I site located in the enzymatically active domain III of the toxin gene provided the template for p77.

### Conversion of antisense toxin RNA to a lethal toxin gene by self primed cDNA synthesis

The Fd-terminator stem loop at the 3' end of the RNAs (SL1, ..ACAAAATTAAAGGCTCCCTTTTGGAGCCTTTTTCG-3') can prime the first strand synthesis, the 3' end of which forms a stem loop (SL2, previous RNA-5'-stemloop 5'-GGGAGACCACAACGGTTTCCC..) initiating second strand synthesis. cDNA multimers might also be obtained by repeated flip-over polymerizations around the stem loops. The cDNAs would represent functional and lethal toxin expression cassettes. Depending on the orientation of the toxin coding region in the T7/CMV promoter cassette, these plasmids contain either a functional eukaryotic toxin expression cassette (p100) or no toxic activity (p77), or a cassette in which the toxin is encoded by the opposite strand (p95). In this latter plasmid p95, the CMV promoter driven expression generates nonsense RNA in eukaryotic cells. Since the T7 and CMV promoters are in opposite orientation, this situation is inverted for the RNAs from T7-promoter driven *in vitro* transcription: RNA produced in vitro from p100 and p77 is nonsense RNA (T7-nixot) while RNA from p95 (T7-Toxin) encodes the toxin.

### Construction of plasmid pCH-391mt

Plasmid pCH-391wt contains the complete HBV2 (ayw) genome (infectious in chimpanzees) under control of a CMV promoter. The CMV promoter allows transcription of an authentic pregenomic RNA. Plasmid pCH-391mt is derived from pCH-391wt. pCH-391mt contains a G2024C mutation that inactivates the endogenous polymerase because the active polymerase motif YMDD in pCH-391mt is mutated to YMDH<sup>[24]</sup>. In pCH-391mt a new *Nsi* I restriction site is created. pCH-391mt was cloned by restriction digest of pCH-391wt with *Xba* I and *Bsr*G I, removing the wild-type *Xba* I / *Bsr*G I fragment and insertion of a PCR fragment containing the YMDH motif using primers HBV-pol G2024C Rev (5'-CTCAAGATGCTGTACAGACTTGCCCCCAATACCACATCATGCA TATACTG-3') and HBV-pol 1400 For (5'-GTCAATCTTCTCGAGGATTGGGGACCCT-3') and pCH-391wt as template for PCR amplification. The mutation was verified by sequencing as well as *Nsi* I digestion.

### Synthesis of antisense toxin RNA by reverse transcription *in vitro*

Templates for *in vitro* transcription were linearized with *EcoR* I to eliminate read-through transcripts. Transcription was performed using a large-scale RNA synthesis kit (RiboMAX, Promega, Mannheim, Germany) without Cap analog. RNA was incubated for 1 h at 37°C with 10 units RNase free DNase I (Promega, Mannheim, Germany) to

remove template DNA, extracted with phenol, phenol/chloroform and chloroform, precipitated with ethanol, dissolved in DEPC-treated water and stored at -70°C. For reverse transcription 1 µg p100 RNA (r100) was incubated for 30 min in 20 µL RT buffer (Advantage RT-PCR-Kit, Clontech, Palo Alto, CA, USA) with or without random hexamer primers and M-MLV RT and subsequently treated with 1 µg RNase A for 1 h at 37°C. One µL of the reaction mixture was used to PCR amplify [25 × (95°C, 1 min; 60°C, 2 min; 72°C, 3 min)] a PE gene fragment with the primers 5'-ATGGTCTCCAGGCGCCCGCCTTCCTC-3' and 5'-GCTATGTGTTTCGTCGGCTACCACGGC-3'. Ethidium bromide stained nondenaturing 2% agarose gel-electrophoresis was performed to identify the PE cDNA PCR fragments.

### Transfection of HepG2 and HepG2.2.15 cells with toxin expression plasmids

Transfections were performed using the calcium phosphate transfection kit (GIBCO, Heidelberg, Germany).  $1.5 \times 10^5$  cells were plated 24 h prior to transfections. Transfections were performed with 7.5 µg β-Gal expressing plasmid p92 and 7.5 µg toxin expressing plasmid. β-Gal activities obtained after cotransfection with the β-Gal expressing plasmid p92 (7.5 µg) and p92 plasmid without insert (7.5 µg) were set to 100%. Activities resulting from coexpression of toxin plasmids (p77, p95, p100) and β-Gal expressing plasmid p92 are expressed as relative levels. RT dependent toxicity was determined by β-Gal activity staining.

In RNA transfection experiments, 7.5 µg β-Gal expression plasmid p92 and 7.5 µg of r95, r100, r77 or r92-RNA obtained by *in vitro* transcription were transfected. In experiments determining the sensitivity of HepG2 cells to sense toxin and antisense RNA a further plasmid (7.5 µg) expressing either wild-type (pCH-391wt) or mutant HBV polymerase (pCH-391mt) was transfected. β-Gal activity was assessed using the β-Gal activity staining set (Invitrogen, Groningen, Netherlands). To assess the sensitivity of Hep G2 cells to sense and antisense toxin RNA in the presence and absence of the RT inhibitor adefovir, Hep G2 cells were transfected with 7.5 µg β-Gal expressing plasmid p92 and 7.5 g of r95, r100, r77 or r92-RNA. After transfection of plasmid p92 and the respective RNA adefovir was added to a final concentration of 10 µmol/L. In addition, a plasmid expressing wild-type or mutant HBV polymerase was transfected (7.5 µg).

### PCR of genomic DNA of HepG2 cells after transfection with *in vitro* transcribed toxin RNA

To demonstrate that HBV polymerase reverse transcribed p100 RNA in HepG2 cells only that were transfected with the HBV polymerase encoding plasmid DNA pCH-391wt, genomic DNA was extracted and purified using the QIA-amp-DNA mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol, following transfection with β-Gal expression plasmid p92 and r100 PE expressing RNA. Genomic DNA was stored at -70°C. For reverse transcription 1 µL of the genomic DNA was used to PCR amplify [25 × (94°C, 1 min; 58°C, 2 min;



72°C, 3 min)] a gene fragment with the primers PE-FOR (5'-GCTATGTGTTTCGTCGGC-3') and 4648 REV (5'-ATTAATGTGAGTTAGCTCACTCAT-3').

#### **Transfection of DHBV infected primary duck hepatocytes with toxin expression plasmids**

DHBV was obtained from the serum of 2-wk to 3-wk-old Pekin ducks congenitally DHBV-infected. One day after hatching, Pekin ducks were infected by intravenous injection of 100 L DHBV DNA positive serum ( $10^9$  virions/mL). Ten days later the ducks were sacrificed. Hepatocytes were obtained by liver perfusion. After the ducks were anesthetized with pentobarbital sodium, the livers were perfused *via* the portal vein with 200 mL of 0.5 mmol/L EGTA [ethyleneglycol-bis ( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid] in Swimms 77 medium (GIBCO Heidelberg, Germany) that was buffered with 20 mmol/L HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) followed by 200 mL of 0.5 mg of collagenase type 1 (Sigma, Munich, Germany) per mL, 2.5 mmol/L  $MgCl_2$  in Swimms 77 medium. To maintain the perfusion at 37°C, all solutions were kept at 39°C, and the livers were removed and cells dispersed in Williams' medium (GIBCO Heidelberg, Germany) containing 5% fetal bovine serum (FBS), 20 mmol/L HEPES (pH 7.4), 300 mg of penicillin per liter, 1.5 mg of glucose per liter,  $10^{-5}$  mol/L hydrocortisone-hemisuccinate (Sigma, Munich, Germany), and nystatin (10 U/mL). Cells were filtered through gauze and centrifuged at  $50 \times g$  for 4 min. The cell pellet was washed three times with Williams' medium supplemented 20 mmol/L HEPES (pH 7.4), 5 mmol/L glutamine, 0.066 mmol/L insulin, 10 mmol/L dexamethasone, 100  $\mu$ g/mL penicillin, 100  $\mu$ g/mL streptomycin and 1.5% dimethyl sulfoxide containing 5% FBS. Cells were counted in a hemacytometer, and 60 mm dishes were seeded with  $1.5 \times 10^5$  cells per dish in Williams' medium containing the above mentioned supplements. Cultures were incubated at 37°C and 5%  $CO_2$  in a humidified incubator.

Transfections were performed using the calcium phosphate transfection kit (GIBCO, Heidelberg, Germany). DNA and RNA transfections were performed identical to "Transfection of HepG2 and HepG2.2.15 cells with toxin expression plasmids".

## **RESULTS**

#### **Transfection of human HepG2 cells with PE antisense expression constructs and HBV reverse transcription**

To study the ability of a RT to reverse transcribe an antisense toxin RNA, we first constructed a series of PE expression plasmids allowing primer-independent synthesis of cDNA by RT. RNA was obtained by *in vitro* transcription from the T7 promoter. The 5' end of the RNA can form 5-end stem loop of T7 gene 10 promoter transcripts<sup>[20]</sup>. The 3' end consists of the Fd-terminator<sup>[21]</sup> followed by a residual *EcoR* I sequence (used to linearize the plasmid templates prior to *in vitro* transcription) that can participate in a 3' stem loop. Primer independent cDNA synthesis was used to verify that these secondary

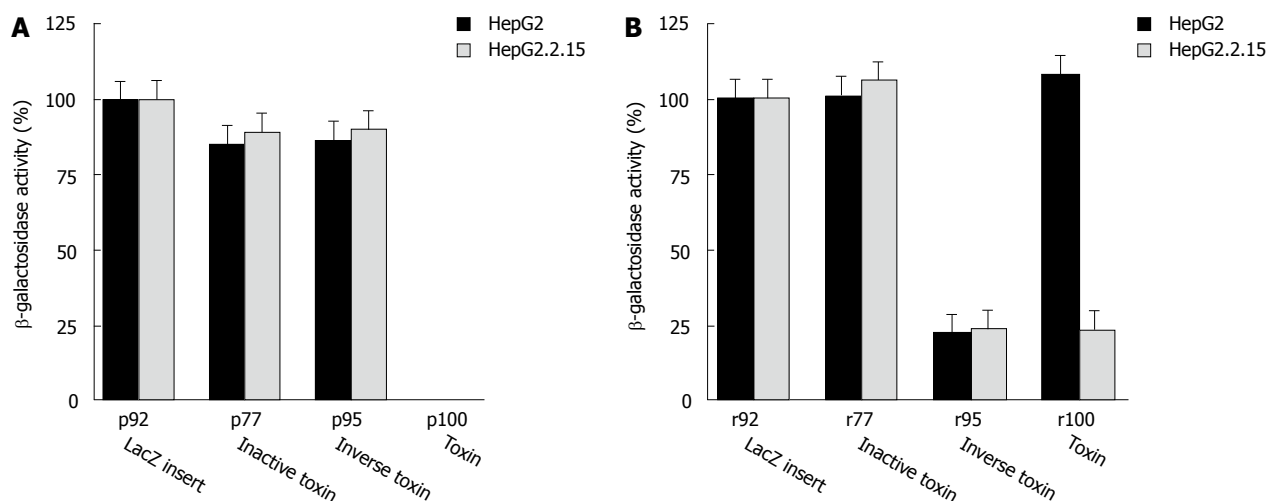
structures at the 5'- and 3'- ends of the RNA allow self-primed, i.e., primer-independent synthesis of cDNA by RT. Figure 1B shows the conversion of these RNAs to cDNA when incubated with dNTPs and RT either with or without primers. The self-primed *in vitro* cDNA synthesis is as effective as random-primed cDNA synthesis under otherwise identical conditions.

Expression of PE or truncated active PE derivatives in animal cells leads to cell death resulting in lack of viable PE expressing transfectants<sup>[16]</sup>. The analysis of cell viability is based upon transfection of toxin expression plasmids, sense or antisense toxin RNA, and a LacZ reporter plasmid (p92) followed by colorimetric quantitation of the LacZ gene product  $\beta$ -galactosidase ( $\beta$ -Gal). The  $\beta$ -Gal activity correlates with the number of transfectants as well as cell viability, because  $\beta$ -Gal protein is synthesized in viable cells only. Thus, reduced  $\beta$ -Gal activity reflects cell death induced by intracellular PE expression. Our data indicate that plasmids expressing an active toxin (p100) kill cells independent from RT (Figure 2A). By contrast, a plasmid expressing a mutated inactive toxin gene (p77) is not toxic. p95 is not toxic, because the PE cassette has an inverse orientation to the CMV promoter resulting in synthesis of nonsense RNA. We conclude that transfection and expression of a fully active or only partially active sense PE gene kills target cells. By contrast, a PE gene inactivated by a frameshift mutation or inversion of the cassette is not toxic.

Next, we analyzed whether PE sense RNA synthesized *in vitro* can be transfected and expressed in target cells resulting in cytotoxicity. The T7 *in vitro* transcription cassette of p95 is in the same orientation as the CMV eukaryotic expression cassette encoding the toxin. Indeed, RNA derived from p95 is cytotoxic even though transfected DNA plasmid itself is not toxic. Cell viability after transfection of r95 toxin RNA is reduced in both HepG2 and HepG2.2.15 cells (Figure 2B compare to p100 DNA in Figure 2A) as well as in DHBV infected cells (Figure 3). These data show that transfected PE RNA is expressed in cells, resulting in cytotoxicity. By comparison transfection of r95 (inverse orientation of PE cassette) is less toxic than transfection of p100.

#### **Reverse transcription of PE antisense RNA by HBV and DHBV**

Transfection of sense RNA kills HBV expressing target cells as well as normal cells in a non-specific manner. Using PE antisense RNA we analyzed the effect of HBV-mediated reverse transcription on cytotoxicity. PE antisense RNA was synthesized by *in vitro* transcription of the plasmids p77 and p100. It contains stem loop sequences at both ends, a toxin gene, poly A signals and CMV promoter sequences, all in inverse orientation to the toxin reading frame and transcription/translation signals (Figure 1A). Antisense RNA should be nontoxic to cells because, similarly to p95 the toxin gene has an inverse orientation. However, if antisense toxin RNA is reverse transcribed in RT positive cells, similar to M-MLV RT *in vitro* (Figure 1B), an active toxin should be synthesized resulting in cell death of RT positive cells only. Indeed,

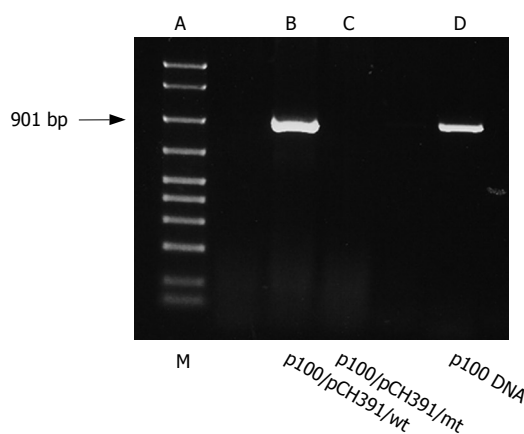


**Figure 2 A:** Cytotoxicity of toxin expression plasmids to HepG2 and HepG2.2.15 cells.  $\beta$ -Gal activities obtained after cotransfection with the  $\beta$ -Gal expressing DNA plasmid p92 (7.5  $\mu$ g) and p92 plasmid without insert (7.5  $\mu$ g) were set to 100%. Activities resulting from coexpression of toxin plasmids (p77, p95, p100) and  $\beta$ -Gal expressing plasmid p92 are expressed as relative levels. p95, coexpression of p95 with the toxin gene in opposite orientation to the CMV promoter; p100, coexpression of p100 with the active toxin fragment under control of the CMV promoter. Cell viability after cotransfection was determined by  $\beta$ -Gal activity from the  $\beta$ -Gal expressing plasmid. Reduced activity reflects cytotoxicity. **B:** Cytotoxicity of toxin of sense and antisense RNA. Transfection of PE sense and antisense RNA in HepG2 and HepG2.2.15 cells. RT dependent toxicity of antisense toxin RNA in HepG2 and HepG2.2.15 cells was determined. The  $\beta$ -Gal activities obtained after cotransfection with the  $\beta$ -Gal expressing DNA plasmid p92 (7.5  $\mu$ g) and *in vitro* transcribed r92 control RNA (7.5  $\mu$ g) were set to 100%. Activities resulting from coexpression of toxin RNA (r77, r95, r100) and  $\beta$ -Gal expressing plasmid p92 are expressed as relative levels. r77, antisense RNA of inactivated toxin from p77; r95, sense toxin RNA from p95; r100, antisense RNA of active toxin from p100.

transfection of antisense RNA into HepG2.2.15 cells or DHBV infected hepatocytes expressing HBV or DHBV polymerase (HBV or DHBV-RT) results in cell death (Figure 2B, Figure 4) while HepG2 cells expressing no HBV or DHBV polymerase are not affected. In contrast to transfection of sense toxin RNA r95 (or toxin genes, Figure 2A), that kills cells independent from the presence of RT, antisense toxin RNA r100 derived *in vitro* from p100 is selectively toxic in HBV or DHBV positive cells. We therefore conclude that HBV and DHBV RT reverse transcribes PE antisense RNA, resulting in cytotoxicity.

To further analyze the selective cytotoxicity to HBV infected cells, HepG2 cells were transfected with the HBV polymerase construct pCH-391wt expressing wild-type HBV polymerase or pCH-391mt encoding an inactive HBV polymerase with a mutation in the YMDD-motif (Figure 5A). A similar effect on cell viability was observed after transfection of HepG2 cells with r100 RNA and pCH-391wt or of HepG2.2.15 cells with r100 RNA (Figure 2B). Again, r95 RNA kills HepG2 cells independent from the cotransfection of pCH-391wt or pCH-391mt (Figure 5A). By contrast, markedly reduced cytotoxicity was observed after HepG2 cells were transfected with PE antisense RNA r100 and pCH-391mt. This finding indicates that cell death caused by toxin antisense RNA r100 depends on the presence of active HBV polymerase.

To further support this conclusion, HepG2 cells were transfected with antisense toxin RNA r100 and wild-type HBV polymerase pCH-391wt in the presence of adefovir - a potent inhibitor of HBV polymerase. Inhibition of HBV polymerase indeed results in reduced cytotoxicity (Figure 5B). A similar result was achieved when adefovir was added to DHBV infected cells after transfection with



**Figure 3** RT-PCR of genomic DNA from HepG2 cells transfected with a HBV polymerase expression plasmid. 7.5  $\mu$ g p77 RNA mixed with 7.5  $\mu$ g p92  $\beta$ -Gal expression plasmid were transfected in each experiment as well as plasmids expressing either wild-type or mutant HBV polymerase (7.5  $\mu$ g each). Genomic DNA was extracted and analyzed by non-denaturing 2% agarose gel electrophoresis. PCR fragments indicate the presence of toxin p77 cDNA. A: Bio-Rad marker 50-2000 base pairs; B: PCR amplified fragment of HepG2 cells cotransfected with pCH391wt and r100; C: PCR amplified fragment of HepG2 cells cotransfected with pCH391mt and r100; D: P77 DNA as control DNA.

antisense toxin RNA r100 (Figure 4). Taken together these findings demonstrate that the toxicity of r100 is dependent on HBV RT.

#### RT-PCR of genomic DNA from HepG2 cells transfected with HBV polymerase expression plasmid

To demonstrate that r100 is reverse transcribed in HBV-infected cells and to visualize the resulting p100 cDNA, genomic DNA was extracted from HepG2 cells transfected with either wild-type or mutant HBV

polymerase expression plasmid (pCH-391wt, pCH-391mt). RNA was transcribed from linearized p100 DNA. RT-PCR (Figure 3) demonstrates that r100 was reverse transcribed from p100 only in cells cotransfected with pCH-391wt, resulting in the amplification of a 901 bp DNA fragment. In cells cotransfected with pCH-391mt p100 DNA could not be amplified.

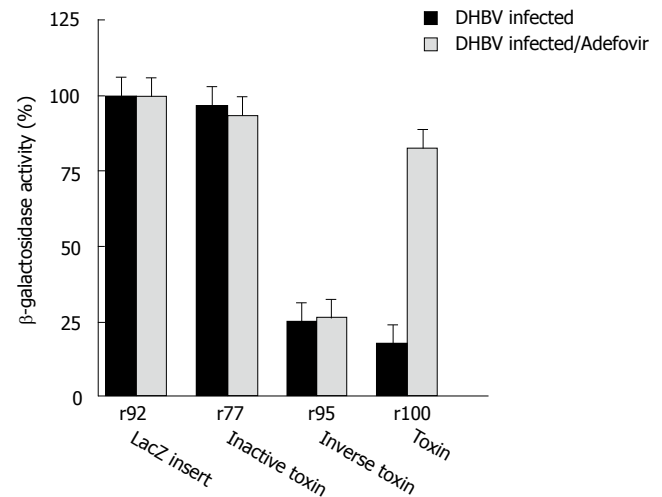
## DISCUSSION

HBV infection is a major cause of chronic hepatitis, liver cirrhosis and HCC worldwide. HCC develops 30-50 years after HBV infection and is a leading cause of death, especially in sub-Saharan Africa and South East Asia where up to 20% of the population are HBV infected.

The HBV genome replicates *via* reverse transcription of a RNA intermediate. This property has been used in a model system of HBV infection based on HBV transfected cell lines to study the selective cytotoxicity of a PE antisense toxin RNA.

Despite the availability of a safe and efficient vaccine, chronic HBV infection remains a major health problem. The problems of non-responding individuals and emergence of vaccine escape mutants are largely still unsolved. New treatment options with nucleotide analogues initially designed for HIV infection have become available. Lamivudine efficiently inhibits HBV infection but drug resistant mutations are frequent. Adefovir is an alternative for the treatment of chronic HBV infection. Different from lamivudine, viral resistance to adefovir is used lesser frequent. Viral variants may influence the course of disease and require special attention during antiviral therapy<sup>[8]</sup>. Antiviral agents such as lamivudine, adefovir, entecavir and others work directly inhibit HBV viral polymerase and thereby suppress viral replication<sup>[25]</sup>. The viral cccDNA template in the cell nucleus, however, is not affected by antiviral therapy. Therefore, HBV infection persists, because the turnover rate of hepatocytes is rather slow. Therefore, cessation of treatment or the development of breakthrough mutations promptly results in eradication of replication. HBV polymerase is highly error-prone and lacks proofreading activity, as do other RT such as HIV-RT. Thus, viral quasiespecies will develop during persistent HBV infection<sup>[26]</sup>. In this setting, the use of nucleotide chain terminators such as lamivudine, adefovir, entecavir and others carry the risk of selecting drug resistant viral mutants by suppressing the replication of the major wild-type population. These agents allow noncompetitive drug-resistant quasiespecies to replicate and become the dominant viral population.

To overcome the problem of drug resistance we explored a novel experimental strategy approach to eliminate HBV infected cells. PE has the advantage as a candidate gene for gene therapy, because only very few molecules are necessary to induce cell death<sup>[16]</sup>. It is widely used as an experimental therapeutic approach as part of immunotoxins for a variety of malignant diseases. A major requirement for *in vivo* gene therapy is to target the therapeutic gene expression to tissues of interest by selecting potent therapeutic genes. The mechanism of



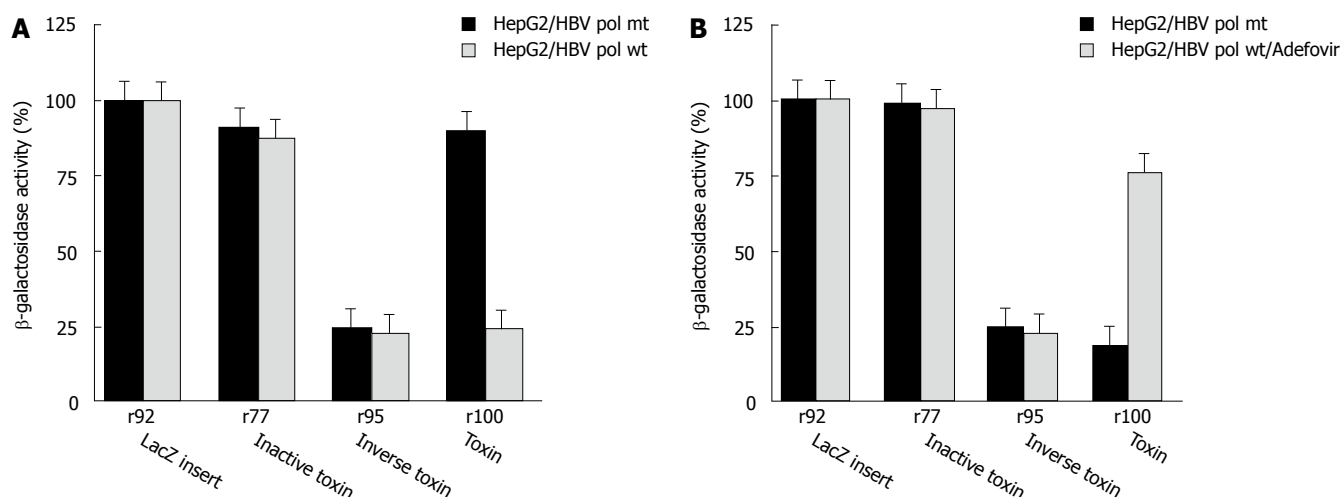
**Figure 4** Cytotoxicity of toxin sense and antisense RNA to DHBV infected duck hepatocytes in the presence or absence of 10 mmol/L adefovir. 7.5 µg RNA mixed with 7.5 µg p92 β-Gal expression plasmid were transfected in each experiment and β-Gal activities were quantified as described in legend to Figure 5. In addition, a wild-type HBV polymerase expression plasmid was transfected (7.5 µg). P92, control RNA from p92; p77, antisense RNA of inactivated toxin from p77; p95, sense toxin RNA from p95; p100, antisense RNA of active toxin from p100.

action of plant or bacterial toxins is internalization into an endosome, and translocation to the cytosol followed by enzyme induced modification or destruction of components critical for the cellular translation machinery resulting in lethal inhibition of protein synthesis or induction of programmed cell death<sup>[27,28]</sup>. An example for a retroviral disease is HIV infection where infected cells were selectively killed by an immunotoxin, consisting of a truncated PE joined to the variable region of a broadly neutralizing antibody (3B3) recognizing the viral envelope glycoprotein (env)<sup>[17]</sup>.

In our experiments PE antisense toxin RNA is less toxic to RT positive cells than toxin DNA (compare Figures 4 and 5: killing of HepG2 cells by p100 DNA *versus* r100 RNA). Possibly, intracellular reverse transcription of toxin antisense RNA is inefficient and the transfected RNA is degraded rather than expressed. Despite these limitations, our data clearly demonstrate that, in principle, it is possible to reverse transcribe a nontoxic RNA in RT positive cells to a toxic cDNA expressing active PE, resulting in cell death.

The elimination of retrovirus infected cells *in vivo* by antisense toxin RNA requires effective delivery to target cells. Thus, RNA instability and low transfection efficacy limit its usefulness and make even the analysis of antisense toxin RNA *in vitro* difficult. It might be possible to deliver such RNA, more effectively using liposomes or recombinant viruses<sup>[29,30]</sup>. Cells with retrotransposon activity could be affected by this approach when retroposon activity would be able to reverse transcribe the antisense-toxin RNA template.

Ultimately, retroviruses which naturally contain and stabilize RNA could be engineered to carry antisense toxin RNA and thus serve as a Trojan horse for the treatment of HBV infection.



**Figure 5** A: Cytotoxicity of toxin sense and antisense RNA to HepG2 cells in the presence or absence of transfected HBV polymerase expression plasmid. The  $\beta$ -Gal activities obtained after cotransfection with the  $\beta$ -Gal expressing DNA plasmid p92 (7.5  $\mu$ g), *in vitro* transcribed r92 control RNA (7.5  $\mu$ g) and plasmids expressing either HBV harbouring a wild-type or mutant HBV polymerase (7.5  $\mu$ g each) were set to 100%. Values resulting from coexpression of toxin RNAs (r77, r95, r100) with p92 and either wild-type HBV polymerase or mutant HBV polymerase expressing plasmids are expressed as relative levels. B: Cytotoxicity of toxin sense and antisense RNA to HepG2 cells in the presence or absence of 10 mmol/L adefovir. 7.5  $\mu$ g RNA mixed with 7.5  $\mu$ g p92  $\beta$ -Gal expression plasmid were transfected in each experiment and  $\beta$ -Gal activities were quantified as described in legend to Figure 3. In addition, a wild-type HBV polymerase expression plasmid was transfected (7.5  $\mu$ g). p92, control RNA from p92; p77, antisense RNA of inactivated toxin from p77; p95, sense toxin RNA from p95; p100, antisense RNA of active toxin from p100.

## COMMENTS

### Background

Hepatitis B virus (HBV) is the prototypic member of the Hepadnaviridae, a DNA-containing virus that replicates by reverse transcription of a pregenomic intermediate. Initiation of reverse transcription is thought to be strictly template specific. Here we present data showing that HBV-reverse transcription of the non-viral template coding for *Pseudomonas* exotoxin (PE) is possible. The RT-activity expressed in hepadnavirus-infected cells is recruited to convert an "antisense-toxin-RNA" into a lethal toxin exclusively in HBV-infected cells. We also present this approach for selectively killing retrovirus infected cells that combines the toxicity of *Pseudomonas* exotoxin (PE) and the presence of RT in infected cells.

### Research frontiers

Current therapies for chronic HBV infection are limited in their effect on viral gene expression and replication. Experimental therapies for HBV-infection are RNA interference (RNAi), ribozyme technology, target-dependent ribozymes, and gene therapeutic approaches using a variety of vectors to deliver therapeutic genes into HBV-infected cells. *Pseudomonas* exotoxin A kills cells either by direct inhibition of protein synthesis, or by concomitant induction of apoptosis.

### Innovations and breakthroughs

Here it is shown for the first time that HBV-and DHBV infected cells can be selectively killed by *Pseudomonas* exotoxin. The explanation for RT dependent toxicity is likely conversion of antisense-toxin-RNA to a toxin expression module by self-primed reverse transcription in the cell. This approach has been previously used by targeting HIV- infected cells by recombinant toxins that bind to HIV-surface antigens and kill infected cells by the toxin moiety. Moreover it is shown that HBV-polymerase can replicate a non-viral template. HBV polymerase can be active in trans outside the HBV nucleocapsid.

### Applications

The data demonstrate that, in principle, it is possible to generate a lethal cDNA copy of a per se nontoxic RNA in RT-containing infected cells. This principle is not limited to toxin genes but can probably be applied to other genes, e.g. inhibitors or ribozymes. The elimination of retrovirus infected cells *in vivo* by antisense-toxin-RNA requires effective delivery to target cells. It might be possible to deliver such RNA more effectively with liposomes or recombinant viruses. Ultimately, retroviruses which naturally contain and stabilize RNA could be loaded with antisense-toxin-RNA to serve as a "Trojan horse" in the battle against AIDS.

### Peer review

Cells with retrotransposon activity could be affected by this approach when retroposon activity would be able to reverse transcribe the antisense-toxin RNA template.

## REFERENCES

- 1 Poiesz BJ, Ruscetti FW, Gazdar AF, Bunn PA, Minna JD, Gallo RC. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. *Proc Natl Acad Sci USA* 1980; **77**: 7415-7419
- 2 Gallo RC. Human retroviruses: a decade of discovery and link with human disease. *J Infect Dis* 1991; **164**: 235-243
- 3 Zoulim F. Hepatitis B virus resistance to entecavir in nucleoside naive patients: Does it exist? *Hepatology* 2006; **44**: 1404-1407
- 4 Matthews G. The management of HIV and hepatitis B coinfection. *Curr Opin Infect Dis* 2007; **20**: 16-21
- 5 Pawlitsky JM, Dusheiko G, Hatzakis A, Lau D, Lau G, Liang TJ, Locarnini S, Martin P, Richman DD, Zoulim F. Virologic monitoring of hepatitis B virus therapy in clinical trials and practice: recommendations for a standardized approach. *Gastroenterology* 2008; **134**: 405-415
- 6 Leemans WF, Ter Borg MJ, de Man RA. Review article: Success and failure of nucleoside and nucleotide analogues in chronic hepatitis B. *Aliment Pharmacol Ther* 2007; **26** Suppl 2: 171-182
- 7 Tan J, Degertekin B, Wong SN, Husain M, Oberhelman K, Lok AS. Tenofovir monotherapy is effective in hepatitis B patients with antiviral treatment failure to adefovir in the absence of adefovir-resistant mutations. *J Hepatol* 2008; **48**: 391-398
- 8 Baumert TF, Thimme R, von Weizsacker F. Pathogenesis of hepatitis B virus infection. *World J Gastroenterol* 2007; **13**: 82-90
- 9 Chaudhary VK, Mizukami T, Fuerst TR, FitzGerald DJ, Moss B, Pastan I, Berger EA. Selective killing of HIV-infected cells by recombinant human CD4-Pseudomonas exotoxin hybrid protein. *Nature* 1988; **335**: 369-372
- 10 Ashorn P, Moss B, Weinstein JN, Chaudhary VK, FitzGerald DJ, Pastan I, Berger EA. Elimination of infectious human immunodeficiency virus from human T-cell cultures by synergistic action of CD4-Pseudomonas exotoxin and reverse transcriptase inhibitors. *Proc Natl Acad Sci USA* 1990; **87**: 8889-8893
- 11 Pincus SH, Cole RL, Hersh EM, Lake D, Masuho Y, Durda PJ,



- McClure J. In vitro efficacy of anti-HIV immunotoxins targeted by various antibodies to the envelope protein. *J Immunol* 1991; **146**: 4315-4324
- 12 **Bell KD**, Ramilo O, Vitetta ES. Combined use of an immunotoxin and cyclosporine to prevent both activated and quiescent peripheral blood T cells from producing type 1 human immunodeficiency virus. *Proc Natl Acad Sci USA* 1993; **90**: 1411-1415
  - 13 **Erice A**, Balfour HH Jr, Myers DE, Leske VL, Sannerud KJ, Kuebelbeck V, Irvin JD, Uckun FM. Anti-human immunodeficiency virus type 1 activity of an anti-CD4 immunoconjugate containing pokeweed antiviral protein. *Antimicrob Agents Chemother* 1993; **37**: 835-838
  - 14 **Hwang J**, Fitzgerald DJ, Adhya S, Pastan I. Functional domains of Pseudomonas exotoxin identified by deletion analysis of the gene expressed in E. coli. *Cell* 1987; **48**: 129-136
  - 15 **Carroll SF**, Collier RJ. Active site of Pseudomonas aeruginosa exotoxin A. Glutamic acid 553 is photolabeled by NAD and shows functional homology with glutamic acid 148 of diphtheria toxin. *J Biol Chem* 1987; **262**: 8707-8711
  - 16 **Wels W**, Baldrich M, Chakraborty T, Gross R, Goebel W. Expression of bacterial cytotoxin genes in mammalian target cells. *Mol Microbiol* 1992; **6**: 2651-2659
  - 17 **McHugh L**, Hu S, Lee BK, Santora K, Kennedy PE, Berger EA, Pastan I, Hamer DH. Increased affinity and stability of an anti-HIV-1 envelope immunotoxin by structure-based mutagenesis. *J Biol Chem* 2002; **277**: 34383-34390
  - 18 **Kennedy PE**, Bera TK, Wang QC, Gallo M, Wagner W, Lewis MG, Berger EA, Pastan I. Anti-HIV-1 immunotoxin 3B3(Fv)-PE38: enhanced potency against clinical isolates in human PBMCs and macrophages, and negligible hepatotoxicity in macaques. *J Leukoc Biol* 2006; **80**: 1175-1182
  - 19 **Sells MA**, Chen ML, Acs G. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc Natl Acad Sci USA* 1987; **84**: 1005-1009
  - 20 **Dunn JJ**, Studier FW. Complete nucleotide sequence of bacteriophage T7 DNA and the locations of T7 genetic elements. *J Mol Biol* 1983; **166**: 477-535
  - 21 **Gentz R**, Langner A, Chang AC, Cohen SN, Bujard H. Cloning and analysis of strong promoters is made possible by the downstream placement of a RNA termination signal. *Proc Natl Acad Sci USA* 1981; **78**: 4936-4940
  - 22 **Theuer CP**, Kreitman RJ, Fitzgerald DJ, Pastan I. Immunotoxins made with a recombinant form of Pseudomonas exotoxin A that do not require proteolysis for activity. *Cancer Res* 1993; **53**: 340-347
  - 23 **Brinkmann U**, Pai LH, Fitzgerald DJ, Willingham M, Pastan I. B3(Fv)-PE38KDEL, a single-chain immunotoxin that causes complete regression of a human carcinoma in mice. *Proc Natl Acad Sci USA* 1991; **88**: 8616-8620
  - 24 **Radziwill G**, Tucker W, Schaller H. Mutational analysis of the hepatitis B virus P gene product: domain structure and RNase H activity. *J Virol* 1990; **64**: 613-620
  - 25 **Koeck J**, Baumert TF, Delaney WE, Blum HE, Weizsaecker F. Inhibitory effect of adefovir and lamivudine on the initiation of hepatitis B virus infection in primary tupaia hepatocytes. *Hepatology* 2003; **38**: 1410-1408
  - 26 **Stuyver LJ**, Locarnini SA, Lok A, Richman DD, Carman WF, Dienstag JL, Schinazi RF. Nomenclature for antiviral-resistant human hepatitis B virus mutations in the polymerase region. *Hepatology* 2001; **33**: 751-757
  - 27 **Kreitman RJ**, Bailon P, Chaudhary VK, Fitzgerald DJ, Pastan I. Recombinant immunotoxins containing anti-Tac(Fv) and derivatives of Pseudomonas exotoxin produce complete regression in mice of an interleukin-2 receptor-expressing human carcinoma. *Blood* 1994; **83**: 426-434
  - 28 **Hafkemeyer P**, Brinkmann U, Gottesman MM, Pastan I. Apoptosis induced by Pseudomonas exotoxin: a sensitive and rapid marker for gene delivery in vivo. *Hum Gene Ther* 1999; **10**: 923-934
  - 29 **Zhu N**, Liggitt D, Liu Y, Debs R. Systemic gene expression after intravenous DNA delivery into adult mice. *Science* 1993; **261**: 209-211
  - 30 **Danos O**, Mulligan RC. Safe and efficient generation of recombinant retroviruses with amphotropic and ecotropic host ranges. *Proc Natl Acad Sci USA* 1988; **85**: 6460-6464

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CLINICAL RESEARCH

# Effect of oophorectomy and exogenous estrogen replacement on liver injury in experimental obstructive jaundice

Hamdi Bülent Uçan, Mehmet Kaplan, Bülent Salman, Utku Yılmaz, B Bülent Menteş, Cemalettin Aybay

Hamdi Bülent Uçan, Department of General Surgery, Karaelmas University Medical School, Zonguldak 67100, Turkey  
Mehmet Kaplan, Department of General Surgery, Gaziantep State Hospital, Gaziantep 27100, Turkey  
Bülent Salman, Utku Yılmaz, B Bülent Menteş, Department of General Surgery, Gazi University Medical School, Ankara 06500, Turkey  
Cemalettin Aybay, Department of Immunology, Gazi University Medical School, Ankara 06500, Turkey  
Author contributions: Uçan HB, Kaplan M and Salman B designed research; Uçan HB, Kaplan M, Salman B and Yılmaz U performed research; Menteş BB and Aybay C contributed new analytic tools; Uçan HB, Salman B and Yılmaz U Menteş BB analyzed data; Uçan HB, Salman B and Yılmaz U wrote the paper; Menteş BB and Aybay C supervised and revised the article.  
Correspondence to: Bülent Salman, MD, Department of General Surgery, Gazi University School of Medicine, Beşevler 06500, Ankara, Turkey. [dr.bsalman@yahoo.com](mailto:dr.bsalman@yahoo.com)  
Telephone: +90-312-2024196 Fax: +90-312-2124647  
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**Peer reviewer:** Valentin Fuhrmann, MD, Department of Internal Medicine 4, Intensive Care Unit, Medical University Vienna, Wahringer Gürtel 18-20, A-1090 Vienna, Austria

Uçan HB, Kaplan M, Salman B, Yılmaz U, Menteş BB, Aybay C. Effect of oophorectomy and exogenous estrogen replacement on liver injury in experimental obstructive jaundice. *World J Gastroenterol* 2008; 14(18): 2818-2824 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2818.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2818>

## Abstract

**AIM:** To investigate the role of estrogen on liver injury in an experimental obstructive jaundice model.

**METHODS:** Three groups of female rats were constituted; group 1 was oophorectomized and given E2 ( $n = 14$ ), group 2 was oophorectomized and given placebo ( $n = 14$ ), and group 3 was sham operated ( $n = 14$ ). Fourteen days following constitution of bile duct ligation, all groups were compared in terms of serum tests, histopathologic parameters, and tissue levels of IFN- $\gamma$  and IL-6.

**RESULTS:** The parameters representing both the injury and/or the reactive response and healing were more pronounced in groups 1 and 2 ( $\chi^2 = 17.2$ ,  $\chi^2 = 10.20$ ;  $\chi^2 = 12.4$ ,  $P < 0.05$ ). In the sham operated or E2 administered groups significantly lower tissue levels of IFN- $\gamma$  and higher IL-6 levels were found. In contrast, high IFN- $\gamma$  and low IL-6 tissue levels were found in the oophorectomized and placebo group ( $P < 0.001$ ). Kupffer cell alterations were observed to be more pronounced in the groups 1 and 3 ( $\chi^2 = 6.13$ ,  $P < 0.05$ ).

**CONCLUSION:** Our study indicates that E2 impaired liver functions, accelerated both the liver damage and healing. In the conditions of bile duct obstruction, estrogen significantly changed the cytokine milieu in the liver.

## INTRODUCTION

It has long been known that there are differences between the two sexes in the occurrence of some diseases or the response that they give to the same disease entity<sup>[1]</sup>, such as cardiovascular disease<sup>[2]</sup>, sepsis<sup>[3]</sup>, various autoimmune and collagen diseases including autoimmune thyroid disease<sup>[4]</sup>, or liver diseases<sup>[5]</sup>. These findings suggest that gender may determine the susceptibility to some diseases whereas it may be preventive to others, which is probably attributed to estrogen(s). The recent identification of estrogen receptor (ER)  $\beta$  in many tissues besides the target organs of estrogen, which mainly possess ER $\alpha$ <sup>[6,7]</sup>, has suggested that a larger tissue area, than previously known, is affected by estrogen through its specific receptors<sup>[8,9]</sup>. The demonstration of ER in the liver<sup>[10]</sup> has led studies to focus on the influence of steroid hormones, particularly estrogens, on liver disorders and regeneration<sup>[11]</sup>. In rats, liver injury induced by alcohol<sup>[12,13]</sup> or carbon tetrachloride<sup>[14]</sup> is more frequently seen in females. In transplanted liver<sup>[15]</sup> and hepatectomy models<sup>[16,17]</sup>, estrogen significantly increases regeneration and proliferation, and reverses inhibitory effect of the estrogen receptor antagonist, tamoxifen, on hepatocyte proliferation. These findings demonstrate that estrogens may aggravate injury or accelerate regeneration, depending on the model used.

The influence of estrogen on liver injury has not been studied in obstructive jaundice before. Biliary stones are found in 10% of adult population, and the risk of occurrence increases in women on oral contraceptives and with pregnancy. Each year 3%-5% of patients with

symptomatic biliary stones become complicated<sup>[18]</sup>. In patients with chronic extrahepatic bile duct obstructions, endotoxemia has been demonstrated and prolonged bile duct obstruction is harmful for phagocytic activity of reticuloendothelial system and increases the risk of mortality in the presence of endotoxemia<sup>[19,20]</sup>.

In this experimental study we have searched the effects of estrogens on liver injury and regeneration with the associated cytokine production using the bile duct ligation model. The aim of the study is to determine the differences, in terms of biochemical and histopathological parameters of liver injury in obstructive jaundice, between the groups of rats that represent women who are postmenopausal or oophorectomized with or without estrogen replacement therapy, and premenopausal; and to demonstrate the possible role of cytokines in these differences; and to assess the clinical importance of these data.

## MATERIALS AND METHODS

Female Wistar albino rats, 12-16 wk old and 180-200 g, were used. They were kept in temperature-controlled environment, and they had free access to standard rat food and water. The study was approved by Gazi University ethic committee.

### *The study design and administration of estrogen*

Forty-two female rats were randomly assigned to three groups of 14 each. In order to nullify the effect of ovarian estrogen, the first two groups, group 1 and 2 ( $n = 14$ ), underwent bilateral oophorectomy, as previously described<sup>[22]</sup> while group 3 ( $n = 14$ ) was sham operated to maintain physiological estrogen levels. Only laparotomy and ovary exploration was performed in group 3. Synthetic estrogen 17  $\beta$ -estradiol (Sigma Chemical Co., Steinheim, Germany) was dissolved in sesame oil (Sigma) and 5 mg/0.1 mL injection doses were prepared. Fourteen days after surgeries, during which effects of ovarian estrogen had disappeared, group 1 was given subcutaneous 5 mg/0.1 mL 17  $\beta$ -estradiol. Group 2 and group 3 were given 0.1 mL sesame oil subcutaneously as a placebo, meanwhile group 1 received 5 mg estrogen in 0.1 mL doses subcutaneous. Second and third doses were given 1 d and 5 d after the first dose.

The bile ducts were ligated 24 h after administration of the last dose of estrogen (or placebo), as previously described<sup>[20]</sup>. The animals were anesthetized by an intramuscular injection of ketamine hydrochloride (50 mg/kg of body weight) under semisterile conditions and through a 3 cm long upper midline abdominal incision, the common bile ducts were isolated, double ligated with 6-0 vicryl (Ethicon, Birmingham, UK) and transected between the sutures. Meticulous attention was paid not to damage the blood supply and neighboring organs.

Fourteen days after bile duct ligation, the abdominal incision was reopened following ketamine anesthesia. The animals were killed by cardiac puncture and blood withdrawn for the measurements of estrogen level and biochemical parameters. The liver was excised and the liver was divided into two samples; one sample was kept in 10% formaldehyde solution for histopathological evaluation and

**Table 1** Study design ( $n = 14$ )

Day	Group 1	Group 2	Group 3
0	Bilateral oophorectomy	Bilateral oophorectomy	Sham operation
15, 16, and 22	5 mg/0.1 mL E2 in sesame oil, SC	0.1 mL sesame oil, SC	0.1 mL sesame oil, SC
23	Bile duct ligation	Bile duct ligation	Bile duct ligation
37	Killed, blood and tissue samples obtained	Killed, blood and tissue samples obtained	Killed, blood and tissue samples obtained

SC: Subcutane.

the other was sent to the laboratory immediately as a fresh tissue for cytokine level measurements by ELISA (Table 1).

### *Measurements of blood biochemical parameters, estrogen and tissue cytokine levels*

Blood estrogen levels were measured by chemiluminescence method in pg/mL with Access Immunoassay System (Beckman Coulter, Inc., CA, USA) analyzer. To show altered liver functions, blood levels of bilirubin in mg/dL, alanine transaminase (ALT), and  $\gamma$ -glutamyl transpeptidase (GGT) in U/L were measured by Synchron CX-7 Clinical System (Beckman).

To assess the effect of estrogen on cytokines, liver IFN (interferon)- $\gamma$  and IL (interleukin)-6 levels were measured by ELISA in pg/mL, because of their significant role on liver injury and regeneration. For the measurements, 0.5 g of the fresh liver tissue was harvested and prepared for ELISA by homogenization as described previously<sup>[22]</sup>. Briefly, each specimen was homogenized for 60 s in 10 mL of phosphate buffered solution containing a cocktail of protease inhibitors including 2 mmol/L phenylmethyl-sulphonyl fluoride and 2  $\mu$ g/mL aprotinin, leupeptin and pepstatin A (Sigma) to inhibit proteolysis of cytokines. The homogenates were collected from the homogenizer (Jencons Scientific Ltd., Bedfordshire, UK), ultra-centrifuged at 10 000 r/min at 4°C for 45 min, the supernatants aliquoted and stored at -70°C. Cytokines were measured by ELISA with rat IFN- $\gamma$  and rat IL -6 kits (BioSource Int. Inc., CA, USA) as recommended.

### *Histopathological evaluation*

Liver fragments were kept in 10% formaldehyde solution for 6-48 h. Following treatment with various concentrations of alcohol and xylene, the specimens were embedded in paraffin blocks. After the preparation, the sections were stained with Hematoxylin & Eosin and Masson's trichrome stains. Evaluations were made by a pathologist, blinded, using the parameters described previously<sup>[20]</sup>. Each of the histopathological parameters listed in Table 2 were evaluated and scored for each slide in terms of the degree of change such that zero was given for no change, 1 for slight, 2 for moderate, and three for severe changes. For some of the parameters that could not be so scored, zero or two was assigned for absence or presence of the pathology, respectively (Table 2).

### *Statistical analysis*

For all parameters the means and standard deviations

Table 2 Histopathologic scoring of liver injury

	Parameters	Score
1	Necrosis	0, 1, 2, 3
2	Regenerative activity	0, 1, 2, 3
3	Portal PMNL infiltration	0, 1, 2, 3
4	Portal MNL infiltration	0, 1, 2, 3
5	Ductular proliferation	0, 1, 2, 3
6	Fibroblastic activity	0, 1, 2, 3
7	Kupffer cell abnormalities	0, 2
8	Sinusoidal PMNL infiltration	0, 2
9	Sinusoidal MNL infiltration	0, 2
10	Portal vascular congestion	0, 2
11	Sinusoidal vascular congestion	0, 2
12	Portal vascular thrombosis	0, 2
13	Sinusoidal vascular thrombosis	0, 2
14	Portal and central venous phlebitis	0, 2
15	Arterial wall changes	0, 1, 2, 3
16	Hydropic degeneration	0, 1, 2, 3
17	Decrease in hepatocyte glycogen content	0, 2

PMNL: Polymorphonuclear leukocytes; MNL: Mononuclear leukocytes.

were calculated. Kruskal-Wallis variance analysis and Chi-squared tests were used for all nonparametric comparisons.

## RESULTS

Blood estrogen levels were significantly different in all groups ( $P < 0.001$ ). The highest values were  $580 \pm 124.01$  in the group 1 in which oophorectomy and E2 replacement had been done, whereas the lowest values were  $61.5 \pm 15.97$  obtained in group 2 given placebo. Blood estrogen levels of group 3 was  $208 \pm 35.02$ .

Serum bilirubin, ALT, and GGT levels increased in all groups, as evidence of obstructive jaundice and liver damage. Serum bilirubin levels were not found to be different among the groups ( $P > 0.05$ ). Blood ALT and GGT levels generally were lower in group 2 given the placebo given group 2 than in the groups 1 and 3 in which blood estrogen levels were normal or high, respectively. The significant differences emerged with its lowest value for ALT in group 2 and its higher values for GGT only between groups 1 and 2 ( $P < 0.05$ , Table 3).

Liver specimens of all rats were examined histopathologically according to the parameters mentioned in table 2 and groups were then compared using the chi-squared test. Among 17 parameters necrosis, regenerative activity, ductular proliferation, fibroblastic activity, Kupffer cell abnormalities, sinusoidal congestion and portal-central venous phlebitis showed statistical difference among the groups ( $P < 0.05$ , Table 4). Progressive rounding, ruffling of cell surface, polarization, appearance of wormlike densities, vacuolization and degranulation were the abnormalities seen in Kupffer cells. The photos of ductal proliferation, fibrosis and sinusoidal congestion of specimens can be seen especially in group 1 and 3 (Figure 1). In terms of portal polymorphonuclear leukocyte and mononuclear leukocyte infiltration, sinusoidal polymorphonuclear leukocyte and mononuclear leukocyte infiltration, portal vascular congestion, portal and sinusoidal vascular thrombosis, arteriolar wall changes, hydropic degeneration,

Table 3 Comparison of liver function tests of the groups

Groups	ALT (U/L)	GGT (U/L)	Bilirubin level (mg/dL)
1	$347.85 \pm 61.12$	$65.35 \pm 20.98^a$	$6.83 \pm 1.32$
2	$256.57 \pm 54.19^a$	$46.50 \pm 12.0$	$5.90 \pm 2.12$
3	$360.50 \pm 65.22$	$58.78 \pm 7.47$	$6.30 \pm 1.57$

There are significant differences when compared with other groups ( $^aP < 0.05$ ).

and hepatocyte glycogen content, there was no significant difference among groups ( $P > 0.05$ , data not shown).

When necrosis was considered there appeared a significant difference, which was due to the lower values in group 2 ( $\chi^2 = 17.2$ ,  $P < 0.05$ , Table 4). Group 2 was also found to be significantly different with its lower regenerative activity ( $\chi^2 = 10.22$ ,  $P < 0.05$ , Table 4).

Ductular proliferation, a reactive response of liver to bile duct obstruction, was statistically more in groups 1 and 3 than in group 2 ( $\chi^2 = 12.43$ ,  $P < 0.05$ , Table 4).

In groups 1 and 3 with higher estrogen levels, fibroblastic activity was also significantly more pronounced ( $\chi^2 = 31.06$ ,  $P < 0.001$ , Table 4). There was a significant difference in terms of Kupffer cell abnormalities between groups 1 and 2 ( $\chi^2 = 6.13$ ,  $P < 0.05$ , Table 4).

Sinusoidal congestion and portal venous phlebitis were significantly lower in group 2 than in group 3 ( $\chi^2 = 8.17$ ,  $P < 0.05$ ,  $\chi^2 = 7.636$ ,  $P < 0.05$ , respectively, Table 4).

To summarize, in all groups, bile duct ligation produced histopathological changes in liver and there appeared a significant difference between group 2 with the lowest estrogen level and the others in terms of at least some of the parameters.

The groups were also compared according to the tissue cytokine measurements of IFN- $\gamma$  and IL-6 and were found to be statistically different. While IFN- $\gamma$  was significantly higher, IL-6 was lower in group 2 with the lowest estrogen level ( $P < 0.001$ , Table 4).

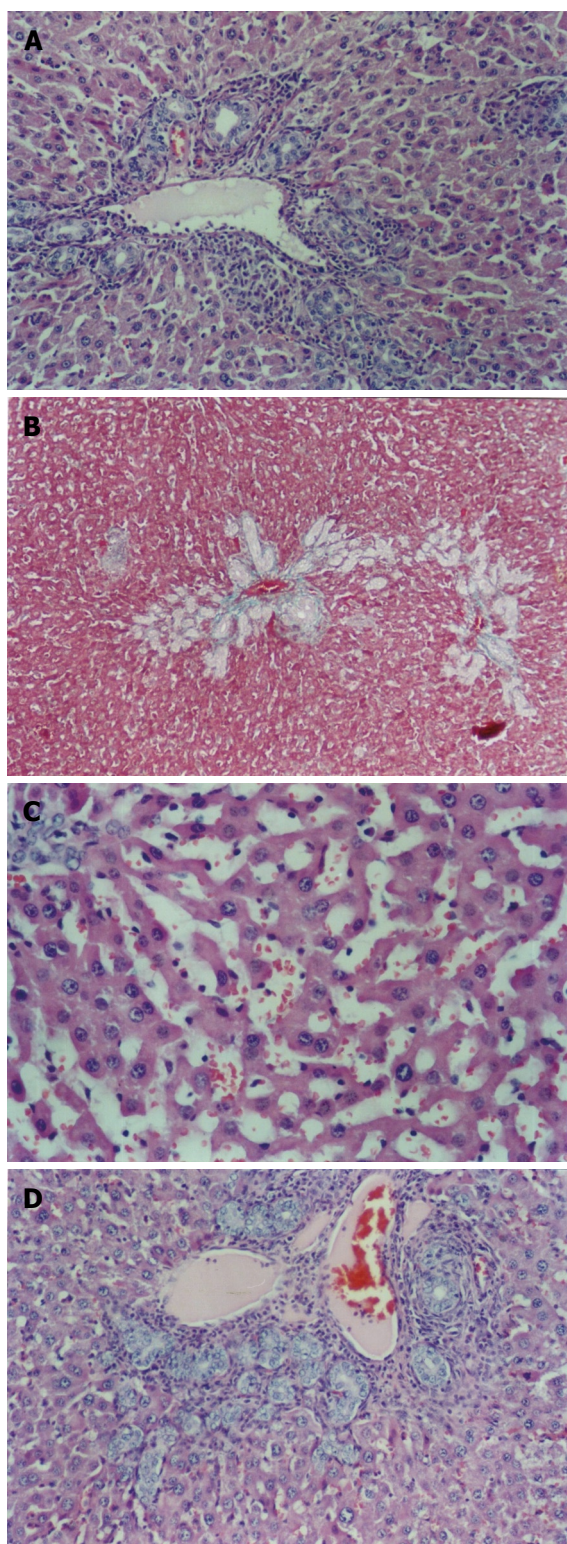
With Pearson's correlation analysis, IFN- $\gamma$  and IL-6 levels were tested and found to be inversely related to each other when group 2 was compared with groups 1 and 3 ( $r = -0.36$ ,  $P < 0.001$ , Figure 2).

## DISCUSSION

Differences exist between the two sexes of humans and animals<sup>[1]</sup>. It is known that in premenopausal period, female gender is a preventive factor against cardiovascular disorders<sup>[2,23]</sup>, peptic ulcer disease<sup>[24]</sup> and some others<sup>[3,23]</sup>, on the other hand, the risk of occurrence of autoimmune and collagen tissue disorders is more frequent in women<sup>[26]</sup>. It has been proposed that this fact may be due to the differences between the two sexes in hormone levels and tissue distribution of hormone receptors.

Because estrogen has influences on many tissues, other than its main target tissues, it has been speculated and shown that estrogen has a larger field of distribution than previously believed<sup>[7,27]</sup>. After the demonstration of estrogen receptors in liver, studies on the interaction of estrogen and its receptor in liver have accumulated<sup>[28,29]</sup>. Estrogen is thought to enhance regeneration after hepatectomy,





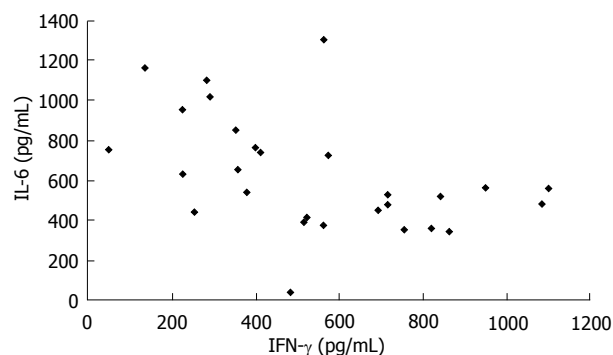
**Figure 1** A: Ductal proliferation and inflammation (HE, × 20); B: Fibrosis and ductal proliferation (Masson trichrom, × 10); C: Sinusoidal congestion (HE, × 40); D: Ductal proliferation and portal vascular congestion (HE, × 20).

due to its potentiative effect on proliferation, and studies have shown that estrogen increases regeneration<sup>[17]</sup> and mitotic activity of hepatocytes<sup>[15]</sup>, significantly. On the other hand, it potentiates liver injury with alcohol and carbon tetrachloride<sup>[4]</sup> administration<sup>[5,13,14,30]</sup>. Estrogen treatment also has an effect in cholangiocyte proliferation<sup>[31]</sup>.

Previous literature comprises data about the influence

**Table 4** Comparison of groups by categories *n* (%)

Categories	Group 1	Group 2	Group 3	$\chi^2/P$
Necrosis				
No change	5 (35.7)	12 (85.7)	7 (50)	$\chi^2 = 17.20$ $P < 0.050$
Slight	7 (50)	0 (0)	1 (7.1)	
Moderate	2 (14.3)	2 (14.3)	6 (42.9)	
Regenerative activity				
Slight	6 (42.9)	13 (92.9)	6 (42.9)	$\chi^2 = 10.22$ $P < 0.050$
Moderate	5 (35.7)	1 (7.1)	6 (42.9)	
Severe	3 (21.4)	0 (0)	2 (14.2)	
Ductular proliferation				
Slight	0 (0)	6 (42.9)	1 (7.1)	$\chi^2 = 12.43$ $P < 0.050$
Moderate	5 (35.7)	5 (35.7)	4 (28.6)	
Severe	9 (64.3)	3 (21.4)	9 (64.3)	
Fibroblastic activity				
Slight	1 (7.1)	9 (64.3)	1 (7.1)	$\chi^2 = 31.06$ $P < 0.001$
Moderate	9 (64.3)	4 (28.6)	11 (78.6)	
Severe	4 (28.6)	1 (7.1)	2 (14.3)	
Kupffer cell abnormalities				
Absence	6 (42.9)	12 (85.7)	7 (50)	$\chi^2 = 6.13$ $P < 0.050$
Presence	8 (57.1)	2 (14.3)	7 (50)	
Sinusoidal congestion				
Absence	13 (92.9)	9 (64.3)	14 (100)	$\chi^2 = 8.17$ $P < 0.050$
Presence	1 (7.1)	5 (35.7)	0 (0)	
Portal central venous phlebitis				
Absence	3 (21.4)	6 (42.9)	0 (0)	$\chi^2 = 7.64$ $P < 0.050$
Presence	11 (78.6)	8 (57.1)	14 (100)	
IFN- $\gamma$ (pg/mL)	349.46 $\pm$ 125.15	764.61 $\pm$ 191.95	450.21 $\pm$ 98.25	$P < 0.001$
IL-6 (pg/mL)	839.84 $\pm$ 245.05	466.5 $\pm$ 106.86	710.02 $\pm$ 167.75	$P < 0.001$



**Figure 2** Correlation between liver IFN- $\gamma$  and IL-6.  $r = -0.36$ ,  $P < 0.001$ .

of estrogen on various types of liver injury other than obstructive jaundice. In this experimental study, effects of estrogen on liver injury in bile duct ligation model were investigated.

Bile stones, 85% of which is cholesterol type, are frequent in surgical practice. Women are affected twice as much as men, and oral contraceptives and pregnancy increase the risk for biliary stone formation. Each year, 3%-5% of symptomatic biliary stones become complicated<sup>[18,32]</sup>. In general, benign and malignant bile duct obstructions are mostly seen in the elderly.

In our study, blood estrogen levels of groups were significantly different, as intended ( $P < 0.001$ , Table 3). While oophorectomized groups 1 and 2 had estrogen levels higher and lower than normal, respectively, group 3 maintained its physiological level. By this way, groups representing postmenopausal or bilaterally oophorectomized women with estrogen replacement (group 1), without

estrogen replacement (group 2) and premenopausal normal women (group 3) were constituted.

Increased serum bilirubin and liver enzyme levels after 14 d of bile duct obstruction proved that bile duct ligation had been successfully performed. The fact that the group with the lowest estrogen level (group 2) had significantly lower levels of liver enzymes than groups 1 and 3 suggested that estrogen enhanced liver injury. To test this, previously described parameters of liver injury were used for the comparison of groups (Table 2).

Histopathological changes were less significant in extent in group 2 with the lowest estrogen level than in the other groups, considering the seven parameters yielding statistical difference among groups (Table 4). This finding has shown that estrogen has an influence on liver injury. Reactive changes secondary to bile duct obstruction, like necrosis, ductular proliferation, Kupffer cell abnormalities, sinusoidal congestion, were significantly more pronounced in groups 1 and 3 than in group 2, which meant that both physiological and supraphysiological levels of estrogen potentiated, not only liver injury, but also reactive responses (Table 4). These data correlate well with the results of studies reporting that estrogen accelerates liver injury caused by alcohol and carbon tetrachloride<sup>[13,15,17]</sup>.

The fact that portal central venous phlebitis was more in both group 1 and 3, with statistical significance in the latter only, suggested that the occurrence of this change might be due to the influence of estrogen on coagulation and venous thromboemboli<sup>[33,34]</sup>.

Another striking result of this study was the statistically significant difference in cytokine levels among groups. There has been an inverse correlation between IFN- $\gamma$  and IL-6 ( $r < -0.5$ ). In group 2, with the lowest estrogen level, IL-6 was significantly lower ( $P < 0.001$ ) while IFN- $\gamma$  was significantly higher ( $P < 0.001$ ) than in the other groups in which IL-6 was higher and IFN- $\gamma$  lower (Table 4). This finding showed the possible strong effect of estrogen on liver cytokine pattern. It has been proposed that for regeneration, hepatocyte needs growth factors like hepatocyte growth factor, transforming growth factor- $\alpha$ , and epidermal growth factor; however, to be responsive to these growth factors, it must be sensitized with tumor necrosis factor- $\alpha$  and IL-6<sup>[35]</sup>. Kupffer cells have important effects on liver regeneration and in one study a decrease in that cell population, and hence in the production and the levels of many cytokines including IL-6, have been shown to slow down regeneration<sup>[36]</sup>. It has been accepted that IL-6 accelerates regeneration<sup>[35,37,38]</sup> and the most important synthesizers of IL-6 are Kupffer cells and macrophages infiltrating liver<sup>[39]</sup>. Besides, it has been suggested that IL-6 increases fibrosis because transforming growth factor- $\beta$ , that enhances fibrosis as a response to liver injury, seems to decrease in rats deprived of IL-6<sup>[40]</sup>. In this study, group 2 with low serum estrogen and liver IL-6 levels showed decreased regeneration and fibrotic activity unlike the other groups with higher IL-6 levels, and this supported the data about IL-6 in the literature.

IFN- $\gamma$  increases fibrosis in liver and in the absence of lymphocytes, responsible for the production of this cytokine, more intense fibrosis results<sup>[41,42]</sup>. In our study, IFN- $\gamma$  was found to be high in group 2 with the lowest

estrogen level and in group 2 regeneration and fibroblastic activity were decreased unlike the other groups with low IFN- $\gamma$  levels. This suggested that IFN- $\gamma$  had a role opposite to that of IL-6.

It has been reported that, estrogen suppresses<sup>[43-45]</sup>, stimulates<sup>[46]</sup> or is ineffective<sup>[3]</sup> on the secretion of IL-6 from various tissues or cells. The mentioned different effects of estrogen on IL-6 secretion probably are due to the distinct features of different tissues. Although it has been shown that, liver<sup>[47]</sup> and serum<sup>[48]</sup> IL-6 levels are increased transiently after obstructive jaundice, the effects of estrogen on liver tissue cytokine environment is not well understood. Influence of estrogen on IFN- $\gamma$  secretion is also contradictory; some reports have suggested suppression<sup>[49,50]</sup>, while others have claimed inhibition<sup>[51,52]</sup>.

In this study, we showed for the first time, even after 14 d of bile duct ligation, secretion of IL-6 was found to be increased while IFN- $\gamma$  was decreased in a reverse correlation under the influence of estrogen. Increased IL-6 and decreased IFN- $\gamma$ , together with the demonstration of accelerated regeneration and fibroblastic activity in liver, suggested the role of estrogen on liver injury might be mediated by these cytokines. Furthermore, determination of significantly more Kupffer cell changes in groups 1 and 3 with high level of estrogen, reminded us that changing of liver cytokine levels might be controlled by these cells. However, demonstration of higher degree of necrosis and other injury parameters in the groups with high level of estrogen, suggests that beside augmentation of regeneration, estrogen also increases existing injury.

As a result, our study indicates that in liver injury that is caused by bile duct obstruction, medications that contain estrogens should be avoided until the underlying cause of the liver damage is eliminated but, thereafter, they may be used because of possible effects of estrogen on liver regeneration. The existence and the considerable effects of estrogen receptors in liver may give rise to experiments studying on specific estrogen receptor modulators, at least, in some liver diseases. This study has also suggested that, in liver disease occurring before menopausal period, detrimental effects of physiological levels of estrogen(s) on the liver can perhaps be prevented by using specific estrogen blockers.

## COMMENTS

### Background

Different responses depend on differences in hormones between the sexes and have been the subject of several studies for several years. The main target hormone, estrogen, plays a critical role in several mechanisms in the body. Among these, estrogen receptors found in liver tissue have a key role for the estrogen effects on liver and related mechanisms. As women are more prone to bile stones and elderly postmenopausal patients have a higher incidence of bile obstruction, a role for estrogen must be considered in these pathologies.

### Research frontiers

It was shown that estrogen has a proliferative effect in partial hepatectomy; however, it increases the susceptibility of the liver to injury. On the other hand, bile duct obstruction, bile stasis and related cytokine changes by Kupffer cells have a distinct entity that must be investigated. Histopathological changes including Kupffer cells and cytokine release related to estrogen is still an unclear mechanism.

## Innovations and breakthroughs

As several studies mentioned the regenerative effect and increased injury due to hepatectomy, our study is performed in bile duct obstruction model. Not only the cholangiocyte proliferation, but also the cytokine levels, liver regeneration and liver function tests were also evaluated. Women are more prone to bile stones and the rate of bile duct obstruction increases with age. Bile duct obstruction related to bile stones is more likely to be seen in postmenopausal women. In this point of view, estrogen status and expected situations must be investigated.

## Applications

Although some points have been mentioned here, there are still unknown mechanisms for the actions of estrogen. Cytokine release, thromboemboli and coagulation are thought to be potential mechanisms. However, no clear definition has been made. We feel that this study will help in further clinical studies and will be an investigative source for other estrogen and bile duct obstruction related studies.

## Peer review

This is an interesting investigation of effects of estrogens on liver injury and regeneration using the bile duct ligation model. The presentation and readability of the manuscript is good. It indicates that E2 impaired the liver functions, accelerated both the liver damage and healing. In the conditions of bile duct obstruction, estrogen significantly changed the cytokine milieu in the liver.

## REFERENCES

- Blair ML. Sex-based differences in physiology: what should we teach in the medical curriculum? *Adv Physiol Educ* 2007; **31**: 23-25
- Villablanca AC. Coronary heart disease in women. Gender differences and effects of menopause. *Postgrad Med* 1996; **100**: 191-196, 201-202
- Schroder J, Kahlke V, Staubach KH, Zabel P, Stuber F. Gender differences in human sepsis. *Arch Surg* 1998; **133**: 1200-1205
- Lahita RG. The connective tissue diseases and the overall influence of gender. *Int J Fertil Menopausal Stud* 1996; **41**: 156-165
- Bradley KA, Badrinath S, Bush K, Boyd-Wickizer J, Anawalt B. Medical risks for women who drink alcohol. *J Gen Intern Med* 1998; **13**: 627-639
- Harris HA. Estrogen receptor-beta: recent lessons from in vivo studies. *Mol Endocrinol* 2007; **21**: 1-13
- Dechering K, Boersma C, Mosselman S. Estrogen receptors alpha and beta: two receptors of a kind? *Curr Med Chem* 2000; **7**: 561-576
- Gustafsson JA. Novel aspects of estrogen action. *J Soc Gynecol Invest* 2000; **7**: S8-S9
- Muramatsu M, Inoue S. Estrogen receptors: how do they control reproductive and nonreproductive functions? *Biochem Biophys Res Commun* 2000; **270**: 1-10
- Viladiu P, Delgado C, Pensky J, Pearson OH. Estrogen binding protein of rat liver. *Endocr Res Commun* 1975; **2**: 273-280
- Eagon PK, Porter LE, Francavilla A, DiLeo A, Van Thiel DH. Estrogen and androgen receptors in liver: their role in liver disease and regeneration. *Semin Liver Dis* 1985; **5**: 59-69
- Muller C. Liver, alcohol and gender. *Wien Med Wochenschr* 2006; **156**: 523-526
- Yin M, Ikejima K, Wheeler MD, Bradford BU, Seabra V, Forman DT, Sato N, Thurman RG. Estrogen is involved in early alcohol-induced liver injury in a rat enteral feeding model. *Hepatology* 2000; **31**: 117-123
- Kulcsar-Gergely J, Kulcsar A, Gomba S. Estrogens as disposing factors in experimental liver injury. *Exp Pathol (Jena)* 1978; **16**: 283-290
- Fujii H, Kotani M. Promoting effect of estrogen on regeneration of the liver transplanted to an ectopic site in mice. *Virchows Arch A Pathol Anat Histopathol* 1986; **409**: 453-460
- Fisher B, Gunduz N, Saffer EA, Zheng S. Relation of estrogen and its receptor to rat liver growth and regeneration. *Cancer Res* 1984; **44**: 2410-2415
- Francavilla A, Polimeno L, DiLeo A, Barone M, Ove P, Coetzee M, Eagon P, Makowka L, Ambrosino G, Mazzaferro V. The effect of estrogen and tamoxifen on hepatocyte proliferation in vivo and in vitro. *Hepatology* 1989; **9**: 614-620
- Strasberg SM, Clavien PA. Cholecystolithiasis: lithotherapy for the 1990s. *Hepatology* 1992; **16**: 820-839
- Holman JM Jr, Rikkers LF. Biliary obstruction and host defense failure. *J Surg Res* 1982; **32**: 208-213
- Mentes BB, Tatlicioglu E, Akyol G, Uluoglu O, Sultan N, Yilmaz E, Celebi M, Taneri F, Ferahkose Z. Intestinal endotoxins as co-factors of liver injury in obstructive jaundice. *HPB Surg* 1996; **9**: 61-69
- Hilf R, Battaglini JW, Delmez JA, Cohen N, Rector WD. Some biochemical changes preceding regression of 7,12-dimethylbenz(a)anthracene-induced mammary tumors following oophorectomy. *Cancer Res* 1971; **31**: 1195-1200
- Wu Y, Kudsk KA, DeWitt RC, Tolley EA, Li J. Route and type of nutrition influence IgA-mediating intestinal cytokines. *Ann Surg* 1999; **229**: 662-667; discussion 667-668
- Contreras I, Parra D. Estrogen replacement therapy and the prevention of coronary heart disease in postmenopausal women. *Am J Health Syst Pharm* 2000; **57**: 1963-1968; quiz 1969-1971
- Chen TS, Chang FY, Lee SD. Smoking and male gender rather than CagA protein are associated with increased risk for duodenal ulcer in *Helicobacter pylori*-infected patients in Taiwan. *Dig Dis Sci* 1999; **44**: 2076-2080
- Salem JE, Kring AM. The role of gender differences in the reduction of etiologic heterogeneity in schizophrenia. *Clin Psychol Rev* 1998; **18**: 795-819
- Dale E, Davis M, Faustman DL. A role for transcription factor NF-kappaB in autoimmunity: possible interactions of genes, sex, and the immune response. *Adv Physiol Educ* 2006; **30**: 152-158
- Barkhem T, Nilsson S, Gustafsson JA. Molecular mechanisms, physiological consequences and pharmacological implications of estrogen receptor action. *Am J Pharmacogenomics* 2004; **4**: 19-28
- Alvaro D, Mancino MG, Onori P, Franchitto A, Alpini G, Francis H, Glaser S, Gaudio E. Estrogens and the pathophysiology of the biliary tree. *World J Gastroenterol* 2006; **12**: 3537-3545
- Kawai T, Yokoyama Y, Kawai S, Yokoyama S, Oda K, Nagasaka T, Nagino M, Chaudry IH, Nimura Y. Does estrogen contribute to the hepatic regeneration following portal branch ligation in rats? *Am J Physiol Gastrointest Liver Physiol* 2007; **292**: G582-G589
- Thurman RG. II. Alcoholic liver injury involves activation of Kupffer cells by endotoxin. *Am J Physiol* 1998; **275**: G605-G611
- Alvaro D, Onori P, Metalli VD, Svegliati-Baroni G, Folli F, Franchitto A, Alpini G, Mancino MG, Attili AF, Gaudio E. Intracellular pathways mediating estrogen-induced cholangiocyte proliferation in the rat. *Hepatology* 2002; **36**: 297-304
- Gillanders W, Strasberg SM. Hepatobiliary disease. 2nd ed. Doherty GM, Meko JB, Olson JA, Peplinski GR, Worrall NK (Eds). The Washington Wilkins, manual of surgery. Lippincott Williams Philadelphia, 1999: 273-296
- Daly E, Vessey MP, Hawkins MM, Carson JL, Gough P, Marsh S. Risk of venous thromboembolism in users of hormone replacement therapy. *Lancet* 1996; **348**: 977-980
- Jick H, Derby LE, Myers MW, Vasilakis C, Newton KM. Risk of hospital admission for idiopathic venous thromboembolism among users of postmenopausal oestrogens. *Lancet* 1996; **348**: 981-983
- Fausto N. Liver regeneration. *J Hepatol* 2000; **32**: 19-31
- Meijer C, Wiezer MJ, Diehl AM, Schouten HJ, Schouten HJ, Meijer S, van Rooijen N, van Lambalgen AA, Dijkstra CD, van Leeuwen PA. Kupffer cell depletion by C12MDP-liposomes alters hepatic cytokine expression and delays liver regeneration after partial hepatectomy. *Liver* 2000; **20**: 66-77
- Kuma S, Inaba M, Ogata H, Inaba K, Okumura T, Saito K, Yamamoto M, Ikehara S. Effect of human recombinant interleukin-6 on the proliferation of mouse hepatocytes in the primary culture. *Immunobiology* 1990; **180**: 235-242
- Selzner M, Clavien PA. Failure of regeneration of the steatotic rat liver: disruption at two different levels in the regeneration



- pathway. *Hepatology* 2000; **31**: 35-42
- 39 **Oyanagi Y**, Takahashi T, Matsui S, Takahashi S, Boku S, Takahashi K, Furukawa K, Arai F, Asakura H. Enhanced expression of interleukin-6 in chronic hepatitis C. *Liver* 1999; **19**: 464-472
- 40 **Natsume M**, Tsuji H, Harada A, Akiyama M, Yano T, Ishikura H, Nakanishi I, Matsushima K, Kaneko S, Mukaida N. Attenuated liver fibrosis and depressed serum albumin levels in carbon tetrachloride-treated IL-6-deficient mice. *J Leukoc Biol* 1999; **66**: 601-608
- 41 **Shi Z**, Wakil AE, Rockey DC. Strain-specific differences in mouse hepatic wound healing are mediated by divergent T helper cytokine responses. *Proc Natl Acad Sci USA* 1997; **94**: 10663-10668
- 42 **Xu Q**, Cao J, Wu F, Hayakawa Y, Saiki I, Koda A. Role of Th1 and Th2 cytokines in regulating the liver injury induced by delayed-type hypersensitivity to picryl chloride. *Liver* 1999; **19**: 473-480
- 43 **Keck C**, Herchenbach D, Pfisterer J, Breckwoldt M. Effects of 17beta-estradiol and progesterone on interleukin-6 production and proliferation of human umbilical vein endothelial cells. *Exp Clin Endocrinol Diabetes* 1998; **106**: 334-339
- 44 **Koka S**, Petro TM, Reinhardt RA. Estrogen inhibits interleukin-1beta-induced interleukin-6 production by human osteoblast-like cells. *J Interferon Cytokine Res* 1998; **18**: 479-483
- 45 **Zuckerman SH**, Bryan-Poole N, Evans GF, Short L, Glasebrook AL. In vivo modulation of murine serum tumour necrosis factor and interleukin-6 levels during endotoxemia by oestrogen agonists and antagonists. *Immunology* 1995; **86**: 18-24
- 46 **Chen CD**, Wu MY, Chen HF, Chen SU, Ho HN, Yang YS. Relationships of serum pro-inflammatory cytokines and vascular endothelial growth factor with liver dysfunction in severe ovarian hyperstimulation syndrome. *Hum Reprod* 2000; **15**: 66-71
- 47 **Plebani M**, Panozzo MP, Basso D, De Paoli M, Biasin R, Infantolino D. Cytokines and the progression of liver damage in experimental bile duct ligation. *Clin Exp Pharmacol Physiol* 1999; **26**: 358-363
- 48 **Kimura F**, Miyazaki M, Suwa T, Sugiura T, Shinoda T, Itoh H, Ambiru S, Shimizu H, Nakagawa K. Serum interleukin-6 levels in patients with biliary obstruction. *Hepatogastroenterology* 1999; **46**: 1613-1617
- 49 **Le N**, Yousefi S, Vaziri N, Carandang G, Ocariz J, Cesario T. The effect of beta-estradiol, progesterone and testosterone on the production of human leukocyte derived interferons. *J Biol Regul Homeost Agents* 1988; **2**: 199-204
- 50 **Salem ML**, Hossain MS, Nomoto K. Mediation of the immunomodulatory effect of beta-estradiol on inflammatory responses by inhibition of recruitment and activation of inflammatory cells and their gene expression of TNF-alpha and IFN-gamma. *Int Arch Allergy Immunol* 2000; **121**: 235-245
- 51 **Correale J**, Arias M, Gilmore W. Steroid hormone regulation of cytokine secretion by proteolipid protein-specific CD4+ T cell clones isolated from multiple sclerosis patients and normal control subjects. *J Immunol* 1998; **161**: 3365-3374
- 52 **Fox HS**, Bond BL, Parslow TG. Estrogen regulates the IFN-gamma promoter. *J Immunol* 1991; **146**: 4362-4367

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## Decreased postprandial gallbladder emptying in patients with black pigment stones

Takakazu Sugo, Kenichi Hakamada, Shunji Narumi, Mutsuo Sasaki

Takakazu Sugo, Kenichi Hakamada, Shunji Narumi, Mutsuo Sasaki, Department of Gastroenterological Surgery, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan

**Author contributions:** Sugo T designed the research; Sugo T, Hakamada K and Narumi S collected the data; Sugo T and Hakamada K carried out the statistical analyses and organized the figures and tables; Hakamada K wrote the paper; and Sasaki M supervised the organization process.

**Correspondence to:** Kenichi Hakamada, MD, Department of Gastroenterological Surgery, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan. [hakamada@cc.hirosaki-u.ac.jp](mailto:hakamada@cc.hirosaki-u.ac.jp)

Telephone: +81-172-395079 Fax: +81-172-395080

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**Peer reviewer:** Sri P Misra, Professor, Gastroenterology, Moti Lal Nehru Medical College, Allahabad 211001, India

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### Abstract

**AIM:** To analyze gallbladder contractility in patients with black pigment stones (BPSs) and to compare this with patients with cholesterol stones (CSs) and healthy volunteers.

**METHODS:** The pattern of bile evacuation from the gallbladder was quantified by computer cholescintigraphy in 28 normal subjects, 22 patients with CSs and 14 with BPSs. The parameters of gallbladder contractility included ejection period (EP), ejection fraction (EF) and ejection rate (ER).

**RESULTS:** A significantly shorter EP was observed in patients with BPSs in comparison to those with CSs ( $t = 2.4$ ,  $P < 0.05$ ). EF in BPS patients significantly decreased in comparison to that in CS and normal subjects ( $t = 6.4$ ,  $P < 0.0001$ ;  $t = 2.1$ ,  $P < 0.05$ ). EF in CS patients also significantly decreased in comparison to that in normal subjects ( $t = -3.0$ ,  $P < 0.005$ ). Consequently, ER in patients with BPSs and CSs was significantly smaller than that in normal subjects ( $t = 3.1$ ,  $P < 0.005$ ;  $t = -3.5$ ,  $P < 0.001$ ). Moreover, in cases where postprandial reflux of a radioisotope into the common hepatic duct from the gallbladder was observed, EF and ER of either CS or BPS patients showed a significant reduction.

**CONCLUSION:** Bile evacuation from the gallbladder is reduced in patients with BPSs, in comparison to those with CSs and to healthy volunteers. Bile stagnation due to impaired gallbladder kinetics seems to be one of the predisposing factors for the development of BPSs.

### INTRODUCTION

The prevalence of gallstone disease has been increasing in Japan over the past five decades, with an increased incidence of cholesterol stones (CSs) as a leading cause<sup>[1-3]</sup>. In addition, the number of patients with black pigment stones (BPSs) has also been increasing, while the incidence of calcium bilirubinate stones is decreasing<sup>[1,2]</sup> and thus becoming less common in comparison to other Asian countries<sup>[2,3]</sup>.

Much effort has been directed to identify the mechanism of gallstone formation, especially in the case of cholesterol and calcium bilirubinate stones. The supersaturation of bile, caused by an increase in cholesterol concentration or a decrease in bile salt concentration, contributes to form a liquid crystalline phase of cholesterol<sup>[4-6]</sup>. Calcium bilirubinate stones (brown pigment stones) develop as the enzyme  $\beta$ -glucuronidase, possibly produced by bacteria such as *Escherichia coli*, generates unconjugated bilirubin, which precipitates as a calcium salt<sup>[5,7]</sup>. Therefore, bilirubinate calcium is closely related to biliary infection<sup>[3,5,7-9]</sup>.

However, the mechanism by which BPSs develop has not been fully elucidated. BPSs sometimes occur in patients with hemolytic anemia<sup>[8,10]</sup>, liver cirrhosis<sup>[5,11,12]</sup>, and histories of cardiac valve replacement<sup>[13]</sup> and gastrectomy<sup>[14-16]</sup>, but the majority of patients have no specific background<sup>[8]</sup>. Contrary to brown pigment stones, BPSs are not associated with biliary infection and almost all occur in the gallbladder<sup>[5,8,9]</sup>. Other reports have suggested that some unique structures of the gallbladder such as the Rokitsansky-Aschoff sinuses<sup>[17]</sup> and the cystic duct<sup>[18]</sup> might be predisposing factors for the formation of BPSs.

A change in gallbladder contractility is another possible mechanism of gallstone formation. Over the past century, many studies have been conducted to disclose a close

association with gallbladder dysmotility and gallstone diseases<sup>[11,19-29]</sup>. Previous studies, however, have yielded conflicting results. The majority of studies have reported impaired gallbladder emptying in patients with gallstones, while others have not. These conflicting results have derived in part from different methodology to assess gallbladder motility<sup>[22]</sup>, and also from the fact that these studies were conducted without taking into account the chemical characteristics of the stones. To date, there have been, according to the PubMed database, very few reports that describe gallbladder motility with special reference to the pathogenesis of BPS formation.

This study analyzed the pattern of postprandial bile evacuation from the gallbladder quantitatively in patients with BPSs, using a non-invasive technique with a radioactive marker <sup>99m</sup>Tc-PMT, and compared that pattern with that seen in patients with CSs and in healthy volunteers.

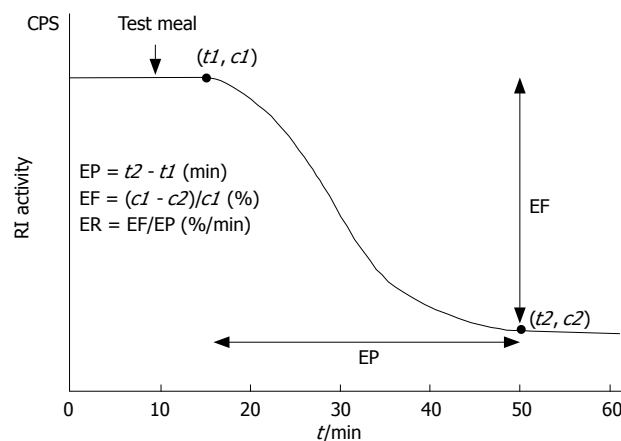
## MATERIALS AND METHODS

### Patients

From 1991 to 2005, 694 patients underwent cholecystectomy for cholelithiasis at Hirosaki University Hospital, Japan. Among these, 22 patients with CSs and 14 with BPSs were included. Patients with positive bile culture obtained at the time of surgery, any sign of cholecystitis, liver cirrhosis or hepatitis, diabetes mellitus, abnormal wall thickening of the gallbladder, and/or a previous surgical history were excluded from the study. The categories of the stones were determined according to the macroscopic characteristics of the specimens and their chemical analyses. Twenty-eight healthy volunteers without any surgical history were also analyzed and included as controls. There was no significant difference in age among the three groups ( $52.4 \pm 13.0$  in CS patients,  $55.8 \pm 14.4$  in BPS patients, and  $54.8 \pm 14.4$  in healthy volunteers,  $P = 0.74$ ). The male-to-female ratios were also comparable (6/16 in CS, 4/10 in PBS, and 15/13 in healthy volunteers,  $P = 0.11$ ). The nature of the study was explained to each subject before cholescintigraphy, and informed consent was obtained under the instruction of the ethical committee for human studies, and the study was approved by the Hirosaki University School of Medicine.

### Cholescintigraphy

The method was a modification of that previously described by Krishnamurthy *et al*<sup>[30]</sup>. All subjects were obliged to fast from 11:00 pm prior to the day of examination. Each subject was injected with 5 mCi <sup>99m</sup>Tc-N-pyridoxal-5-methyltryptophan (<sup>99m</sup>Tc-PMT) *via* the median cubital vein in the morning. After 60 min bed rest in a semi-reclining position, a scintillation camera with a low energy, high-resolution collimator was placed on the right hypochondrium and positioned to enable the separate visualization of the common hepatic duct, common bile duct, descending portion of the duodenum, and gallbladder. The radioactivity gradually increased and reached a plateau in 60 min. Serial views of the abdomen were obtained at 1-min intervals from this point up to 60 min. The information was simultaneously digitalized



**Figure 1** Definition of postprandial contractility indices of the gallbladder. Postprandial gallbladder contractility was expressed by EF, which is determined as a percentage decrease in radioactivity, and ER, which is determined by EF/EP. Radioactivity was measured in three regions of interest in the gallbladder and EP was determined as the time of decrease on the time-activity curve. CPS: Counts per second.

and recorded in the mode on  $64 \times 64$  computer matrices at 1 frame per 15 s. At 10 min, 200 mL Calorie Mate, which was composed of 33.4 g glucose, 6.7 g protein, 4.4 g fat, and 200 kcal (Otuka, Tokyo, Japan), was given to each patient as a test meal, and observation was continued for an additional 50 min. To avoid the effect of respiratory movement on images, each subject was instructed to breathe smoothly throughout the data collection.

### Computer data analysis

Serial images were created by a data-analyzing computer. Four regions of interest on a composite image were chosen. The first included the entire gallbladder; the second, the common hepatic duct; the third, the descending part of the duodenum; and the fourth, the liver as a background. Time-activity curves were generated for each region, background counts were subtracted, and net count decay was corrected and normalized. In a similar manner, time-radioactivity curves were obtained.

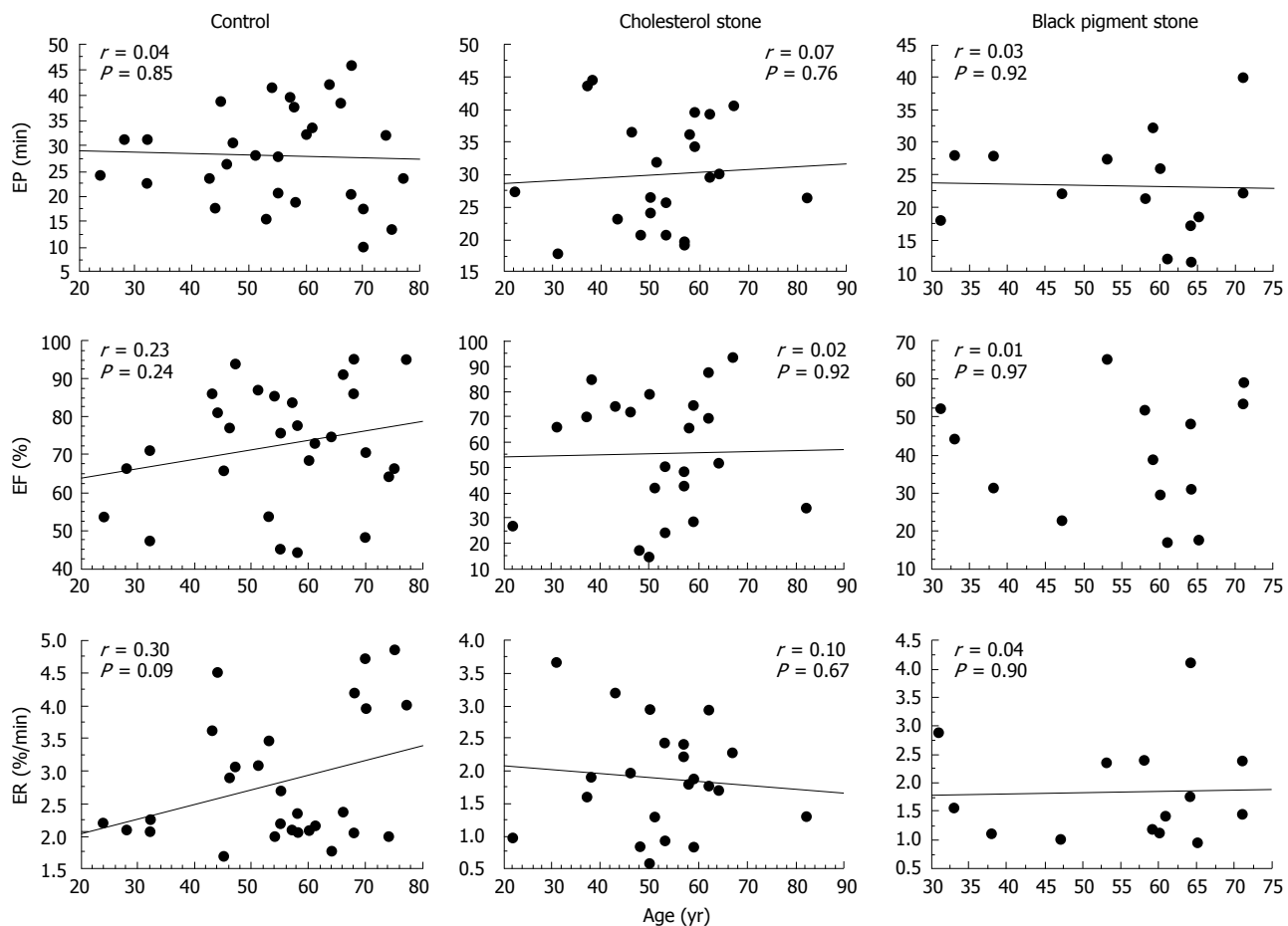
Next, the times when the gallbladder began ejecting ( $t1$ ) and when the gallbladder stopped ejecting ( $t2$ ), and their corresponding counts ( $c1$ ,  $c2$ ) were set (Figure 1). The ejection period (EP), ejection fraction (EF) and ejection rate (ER) were calculated according to the formulae below:  $EP = t2 - t1$  (min);  $EF = (c1 - c2)/c1$  (%);  $ER = EF/EP$  (%/min).

### Statistical analysis

For statistical examinations an analysis of variance, Student's *t* test,  $\chi^2$  test and linear regression analysis were all used appropriately, and the SPSS 11.0J software program for Windows was used for the analysis. The continuous variables were reported as the mean  $\pm$  SD.  $P < 0.05$  was considered to be significantly different.

## RESULTS

Gender did not affect EP, EF and ER in all three groups (Table 1). Age also showed no correlation with EP, EF and



**Figure 2** Correlation of age with contractility indices of the gallbladder. There was no correlation between age and EP, EF and ER in all three groups.

ER (Figure 2). Moreover, the size and the number of the stones correlated with none of the indices in either group with CSs or BPSs (Figures 3 and 4).

Postprandial changes in gallbladder contractility are summarized in Figure 5. The EP values of normal subjects ( $28.2 \pm 9.5$  min) and CS patients ( $30.0 \pm 8.4$  min) were comparable but the value in BPS patients ( $23.3 \pm 7.8$  min) was significantly shorter than that in CS patients ( $P < 0.05$ ). The EF of CS patients ( $55.5\% \pm 24.0\%$ ) was significantly smaller than that of normal subjects ( $72.5\% \pm 15.5\%$ ,  $P < 0.05$ ), and the value of BPS patients ( $40.2\% \pm 15.5\%$ ) was significantly reduced in comparison to that of CS patients ( $P < 0.05$ ), and to that of normal subjects ( $P < 0.0001$ ). Consequently, the ER in the CS group ( $1.9\% \pm 0.8\%/min$ ) and in the BPS group ( $1.9\% \pm 0.9\%/min$ ) significantly decreased in comparison to that of normal subjects ( $2.8\% \pm 1.0\%/min$ ,  $P < 0.001$ ,  $P < 0.005$ ). The ER values were comparable between the CS and BPS groups.

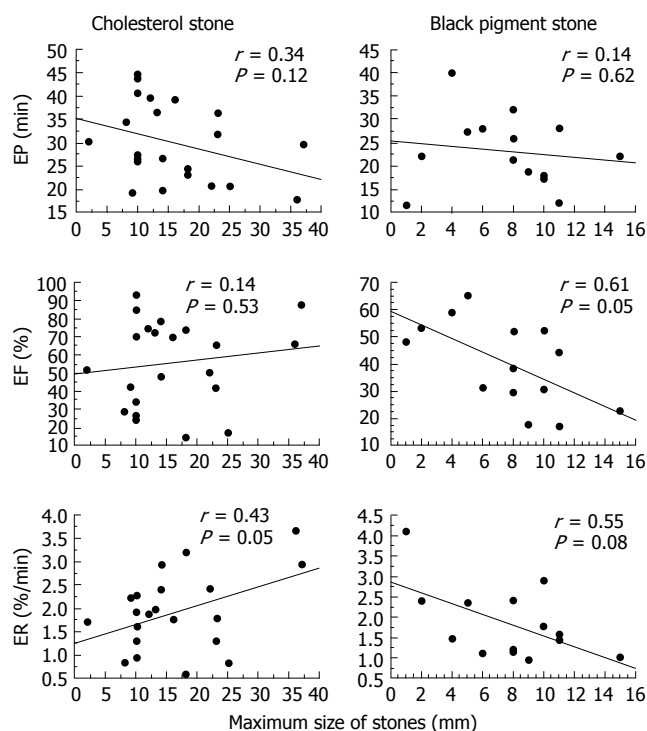
Postprandial bile reflux from the gallbladder to the common hepatic duct was observed in 22/28 (76%) normal subjects, 11/22 (50%) CS patients, and 8/14 (57%) BPS patients. Comparisons between the cases with and without reflux are summarized in Figure 6. In normal subjects, EP and ER were comparable between 22 reflux ( $28.5 \pm 9.5$  min,  $3.0\%/min$ ) and six non-reflux cases ( $27.0 \pm 10.0$  min,  $2.3\% \pm 1.0\%/min$ ), while EF was significantly larger in the reflux group ( $76.3\% \pm 13.6\%$ ) than in the non-reflux group ( $58.4\% \pm 14.3\%$ ,  $P < 0.01$ ).

**Table 1** Effects of gender on postprandial motility indices of the gallbladder

	Male	Female	P
Normal subjects (n)	15	13	
EP (min)	$26.6 \pm 10.3$	$30.1 \pm 8.4$	0.34
EF (%)	$68.7 \pm 14.6$	$77.1 \pm 15.9$	0.16
ER (%/min)	$2.9 \pm 1.2$	$2.7 \pm 0.7$	0.51
CS (n)	6	16	
EP (min)	$29.1 \pm 6.1$	$30.4 \pm 9.2$	0.76
EF (%)	$54.3 \pm 27.1$	$56.0 \pm 23.7$	0.89
ER (%/min)	$1.9 \pm 1.0$	$1.9 \pm 0.8$	0.99
BPS (n)	4	10	
EP (min)	$22.4 \pm 5.6$	$23.6 \pm 8.7$	0.80
EF (%)	$39.3 \pm 10.9$	$40.6 \pm 17.6$	0.89
ER (%/min)	$1.9 \pm 0.8$	$1.9 \pm 1.0$	0.99

In CS patients, EP was significantly longer in 11 reflux cases ( $33.5 \pm 7.4$  min) than in 11 non-reflux cases ( $26.6 \pm 8.1$  min,  $P < 0.05$ ). EF and ER were comparable between reflux and non-reflux groups. In BPS patients, EP, EF and ER were comparable, despite reflux state.

In a subgroup analysis limited to patients with postprandial bile reflux from the gallbladder to the common hepatic duct, EF and ER in the BPS CS groups were significantly smaller than those in normal subjects. EP in the BPS group was also significantly shorter than that in the CS group (Figure 6).

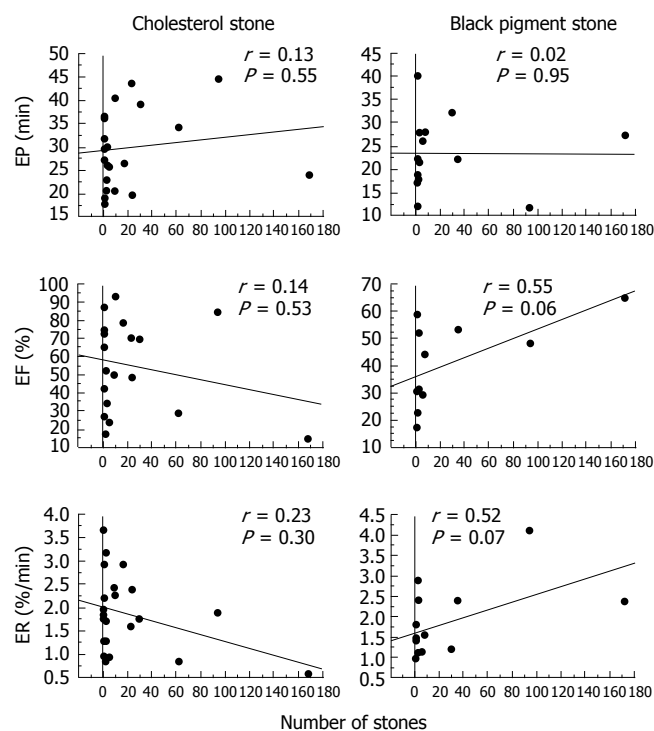


**Figure 3** Correlation of the size of stones with contractility indices of the gallbladder. There was no correlation between stone size and EP, EF and ER in all groups.

## DISCUSSION

Many studies have reported a high incidence of BPSs in patients with hemolytic jaundice<sup>[8,10]</sup>, heart valve replacement<sup>[13]</sup>, liver cirrhosis<sup>[5,11,12]</sup> and a previous history of gastrectomy<sup>[14-16]</sup>. The first two are primarily due to bilirubin overproduction, while the latter are not. However, despite similar biochemical and clinical features, many patients with hemolytic anemia do not develop BPSs. Moreover, Portincasa *et al*<sup>[10]</sup> have reported that impaired gallbladder motility is observed in patients with  $\beta$ -thalassemia major, and have suggested that hemolytic hyperbilirubinemia and dysmotility might contribute to the process of pigment stone formation. In patients with liver cirrhosis, Acalovshi *et al*<sup>[11]</sup> have reported that gallbladder contractility is impaired and hypomotility is proportional to disease severity. A close relationship between high incidence of gallstones and changes in gallbladder kinetics after gastrectomy has also been described<sup>[15]</sup>. Taking into consideration that BPSs also occur without these complications<sup>[17]</sup>, impaired gallbladder function may be associated with BPS formation. However, there have so far been very few reports on gallbladder kinetics and the mechanism of stone formation with special reference to BPSs.

In this study, biliary scintigraphy was used to analyze the postprandial dynamics of bile flow in patients with BPSs in comparison to those with CSs and normal subjects, because it was essentially risk-free, with very slight radiation exposure and yielded a precise assessment of the ejection fraction of the gallbladder motility, as previously reported<sup>[22,30]</sup>. First, the effects of age, gender and the characteristics of the stones on gallbladder



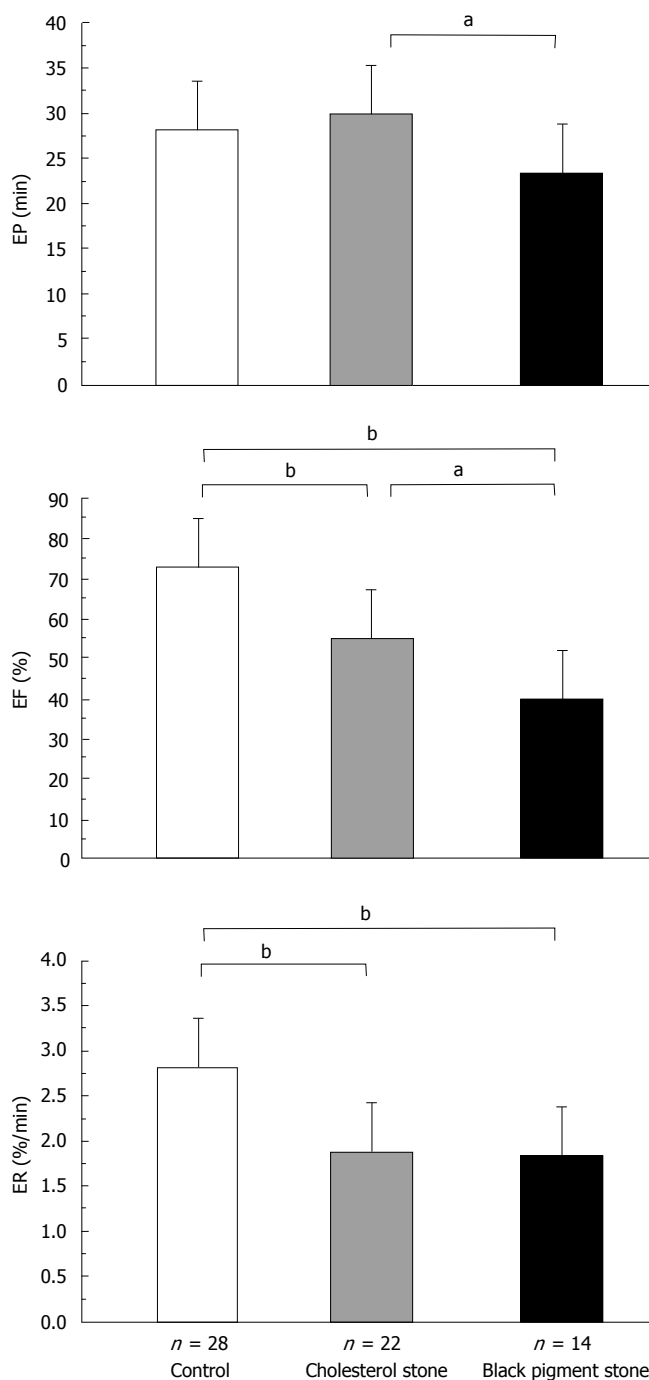
**Figure 4** Correlation between the number of stones with the contractility indices of the gallbladder. There was no correlation between the number of stones and EP, EF and ER in all groups.

contractility were analyzed, because there have been some previous studies on the effects of age and sex on gallbladder emptying<sup>[21,31]</sup>. In all groups, age and gender, and size and number of stones did not affect EP, EF and ER. Other studies have also found that size and number of stones have no influence upon postprandial gallbladder emptying<sup>[11,27]</sup>. Therefore, gallbladder contractility was analyzed without taking these backgrounds into consideration.

It has been reported that EF in normal subjects is 80% in response to postprandial intrinsic loading of cholecystikinin (CCK)<sup>[19]</sup>, and it ranges from 50%-80% in response to extrinsic administration of CCK<sup>[24,27]</sup>. In patients with gallstones, the contractility is reduced to 52% by intrinsic<sup>[19]</sup> and to 32%-50% by extrinsic stimulus of CCK<sup>[24,27]</sup>. These reports, however, included all kinds of gallstones with different etiology, while also neglecting the presence or absence of acute or chronic inflammatory processes, such as gallbladder-wall thickening. In this study, gallbladder contractility was analyzed according to the predominant chemical component of the gallstones. Patients with any sign of cholecystitis, liver cirrhosis or hepatitis, diabetes mellitus, abnormal gallbladder-wall thickening, and/or previous surgical history which might affect contractility were excluded from the study.

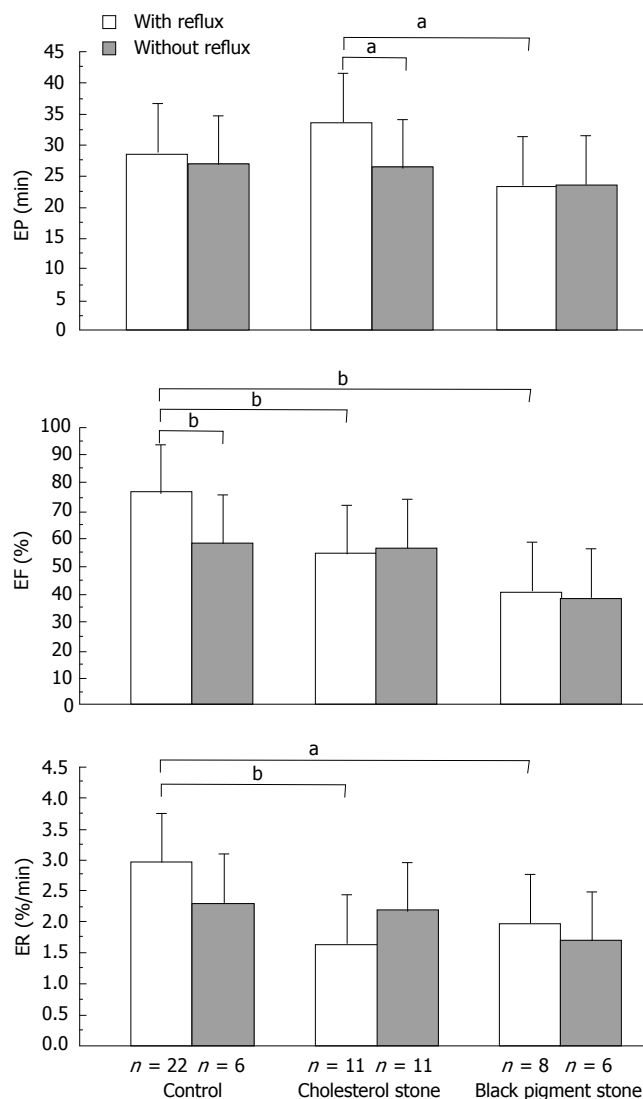
In BPS patients, EP was significantly shorter than that in CS patients. Simultaneously, EF was reduced significantly in BPS patients, in comparison to those with CSs and healthy volunteers. These findings suggested that bile clearance from the gallbladder was significantly reduced in patients with BPSs. Krishnamurthy *et al*<sup>[24]</sup> have reported that there is no difference in EP between





**Figure 5** Postprandial contractility indices of the gallbladder. A significantly shorter EP was observed in patients with BPSs than in those with CSs. EF in BPS patients was significantly decreased in comparison to that in CS patients and normal subjects. EF in CS patients also significantly decreased in comparison to that in normal subjects. Consequently, ER in patients with BPSs and CSs was significantly smaller than that in normal subjects. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ .

gallstone patients and normal individuals, but they do not distinguish patients according to the chemical characteristics of their stones. Such results may come from an increased proportion of CSs in their study, because the EP of normal subjects and gallstone patients did not differ in the current study. Portincasa *et al.*<sup>[28]</sup> have conducted a comparative analysis of gallbladder contractility in patients with BPSs or CSs, and observed an increased fasting volume of the gallbladder in CS but not in BPS patients.



**Figure 6** Postprandial contractility indices of the gallbladder according to the reflux state of a radioisotope into the common hepatic duct. In normal subjects, EF was significantly larger in patients with reflux. In a subgroup analysis of such patients, EF and ER in those with either CSs or BPSs showed a significant reduction in comparison to those in normal subjects. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ .

Postprandial emptying was delayed and incomplete in both CS and BPS patients in comparison to control subjects. However, these observations were made based on the findings of ultrasonography, and not by biliary scintigraphy.

It has been demonstrated that CCK simultaneously induces contraction of the gallbladder and relaxation of the sphincter of Oddi, which facilitates secretion of the bile duct into the duodenum. If this contraction-relaxation coordination fails, bile reflux from the gallbladder to the common hepatic duct may occur because of transient resistance of the sphincter. Itoh *et al.*<sup>[32]</sup> have reported that this phenomenon is observed in patients with chronic pancreatitis and that the degree of reflux is proportional to disease progression. This means that one of the causes of such reflux is relative stenosis of the distal bile duct. Bile reflux, however, was observed in half of the patients with gallstones, in whom EF was significantly reduced in comparison to that in healthy volunteers. This phenomenon

suggests that minute discordance of gallbladder emptying and relaxation of the sphincter of Oddi predisposes to bile reflux and thus causes bile stagnation in the gallbladder.

Chemically, the structure of BPSs is polymerized bilirubin with copper, iron, calcium and other metals, and almost all the stones are formed in the gallbladder<sup>[33]</sup>. Therefore, an environment suitable for the formation of BPSs comprises conditions that allow chemical polymerization with sufficient time. In fact, EF was reduced in patients with gallstones, especially with BPSs in the present study. Moreover, bile reflux from the gallbladder to the common hepatic duct may cause subclinical transient bile stagnation, thereby contributing to stone formation.

In conclusion, bile evacuation from the gallbladder is reduced in patients with BPSs, in comparison to those with CSs and healthy volunteers. We therefore postulate that impaired gallbladder motor function and discordance of bile evacuation is not the result of the presence of gallstones, but instead it is considered to precede the development of BPSs.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

The incidence of BPSs and CSs is increasing in Japan. However, the mechanism of BPS formation is still not completely understood. The authors hypothesize that reduced motor function of the gallbladder and dysfunction of bile evacuation is associated with BPS formation.

### Research frontiers

To date, there have been very few reports that describe gallbladder motility with special reference to the pathogenesis of BPS formation. The authors found impaired gallbladder kinetics in patients with BPSs, in comparison to those with CSs and to healthy volunteers.

### Innovations and breakthroughs

The authors strictly selected the patients without any background diseases such as cholecystitis, liver cirrhosis or hepatitis, diabetes mellitus, abnormal gallbladder-wall thickening, and/or a previous surgical history, and then analyzed physiological patterns of bile evacuation from the gallbladder quantitatively by a less-invasive method of computer cholelscintigraphy.

### Applications

This research is expected to contribute to understanding the pathogenesis of gallstone diseases.

### Peer review

The authors analyzed gallbladder contractility in patients with BPSs in comparison to those with CSs and healthy volunteers. The study is very interesting.

## REFERENCES

- 1 Japan-gallstone-study-group. National survey for gallstone in Japan. *J Jpn Biliary Assoc* 1998; **12**: 276-293
- 2 Nakayama F, Miyake H. Changing state of gallstone disease in Japan. Composition of the stones and treatment of the condition. *Am J Surg* 1970; **120**: 794-799
- 3 Tazuma S. Gallstone disease: Epidemiology, pathogenesis, and classification of biliary stones (common bile duct and intrahepatic). *Best Pract Res Clin Gastroenterol* 2006; **20**: 1075-1083
- 4 Holan KR, Holzbach RT, Hermann RE, Cooperman AM, Claffey WJ. Nucleation time: a key factor in the pathogenesis of cholesterol gallstone disease. *Gastroenterology* 1979; **77**: 611-617
- 5 Sherlock S, Dooley J. Diseases of the liver and biliary system. Oxford Blackwell Science: London, 2002: 242
- 6 Small DM. Gallstones. *N Engl J Med* 1968; **279**: 588-593
- 7 Maki T. Pathogenesis of calcium bilirubinate gallstone: role of E. coli, beta-glucuronidase and coagulation by inorganic ions, polyelectrolytes and agitation. *Ann Surg* 1966; **164**: 90-100
- 8 Soloway RD, Trotman BW, Maddrey WC, Nakayama F. Pigment gallstone composition in patients with hemolysis or infection/stasis. *Dig Dis Sci* 1986; **31**: 454-460
- 9 Tabata M, Nakayama F. Bacteria and gallstones. Etiological significance. *Dig Dis Sci* 1981; **26**: 218-224
- 10 Portincasa P, Moschetta A, Berardino M, Di-Ciula A, Vacca M, Baldassarre G, Pietrapertosa A, Cammarota R, Tannoia N, Palasciano G. Impaired gallbladder motility and delayed orocecal transit contribute to pigment gallstone and biliary sludge formation in beta-thalassemia major adults. *World J Gastroenterol* 2004; **10**: 2383-2390
- 11 Acalovschi M, Dumitrascu DL, Nicoara CD. Gallbladder contractility in liver cirrhosis: comparative study in patients with and without gallbladder stones. *Dig Dis Sci* 2004; **49**: 17-24
- 12 Fornari F, Imberti D, Squillante MM, Squassante L, Civardi G, Buscarini E, Cavanna L, Caturelli E, Buscarini L. Incidence of gallstones in a population of patients with cirrhosis. *J Hepatol* 1994; **20**: 797-801
- 13 Ai T, Azemoto R, Saisho H. Prevention of gallstones by ursodeoxycholic acid after cardiac surgery. *J Gastroenterol* 2003; **38**: 1071-1076
- 14 Chijiwa K, Makino I, Kozaki N, Tanaka M. Differences in gallbladder bile lithogenicity in patients after gastrectomy and colectomy. *Eur Surg Res* 1996; **28**: 1-7
- 15 Inoue K, Fuchigami A, Higashide S, Sumi S, Kogire M, Suzuki T, Tobe T. Gallbladder sludge and stone formation in relation to contractile function after gastrectomy. A prospective study. *Ann Surg* 1992; **215**: 19-26
- 16 Kobayashi T, Hisanaga M, Kanehiro H, Yamada Y, Ko S, Nakajima Y. Analysis of risk factors for the development of gallstones after gastrectomy. *Br J Surg* 2005; **92**: 1399-1403
- 17 Cariati A, Cetta F. Rokitansky-Aschoff sinuses of the gallbladder are associated with black pigment gallstone formation: a scanning electron microscopy study. *Ultrastruct Pathol* 2003; **27**: 265-270
- 18 Li WG, Luo XY, Johnson AG, Hill NA, Bird N, Chin SB. One-dimensional models of the human biliary system. *J Biomech Eng* 2007; **129**: 164-173
- 19 Fisher RS, Stelzer F, Rock E, Malmud LS. Abnormal gallbladder emptying in patients with gallstones. *Dig Dis Sci* 1982; **27**: 1019-1024
- 20 Forgacs IC, Maisey MN, Murphy GM, Dowling RH. Influence of gallstones and ursodeoxycholic acid therapy on gallbladder emptying. *Gastroenterology* 1984; **87**: 299-307
- 21 Howard PJ, Murphy GM, Dowling RH. Gall bladder emptying patterns in response to a normal meal in healthy subjects and patients with gall stones: ultrasound study. *Gut* 1991; **32**: 1406-1411
- 22 Jazrawi RP, Pazzi P, Petroni ML, Prandini N, Paul C, Adam JA, Gullini S, Northfield TC. Postprandial gallbladder motor function: refilling and turnover of bile in health and in cholelithiasis. *Gastroenterology* 1995; **109**: 582-591
- 23 Koruk M, Ozkiliç S, Savas MC, Celen Z, Kadayıfci A, Ozkiliç C. Evaluation of hepatic functions and biliary dynamics in patients with liver cirrhosis by quantitative scintigraphy. *Hepatogastroenterology* 2003; **50**: 1803-1805
- 24 Krishnamurthy GT, Bobba VR, McConnell D, Turner F, Mesgarzadeh M, Kingston E. Quantitative biliary dynamics: introduction of a new noninvasive scintigraphic technique. *J Nucl Med* 1983; **24**: 217-223

- 25 **Maudgal DP**, Kupfer RM, Zentler-Munro PL, Northfield TC. Postprandial gall-bladder emptying in patients with gall stones. *Br Med J* 1980; **280**: 141-143
- 26 **Pauletzki J**, Cicala M, Holl J, Sauerbruch T, Schafmayer A, Paumgartner G. Correlation between gall bladder fasting volume and postprandial emptying in patients with gall stones and healthy controls. *Gut* 1993; **34**: 1443-1447
- 27 **Pomeranz IS**, Shaffer EA. Abnormal gallbladder emptying in a subgroup of patients with gallstones. *Gastroenterology* 1985; **88**: 787-791
- 28 **Portincasa P**, Di Ciaula A, Vendemiale G, Palmieri V, Moschetta A, Vanberge-Henegouwen GP, Palasciano G. Gallbladder motility and cholesterol crystallization in bile from patients with pigment and cholesterol gallstones. *Eur J Clin Invest* 2000; **30**: 317-324
- 29 **Spengler U**, Sackmann M, Sauerbruch T, Holl J, Paumgartner G. Gallbladder motility before and after extracorporeal shock-wave lithotripsy. *Gastroenterology* 1989; **96**: 860-863
- 30 **Krishnamurthy GT**, Bobba VR, Kingston E. Radionuclide ejection fraction: a technique for quantitative analysis of motor function of the human gallbladder. *Gastroenterology* 1981; **80**: 482-490
- 31 **Khalil T**, Walker JP, Wiener I, Fagan CJ, Townsend CM Jr, Greeley GH Jr, Thompson JC. Effect of aging on gallbladder contraction and release of cholecystokinin-33 in humans. *Surgery* 1985; **98**: 423-429
- 32 **Itoh H**, Shimono R, Hamamoto K. Evaluation of common bile duct stenosis in chronic pancreatitis using cholescintigraphy. *Eur J Nucl Med* 1988; **14**: 137-140
- 33 **Suzuki N**, Nakamura Y, Kobayashi N, Sato T. On metal elements in pure pigment gallstones. *Tohoku J Exp Med* 1975; **116**: 233-240

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BASIC RESEARCH

## Protective effects of apocynin and allopurinol on ischemia/reperfusion-induced liver injury in mice

Ping-Guo Liu, Song-Qing He, Yan-Hong Zhang, Jian Wu

Ping-Guo Liu, Song-Qing He, Yan-Hong Zhang, Jian Wu, Department of Internal Medicine, Transplant Research Program, University of California, Davis Medical Center, Sacramento, CA 95817, United States

Ping-Guo Liu, Department of Hepatic-Biliary Surgery, Zhongshan Hospital, Xiamen University, Xiamen 361004, Fujian Province, China

**Author contributions:** Liu PG designed and performed the most of the study; He SQ contributed to an initial idea and helped with MCLA measurement; Zhang YH helped with apoptosis staining; Wu J is the principal investigator, conducted data analysis and manuscript preparation.

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**Correspondence to:** Jian Wu, MD, PhD, UC Davis Medical Center, Transplant Research Institute, 4635 2nd Ave. Suite 1001, Sacramento, CA 95817, United States. [jdwu@ucdavis.edu](mailto:jdwu@ucdavis.edu)

Telephone: +1-916-7348044 Fax: +1-916-7348097

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of superoxide anions during the hepatic I/R procedure by inhibiting xanthine oxidase and NADPH oxidase activity.

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**Key words:** Ischemia/reperfusion; Reactive oxygen species; Allopurinol; Apocynin; NADPH oxidase; Xanthine oxidase

**Peer reviewer:** Paul E Sijens, PhD, Associate Professor, Department of Radiology, UMCG, Groningen 9713GZ, The Netherlands

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### Abstract

**AIM:** To determine the effects of allopurinol, an inhibitor of xanthine oxidase, and apocynin, an inhibitor of NADPH oxidase, on oxidant stress and liver injury caused by hepatic ischemia/reperfusion (I/R) procedure in mice.

**METHODS:** Mice were pretreated with a xanthine oxidase inhibitor, allopurinol, or NADPH oxidase (NOX) inhibitor, apocynin before the hepatic I/R procedure. Then treated or untreated mice underwent the hepatic I/R procedure. The effects on hepatic injury and superoxide anions were determined after starting reperfusion.

**RESULTS:** A standard warm hepatic I/R procedure led to a marked increase in superoxide anion production as indicated by a superoxide anion tracer, MCLA. At the same time, the procedure caused profound acute liver injury, as indicated by elevated serum alanine aminotransferase and tumor necrosis factor- $\alpha$  levels, reduced liver glutathione levels and elevated malondialdehyde contents, as well as a high apoptotic cell count. All these changes were reversed by the use of apocynin or allopurinol prior to the hepatic I/R procedure.

**CONCLUSION:** Allopurinol and apocynin exerted protective effects on hepatic ischemia/reperfusion injury. The protection is associated with blocking the generation

### INTRODUCTION

Excessive reaction oxygen species (ROS) cause tissue damage and cell death by binding and altering cellular macromolecules, including DNA, proteins and lipids, and affect their function. One main chemical source which has been shown to contribute significantly to overall pronounced oxidant stress during hepatic ischemia/reperfusion (I/R) procedure is xanthine oxidase (XO), which generates superoxide anions ( $O_2^-$ ) during the conversion of hypoxanthine to xanthine. It is known from many studies that much of the sustained injury during organ transplantation, including the liver, is triggered by ROS *via* activated XO, because allopurinol, an XO inhibitor, provided some protection against the hepatic I/R-induced injury<sup>[1]</sup>.

NADPH oxidase (NOX), using NADPH as the source of electrons, catalyzes one electron reduction of molecular oxygen to generate  $O_2^{1-2}$ , which is a central and initial ROS molecule and may convert to more active and toxic ROS, such as hydrogen peroxide ( $H_2O_2$ ), hydroxyl radical ( $HO^\bullet$ ), or peroxide nitrite ( $ONOO^-$ ) in the presence of  $H^+$ ,  $H_2O_2$ , and nitric oxide ( $NO$ )<sup>[3]</sup>. These  $O_2^-$ -derived ROS participating in the inflammatory process, are thought to be key mediators for the activation of Kupffer cells<sup>[4]</sup> and thus, are crucial in the apoptotic and/or necrotic cell death of the parenchymal cells and sinusoidal endothelial cells (SEC) in the liver<sup>[5,6]</sup>. The generation of superoxide anions by NADPH oxidase serves as a host defense mechanism



against invading microorganism infection and the enzyme is present in phagocytic cells, such as monocytes and neutrophils<sup>[7]</sup>. Although the evidence exists that ROS generated by NOX participate in many cellular responses, and may be involved in many injuring processes, few studies are available that investigate the role of NOX in the contribution to pronounced oxidant stress during the ischemia/reperfusion-induced hepatic injury<sup>[7,8]</sup>. Apocynin is a naturally occurring methoxy-substituted catechol that effectively inhibits NADPH oxidase through preventing the assembly of its multi-subunits<sup>[9]</sup>. Thus, we used this inhibitor to explore the role of NADPH oxidase in the generation of superoxide anions during the hepatic I/R procedure and investigate whether apocynin confers any protection against the injury in a mouse model of warm ischemia/reperfusion-induced acute liver injury.

## MATERIALS AND METHODS

### Animals and treatments

ICR mice, from Charles River Laboratory, Wilmington, MA, were fed a pellet diet and water ad libitum and maintained on a 12 h-light/dark cycle. The animal experiment was performed according to a protocol approved by the UC Davis Institutional Animal Care and Use Committee (IACUC). The protocol was prepared in accordance with the National Institutes of Health animal use guidelines. Mice were pretreated with either an XO inhibitor, allopurinol (50 mg/kg, i.p. from Sigma Chemical Co. St. Louis, MO) or a NOX inhibitor, apocynin (3 mg/kg, i.p. from Acros Organics, Geel, Belgium) one day and one hour before the hepatic I/R procedure. A warm hepatic I/R procedure was performed as reported previously by us<sup>[10]</sup>. In brief, mice were anesthetized with pentobarbital sodium (60 mg/kg, i.p.). Laparotomy was made with a middle incision to expose the lobes of the liver. Following surgical exposure of the portal vein, mice were injected with heparin (100 unit/kg) *via* tail vein to prevent the formation of blood clot during the ischemia duration. The portal vein and hepatic artery were occluded for 30 min with a microaneurysm clamp to induce hepatic ischemia. Then, the clamp was removed to allow blood to flow through the liver again (reperfusion).

A total of six groups (6 mice in each group) were included in the experiment. Group A: sham-operated group, in which mice received normal saline (N.S.), and underwent a sham operation without I/R procedure; Group B (N.S. control group) in which mice underwent hepatic I/R procedure plus N.S. injection; Group C (allopurinol treatment group), in which animals received prior allopurinol injections and subsequent hepatic I/R procedure; Group D (apocynin treatment group), in which animals received prior apocynin injections and subsequent hepatic I/R procedure. Six and twenty four hours after starting the reperfusion, blood samples were collected from the vena cava before sacrifice. The liver was rapidly excised after drawing blood from the vena cava. Portions of liver tissue were fixed in 10% neutralized formalin for histological evaluation or snap frozen in liquid nitrogen, and maintained at -80°C until homogenization for the various biochemical assays.

### Serum alanine aminotransferase assay

Serum levels of alanine aminotransferase (ALT) served as an indicator of liver injury and were analyzed using a commercially available diagnostic kit (Catachem Inc. Bridgeport, Connecticut), and expressed as unit/L.

### Measurement of ROS generation in liver homogenates

2-Methyl-6-(P-methoxyphenyl)-3,7-dihydroimidazo(1,2- $\alpha$ )pyrazine-3-one (MCLA) enhanced chemiluminescence was used to determine  $O_2^-$  generation, as reported by us previously<sup>[10]</sup>. Approximately, 10 mg of frozen liver tissue was homogenized on ice in 1 mL of homogenization buffer containing 20 mmol/L of N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 10 mmol/L ethylene diamine tetraacetic acid (EDTA). The homogenate was subjected to low speed centrifugation (1000 g) for 10 min to remove debris. Luminometer vials containing 2 mL of prewarmed Krebs-HEPES buffer (99 mmol/L NaCl, 4.7 mmol/L KCl, 1.2 mmol/L  $MgSO_4$ , 1 mmol/L  $KH_2PO_4$ , 1.9 mmol/L  $CaCl_2$ , 25 mmol/L  $NaHCO_3$ , 11.1 mmol/L glucose, and 20 mmol/L HEPES, pH 7.4) with 1.0  $\mu$ mol/L of MCLA were placed in the dark for at least 20 min. After the dark adaptation, background readings were recorded in a luminometer (Lumat LB; Berthold Technologies, BmbH & Co. KG, Germany), and then 20  $\mu$ L of homogenate supernatant was added to each vial containing MCLA. Chemiluminescent emission in relative light units (RLU) was recorded during a plateau phase of each recording period, and corrected by subtracting the background reading. The calculated value was used to express the integrated values of chemiluminescence (RLU/second/ $\mu$ g protein).

### Assays of liver glutathione and malondialdehyde content

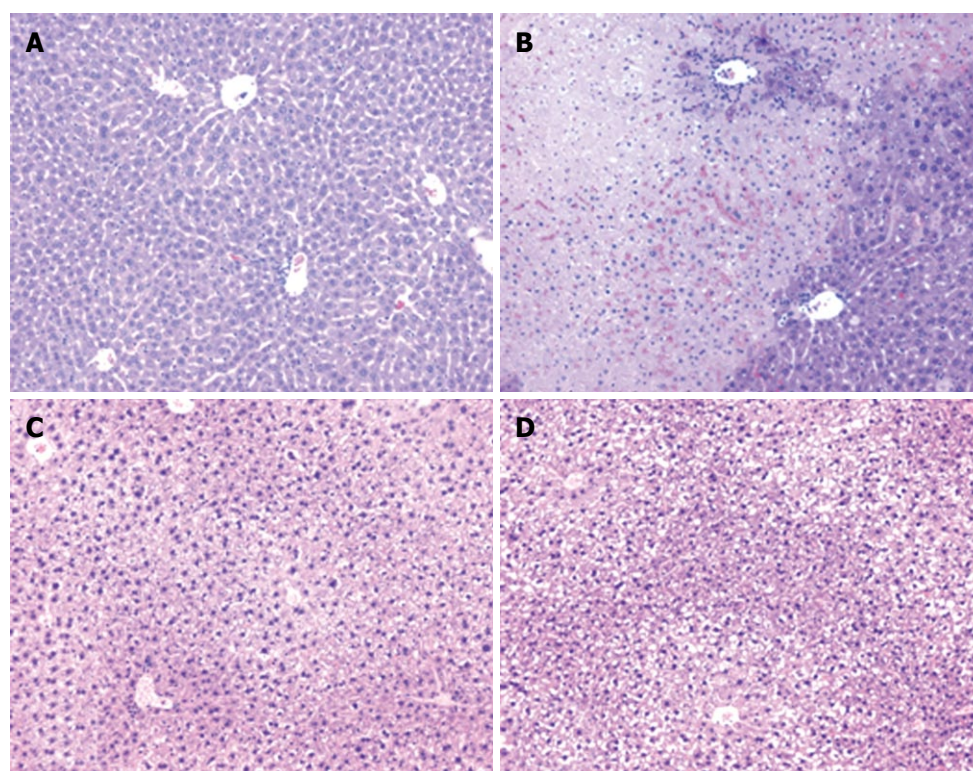
Levels of the reduced form of glutathione (GSH) and the lipid peroxidation product, malondialdehyde (MDA) in liver tissue were measured spectrophotometrically 6 h and 24 h after starting reperfusion by commercially available kits (OXIS Research, Portland, OR). The levels of GSH and MDA are expressed as nanomoles per milligram of protein.

### Histological examination

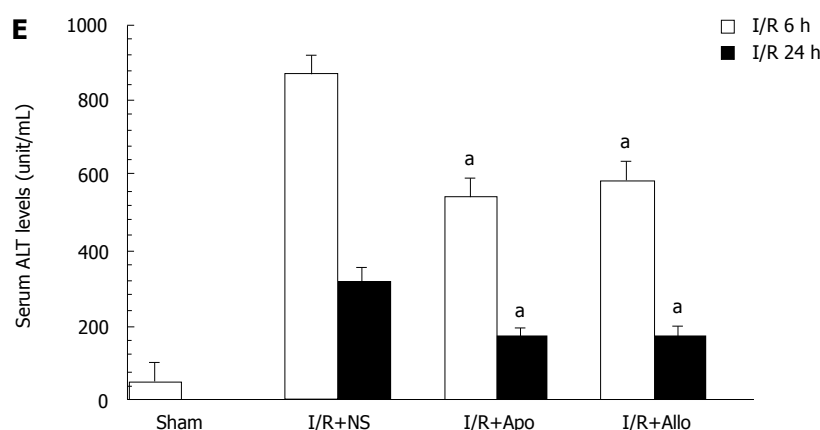
Fixed liver specimens were embedded in paraffin, sectioned in 4  $\mu$ m thickness, and stained with hematoxylin and eosin (HE) for the evaluation of liver injury. Photomicrographs were taken with a digital camera under a microscope. In addition, frozen sections of the livers were stained with the terminal deoxynucleotidyl transferase (TdT) for detecting of apoptotic cells by an *in situ* Apoptosis Detection Kit according to the manufacturer's instructions (Chemicon Inc., Temecula, CA), and reported previously by us<sup>[11]</sup>.

### Determination of serum tumor necrosis factor- $\alpha$ levels

Serum tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) levels after the hepatic I/R procedure were determined with an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) and expressed as nanograms per milliliter.



**Figure 1** Attenuation of I/R-induced liver injury by apocynin and allopurinol in mice. **A-D**: Representative micrographs of liver histology ( $\times 100$ ). **A**: Normal liver; **B**: I/R-induced liver injury 6 h after starting reperfusion; **C** and **D**: Attenuation of liver injury with apocynin (APO) (**C**) or Allo (allopurinol) (**D**); **E**: Serum ALT levels in mice receiving apocynin or allopurinol and subsequently the hepatic I/R procedure. Serum ALT levels were determined 6 h and 24 h after starting reperfusion ( $n = 6$  each group), and expressed as mean  $\pm$  SEM.  $^*P < 0.01$  vs I/R procedure plus normal saline (N.S.) at 6 h or 24 h.



### Statistical analysis

Most data were expressed as mean  $\pm$  SD of the mean (SEM), and evaluated with an ANOVA analysis and Newman-Keuls tests for multiple comparisons among groups. A  $P$  value less than 0.05 was considered statistically significant.

## RESULTS

### Amelioration of I/R-induced acute liver injury

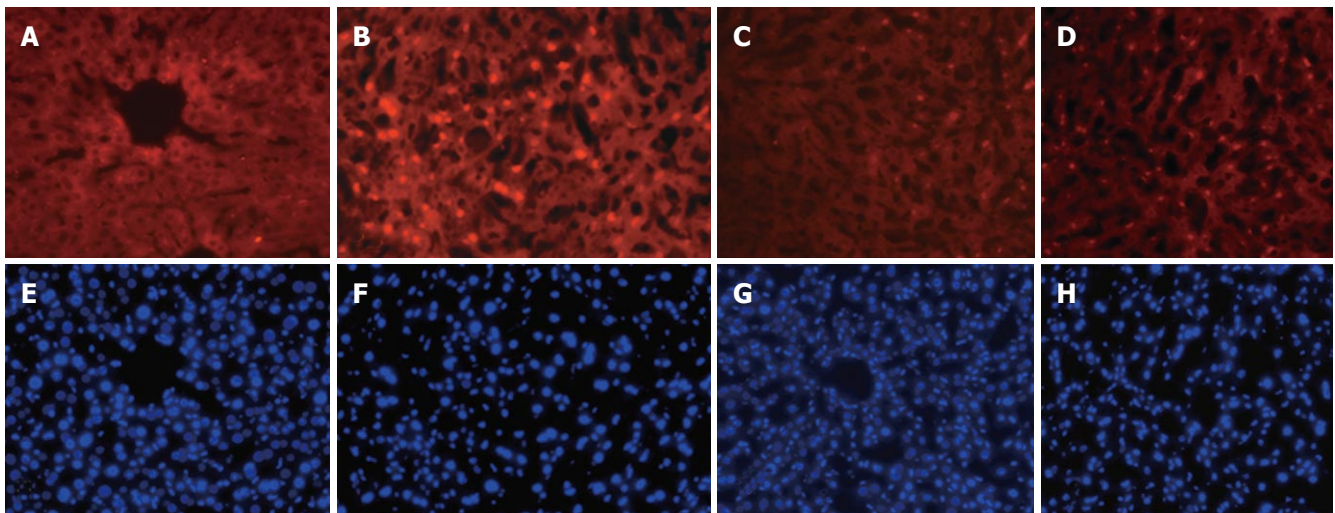
As shown in Figure 1E, the hepatic I/R procedure caused a marked increase in serum ALT levels 6 h to 24 h after starting the reperfusion. Prior intraperitoneal injection of either allopurinol or apocynin led to a marked decrease in serum ALT levels compared to animals with I/R plus N.S. injection at both 6-h and 24-h points ( $P < 0.01$ ). It appeared that either allopurinol or apocynin tended to exert an improved protection to the same extent, and that the differences were not statistically significant at both time points between these two groups. These findings

were consistent with the histological findings as shown in Figure 1A-D. The hepatic I/R procedure led to marked necrosis in the central lobule (Zone III) with significant inflammatory infiltration (Figure 1B). A significant reduction in necrosis was found in the livers of animals receiving injections of allopurinol or apocynin (Figure 1C and D) compared to the control group (Figure 1A). For controls, additional animals were injected with allopurinol or apocynin without the hepatic I/R procedure. Serum ALT levels in animals receiving apocynin ( $65 \pm 17$  unit/mL,  $n = 3$ ) or allopurinol ( $57 \pm 14$  unit/mL,  $n = 3$ ) were similar to levels in N.S. controls ( $46 \pm 27$  unit/mL,  $n = 3$ ). These data indicate that neither apocynin nor allopurinol is toxic to the liver.

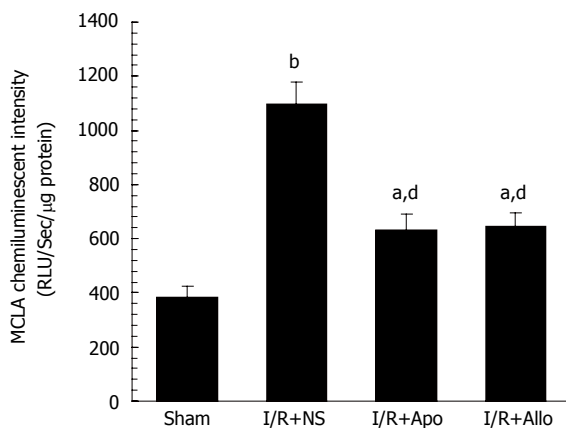
### Enhanced apoptosis in hepatic I/R-induced liver injury

Apoptotic cells in liver sections were detected by in situ staining using TUNEL assay. As shown in Figure 2, 6 h after starting reperfusion, profound apoptotic cells with positive TUNEL red fluorescent staining in nuclei were





**Figure 2** Representative micrographs of *in situ* TUNEL staining of apoptotic cells in liver tissue after the hepatic ischemia/reperfusion procedure. **A-D:** The TUNEL staining assay was performed as described in the text, and the positive apoptotic cells were stained in red ( $\times 100$ ). **A:** Control; **B:** I/R procedure plus N.S. (6 h after starting reperfusion); **C:** I/R plus apocynin; **D:** I/R plus allopurinol. **E-H:** The corresponding liver sections stained with DAPI to illustrate nuclei as a cell number control.



**Figure 3** The hepatic I/R-induced superoxide anion production and the effects of apocynin or allopurinol in mouse liver. The superoxide anion production was determined in mouse liver homogenates with a superoxide anion tracer, MCLA, and the MCLA chemiluminescent emission was recorded in a luminometer and expressed as relative light unit per second per micrograms of protein (mean  $\pm$  SEM). <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs the sham controls, <sup>d</sup> $P < 0.01$  vs the I/R procedure plus N.S. at 6 h time point.

visualized in animals with the hepatic I/R procedure (Figure 2B) compared to sham-operated controls (Figure 2A). Prior treatment of allopurinol or apocynin prevented apoptotic cell death caused by the hepatic I/R procedure (Figure 2C and D).

#### Enhanced superoxide anion release during the hepatic I/R procedure

We employed MCLA, which is proportional to levels of  $O_2^-$  and singlet oxygen, to investigate whether the hepatic I/R procedure enhanced the chemiluminescent emission from the liver homogenates. We found that light emission from the liver homogenates was elevated 3-fold 6 h after starting the perfusion, compared to animals with sham operation. The enhanced chemiluminescent emission, caused by the I/R procedure, was markedly inhibited by prior administration of allopurinol or apocynin (Figure 3).

This finding provides convincing evidence that NADPH oxidase or xanthine oxidase is the equally important source of superoxide anions, which contributes to pronounced oxidant stress during the hepatic I/R procedure.

#### Restored GSH and reduced MDA levels by allopurinol or apocynin treatment

Liver GSH and MDA levels were determined as indicators of oxidant stress and lipid peroxidation during the hepatic I/R procedure. As shown in Figure 4, both allopurinol or apocynin led to a restoration of decreased GSH levels and a reversion of elevated MDA levels in the liver ( $P < 0.05-0.01$ ). The GSH levels in the treated groups were close to controls 6 h after starting the reperfusion.

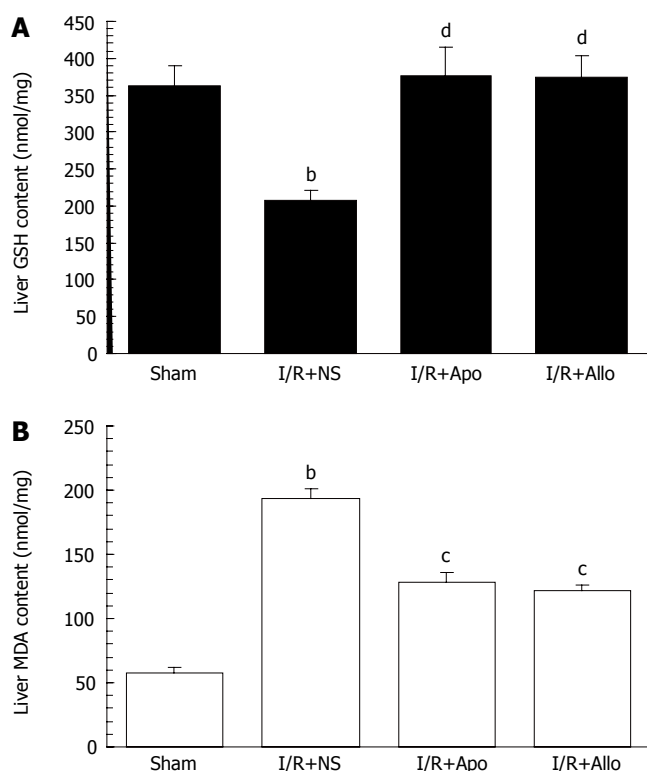
#### Serum TNF- $\alpha$ level

Serum TNF- $\alpha$  was determined using an ELISA kit 6 and 24 h after starting the reperfusion. It is clear that the hepatic I/R procedure caused an elevation of serum TNF- $\alpha$  levels, and that the prior administration of either allopurinol or apocynin reduced the TNF- $\alpha$  levels when compared to the I/R+N.S. controls ( $P < 0.05-0.01$ , Figure 5).

## DISCUSSION

In the present study, we demonstrated that prior administration of either allopurinol or apocynin prevented the hepatic I/R-induced acute liver injury in mice; the protection was due to a blockage of xanthine oxidase (XO) and NADPH oxidase (NOX) by allopurinol and apocynin, respectively. The findings indicate that both XO and NOX play a critical role in the generation of superoxide anion and superoxide anion-derived ROS during the hepatic I/R procedure. Thus, our findings further confirm the involvement of NOX activation in the hepatic I/R-induced acute injury.

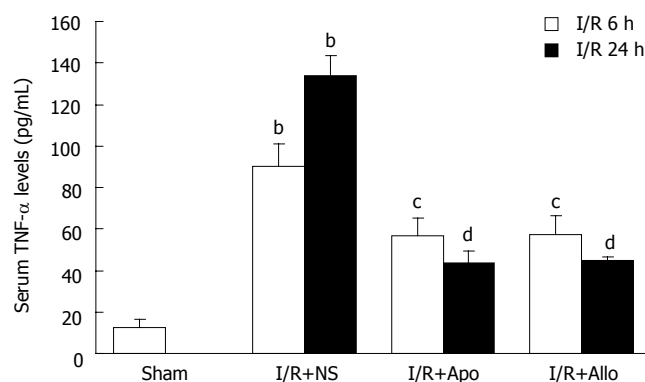
Our previous study showed that pronounced oxidant stress is the major cause of the hepatic I/R injury, and the delivery of either extracellular superoxide dismutase or



**Figure 4** Hepatic I/R-induced depletion of reduced form of glutathione and enhanced lipid peroxidation, and effects of apocynin and allopurinol. **A:** Effect of apocynin or allopurinol on liver GSH levels with the hepatic I/R procedure. Liver GSH content was determined spectrophotometrically and expressed as nanomoles per milligram of protein of the tissue; **B:** Effects of apocynin or allopurinol on liver MDA levels with I/R procedure. Liver MDA content was determined spectrophotometrically and expressed as nanomoles per milligram of tissue protein (mean  $\pm$  SEM). <sup>b</sup> $P < 0.01$  vs Sham controls; <sup>c</sup> $P < 0.05$ , <sup>d</sup> $P < 0.01$  vs the hepatic I/R procedure plus N.S. at 6 h time point ( $n = 6$  in each group).

catalase gene markedly attenuated the hepatic I/R-induced injury in mice<sup>[10]</sup>. It is known that XO plays a critical role in generating superoxide anions and administering allopurinol, a blocker of XO, reduced the injury. It is not clear whether other pathways of oxidative reactions also contribute to the enhanced oxidant stress based on the fact that allopurinol failed to improve the progression of remote hepatic parenchymal injury caused by hind limb I/R in mice<sup>[7]</sup>. This drove us to identify other possible pathologic pathways which lead to enhanced oxidant stress in the hepatic I/R process.

Apocynin is a potent inhibitor of NADPH oxidase by reacting with thiol groups required for enzyme assembly. Apocynin is thought to acquire activation by reacting with hydrogen peroxide and extracellular peroxidase. Active NADPH oxidase is comprised of five subunits (p22phox, p40phox, p47phox, p67phox and gp91phox)<sup>[12]</sup>. In resting cells, three of these subunits (p22phox, p47phox, and p67phox) form a cytosolic complex. Activation of NOX initiates with the phosphorylation of p47phox, which in turn results in the migration of the cytosolic complex to the membrane and subsequent association with membrane-bound p22phox and gp91 to form the active NADPH oxidase<sup>[13]</sup>. Several homologues of gp91phox-Nox1, Nox3, Nox4 and Nox5 have been identified in non-phagocytic cells<sup>[14]</sup>. NADPH oxidase has been shown to



**Figure 5** Effects of apocynin or allopurinol on serum TNF- $\alpha$  levels. Serum TNF- $\alpha$  in mice with hepatic I/R-induced liver injury was measured by an enzyme-linked immunosorbent assay kit. <sup>b</sup> $P < 0.01$  vs Sham controls (mean  $\pm$  SEM); <sup>c</sup> $P < 0.05$ ; <sup>d</sup> $P < 0.01$  vs I/R procedure plus N.S. at 6 or 24 h time point ( $n = 6$  in each group).

have specific subcellular localizations. The mechanisms by which receptors activate NADPH oxidase and regulate ROS production are poorly understood. One may speculate that ROS may be generated during the early and late phases of the hepatic I/R procedure by xanthine oxidase (XO), mitochondrial respiration, or by Kupffer cell-associated and polymorphonuclear neutrophil (PMN)-associated NADPH oxidase<sup>[5,15]</sup>. Thus, blockage of either XO or NOX with their specific inhibitors decreased the production of superoxide anions, as indicated by less chemiluminescent emission with MCLA, and in turn reduced oxidant stress as reflected by restoration of GSH levels and reversed MDA content in the liver. Consequently, lower levels of serum ALT and TNF- $\alpha$ , improved liver histology, as well as fewer apoptotic cell counts, demonstrating the protective effects of these two inhibitors in the hepatic I/R model system. Our results are consistent with a recent report showing that inhibition of NADPH oxidase prevented alcohol-induced liver injury<sup>[16]</sup>, hemorrhagic shock-induced organ injury<sup>[17]</sup> and hepatic I/R injury<sup>[18]</sup>, likely by inhibiting ROS formation *via* NADPH oxidase. However, in the study by Hasegawa *et al*<sup>[18]</sup>, a less specific NADPH oxidase inhibitor, diphenyleneiodonium chloride (DPI), was used, and superoxide anion levels in the liver were not determined. The cell types which often have high levels of NADPH oxidase include neutrophils, monocytes and Kupffer cells. The former two cell types are recruited during the I/R process and contribute to the damaging process by releasing superoxide anions and proteases<sup>[19]</sup>. Activation of Kupffer cells also participates in the enhanced oxidant stress, and increases the production of TNF- $\alpha$  and other cytotoxic factors because the depletion of Kupffer cells resulted in reduced hepatic I/R-associated injury in rodent models<sup>[20,21]</sup>. Nevertheless, further studies are needed to elucidate the role of each cell type that significantly produces superoxide anions *via* the activation of NADPH oxidase during the hepatic I/R process.

In conclusion, the findings in the present study demonstrate that the pretreatment of both allopurinol and apocynin prevented ischemia/reperfusion-induced acute liver injury and that the protection is associated with the blockage of either xanthine oxidase or NADPH oxidase



activity. The activation of both enzymes contributes to the generation of superoxide anions and related reactive oxygen species during the hepatic ischemia/reperfusion-induced liver injury. This study implies a new pharmacological intervention approach by blocking either xanthine oxidase or NADPH oxidase, or in combination to improve the donor organ quality and survival after the transplantation in recipients.

## ACKNOWLEDGMENTS

The authors are grateful to Drs. Mark A Zern and Senthil K Venugopal for their invaluable comments and assistance.

## COMMENTS

### Background

Ischemia/reperfusion-associated donor liver damage is inevitable during the harvest, preservation, transportation and implantation to a recipient. Enhanced oxidant stress has been linked to the donor organ damage; however, the source of reactive oxygen species (ROS) for enhanced oxidant stress has not been well defined. Superoxide anion generated from the reaction of hypoxanthine to xanthine catalyzed by xanthine oxidase (OX) has been thought to be one source of ROS, but an intrinsic source of ROS, via the activation of NADPH oxidase (NOX), has not been well studied in a hepatic ischemia/reperfusion model system.

### Research frontiers

We employed a sensitive chemiluminescent tracer, MCLA, which reflects tissue levels of superoxide anion, one initial and very active oxygen species, to investigate the importance of superoxide anion via the NADPH oxidase reaction in a model of standard hepatic ischemia/reperfusion procedure in mice.

### Innovations and breakthroughs

We found that there was a marked increase in superoxide anion release during the ischemia/reperfusion procedure, and that the use of apocynin, a specific inhibitor of NADPH oxidase, effectively minimized the superoxide anion release, oxidant stress and liver damage caused by a hepatic ischemia/reperfusion procedure in mice.

### Applications

The study implies a new pharmacological intervention approach by blocking either xanthine oxidase or NADPH oxidase, or in combination to improve the donor organ quality, function and survival after the transplantation in recipients.

### Peer review

This study of the beneficial effects of allopurinol and apocynin on the healing of ischemia/reperfusion-induced liver injury is original. It is a well-designed and well-written paper. Allopurinol and apocynin exerted protective effects on hepatic ischemia/reperfusion injury. The protection is associated with blocking the generation of superoxide anions during the hepatic I/R procedure by inhibiting xanthine oxidase and NADPH oxidase activity.

## REFERENCES

- 1 Rhoden E, Pereira-Lima L, Lucas M, Mauri M, Rhoden C, Pereira-Lima JC, Zettler C, Petteffi L, Bello-Klein A. The effects of allopurinol in hepatic ischemia and reperfusion: experimental study in rats. *Eur Surg Res* 2000; **32**: 215-222
- 2 Yamamori T, Inanami O, Nagahata H, Kuwabara M. Phosphoinositide 3-kinase regulates the phosphorylation of NADPH oxidase component p47(phox) by controlling cPKC/PKCdelta but not Akt. *Biochem Biophys Res Commun* 2004; **316**: 720-730
- 3 Arteel GE. Oxidants and antioxidants in alcohol-induced liver disease. *Gastroenterology* 2003; **124**: 778-790
- 4 Czaja MJ. Induction and regulation of hepatocyte apoptosis

- by oxidative stress. *Antioxid Redox Signal* 2002; **4**: 759-767
- 5 Mochida S, Arai M, Ohno A, Masaki N, Ogata I, Fujiwara K. Oxidative stress in hepatocytes and stimulatory state of Kupffer cells after reperfusion differ between warm and cold ischemia in rats. *Liver* 1994; **14**: 234-240
- 6 Jaeschke H, Lemasters JJ. Apoptosis versus oncotic necrosis in hepatic ischemia/reperfusion injury. *Gastroenterology* 2003; **125**: 1246-1257
- 7 Dorman RB, Wunder C, Saba H, Shoemaker JL, MacMillan-Crow LA, Brock RW. NAD(P)H oxidase contributes to the progression of remote hepatic parenchymal injury and endothelial dysfunction, but not microvascular perfusion deficits. *Am J Physiol Gastrointest Liver Physiol* 2006; **290**: G1025-G1032
- 8 Harada H, Hines IN, Flores S, Gao B, McCord J, Scheerens H, Grisham MB. Role of NADPH oxidase-derived superoxide in reduced size liver ischemia and reperfusion injury. *Arch Biochem Biophys* 2004; **423**: 103-108
- 9 Stolk J, Hiltermann TJ, Dijkman JH, Verhoeven AJ. Characteristics of the inhibition of NADPH oxidase activation in neutrophils by apocynin, a methoxy-substituted catechol. *Am J Respir Cell Mol Biol* 1994; **11**: 95-102
- 10 He SQ, Zhang YH, Venugopal SK, Dicus CW, Perez RV, Ramsamooj R, Nantz MH, Zern MA, Wu J. Delivery of antioxidative enzyme genes protects against ischemia/reperfusion-induced liver injury in mice. *Liver Transpl* 2006; **12**: 1869-1879
- 11 Wu J, Liu L, Yen RD, Catana A, Nantz MH, Zern MA. Liposome-mediated extracellular superoxide dismutase gene delivery protects against acute liver injury in mice. *Hepatology* 2004; **40**: 195-204
- 12 Babior BM. NADPH oxidase: an update. *Blood* 1999; **93**: 1464-1476
- 13 Cheng G, Cao Z, Xu X, van Meir EG, Lambeth JD. Homologs of gp91phox: cloning and tissue expression of Nox3, Nox4, and Nox5. *Gene* 2001; **269**: 131-140
- 14 Lentsch AB, Kato A, Yoshidome H, McMasters KM, Edwards MJ. Inflammatory mechanisms and therapeutic strategies for warm hepatic ischemia/reperfusion injury. *Hepatology* 2000; **32**: 169-173
- 15 Kono H, Rusyn I, Uesugi T, Yamashina S, Connor HD, Dikalova A, Mason RP, Thurman RG. Diphenyleneiodonium sulfate, an NADPH oxidase inhibitor, prevents early alcohol-induced liver injury in the rat. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G1005-G1012
- 16 Abdelrahman M, Mazzon E, Bauer M, Bauer I, Delbosc S, Cristol JP, Patel NS, Cuzzocrea S, Thiemermann C. Inhibitors of NADPH oxidase reduce the organ injury in hemorrhagic shock. *Shock* 2005; **23**: 107-114
- 17 Yabe Y, Kobayashi N, Nishihashi T, Takahashi R, Nishikawa M, Takakura Y, Hashida M. Prevention of neutrophil-mediated hepatic ischemia/reperfusion injury by superoxide dismutase and catalase derivatives. *J Pharmacol Exp Ther* 2001; **298**: 894-899
- 18 Hasegawa T, Malle E, Farhood A, Jaeschke H. Generation of hypochlorite-modified proteins by neutrophils during ischemia-reperfusion injury in rat liver: attenuation by ischemic preconditioning. *Am J Physiol Gastrointest Liver Physiol* 2005; **289**: G760-G767
- 19 Gujral JS, Hinson JA, Farhood A, Jaeschke H. NADPH oxidase-derived oxidant stress is critical for neutrophil cytotoxicity during endotoxemia. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G243-G252
- 20 Vega VL, Mardones L, Maldonado M, Nicovani S, Manriquez V, Roa J, Ward PH. Xanthine oxidase released from reperfused hind limbs mediate kupffer cell activation, neutrophil sequestration, and hepatic oxidative stress in rats subjected to tourniquet shock. *Shock* 2000; **14**: 565-571
- 21 von Frankenberg M, Golling M, Mehrabi A, Nentwich H, Thies J, Schaeffer F, Jahnke C, Bud O, Gebhard MM, Otto G, Thurman RG, Herfarth C, Klar E. Destruction of Kupffer's cells increases total liver blood flow and decreases ischemia reperfusion injury in pigs. *Transplant Proc* 1999; **31**: 3253-3254

RAPID COMMUNICATION

## Is a 7-day *Helicobacter pylori* treatment enough for eradication and inactivation of gastric inflammatory activity?

Carlos Robles-Jara, Carlos Robles-Medrand, Manuel Moncayo, Byron Landivar, Johnny Parrales

Carlos Robles-Jara, Carlos Robles-Medrand, Gastroenterology Division, Instituto Ecuatoriano de Enfermedades Digestivas y Pélvicas (IECED), Portoviejo 1301266, Ecuador

Carlos Robles-Medrand, Gastroenterology Division, Hôpital Edouard Herriot, Lyon 69003, France

Manuel Moncayo, Gastroenterology Division, Instituto Ecuatoriano del Seguro Social, Portoviejo 1301266, Ecuador

Byron Landivar, Epidemiology Division, Hospital Oncológico Julio Villacreses Colmont, Sociedad de Lucha contra el Cancer (SOLCA) or Registro Nacional de Tumores, Portoviejo 1301266, Ecuador

Johnny Parrales, Laboratorio de Histopatología (LABOPAT), Portoviejo 1301266, Ecuador

Author contributions: Robles-Jara C and Robles-Medrand C designed research; Robles-Jara C, Moncayo M and Parrales J performed research; Robles-Medrand C and Landivar B analyzed data; Robles-Medrand C and Robles-Jara C wrote the paper.

Correspondence to: Carlos Robles-Jara, MD, Instituto Ecuatoriano de Enfermedades Digestivas y Pélvicas (IECED), Hospital Clinica San Antonio. Av. Paulo Emilio Macias y Tennis club, Portoviejo 1301266, Ecuador. [carlosroblesj@hotmail.com](mailto:carlosroblesj@hotmail.com)  
Telephone: +593-5-2637672 Fax: +593-5-2633265

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96% in group 2 for PP ( $P = 0.0002$ ; OR = 7.25; 95% CI, 2-26).

**CONCLUSION:** In this Ecuadorian population, the 10-d therapy was more effective than the 7-d therapy for *H pylori* eradication as well as for gastric mucosa inflammatory inactivation.

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**Key words:** *Helicobacter pylori* treatment; *Helicobacter pylori* infection; Gastric inflammatory inactivation; Triple therapy eradication; Randomized study

**Peer reviewer:** Colin W Howden, Professor of Medicine, Division of Gastroenterology, Division of Gastroenterology, Northwestern University Feinberg School of Medicine, 676 N. St. Clair Street, Suite 1400, Chicago, IL 60611, United States

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### Abstract

**AIM:** To compare the efficacy of a 7-d vs 10-d triple therapy regarding *H pylori* eradication, endoscopic findings and histological gastric inflammatory inactivation in the Ecuadorian population.

**METHODS:** 136 patients with dyspepsia and *H pylori* infection were randomized in 2 groups (68 per group): group 1, 7-d therapy; group 2, 10-d therapy. Both groups received the same medication and daily dosage: omeprazole 20 mg bid, clarithromycin 500 mg bid and amoxicillin 1 g bid. Endoscopy was performed for histological assessment and *H pylori* infection status before and 8 wk after treatment.

**RESULTS:** *H pylori* was eradicated in 68% of group 1 vs 83.8% of group 2 for the intention-to-treat analysis (ITT) ( $P = 0.03$ ; OR = 2.48; 95% CI, 1.1-5.8), and 68% in group 1 vs 88% in group 2 for the per-protocol analysis (PP) ( $P = 0.008$ ; OR = 3.66; 95% CI, 1.4-10). Endoscopic gastric mucosa normalization was observed in 56.9% in group 1 vs 61.2% in group 2 for ITT, with similar results for the PP, the difference being statistically not significant. The rate of inflammatory inactivation was 69% in group 1 vs 88.7% in group 2 for ITT ( $P = 0.007$ ; OR = 3.00; 95% CI, 1.2-7.5), and 69% in group 1 vs

### INTRODUCTION

*H pylori* bacterial infection is worldwide in humans. It is the main etiologic factor in chronic gastritis and gastroduodenal ulcer disease<sup>[1]</sup>. It is also closely related to gastric adenocarcinoma and low grade gastric lymphoma of mucosa-associated lymphoid tissue (MALT). Forty percent to 50% of the world population is estimated to be infected, with the highest incidence occurring in older people and in those living in areas with low standards of sanitation<sup>[2]</sup>. In South American countries, the prevalence of *H pylori* infection ranges from 70% to 90%<sup>[3]</sup>, being 89.5% in Ecuador<sup>[4]</sup>.

A proton pump inhibitor (PPI) associated with two antibiotics is considered the standard therapy for *H pylori* eradication<sup>[5]</sup>. Clinical trials show *H pylori* eradication rates varying from 45% to 92.3% using PPI associated with amoxicillin and clarithromycin. However, duration of eradication therapy continues to be controversial<sup>[6]</sup>. In many developed countries, a 7-d therapy is considered enough for bacterial eradication. On the other hand, there is still an argument for increasing the duration of treatment to 10 d or even 14 d<sup>[6]</sup>.

In Europe, a 7-d triple therapy is still recommended because 14-d therapy had an insignificant advantage in terms of treatment success rate<sup>[7]</sup>. On the other hand, guidelines from North America recommend 10-d to 14-d therapy, as some studies have reported superior cure rates with prolonged therapy using triple regimens<sup>[8]</sup>. In Asia, an Indian study reported that prolonged triple therapy with lansoprazole, amoxicillin and tinidazole achieved a significant increase in eradication rates: 47.6% *vs* 80% *vs* 91.3% for 1 wk, 2 wk and 3 wk of therapy, respectively<sup>[9]</sup>.

However, in a recent large multicenter randomized trial based on a Korean population, no differences were observed between 7 d *vs* 14 d of triple therapy with omeprazole associated with amoxicillin and clarithromycin<sup>[10]</sup>.

Two studies from Turkey using PPI-based triple therapy with amoxicillin and clarithromycin have shown low eradication rates<sup>[11,12]</sup>. The first study with a 7-d triple therapy showed an eradication rate of 63.6%<sup>[11]</sup>. The second one using a 14-d therapy showed a 45% eradication rate<sup>[12]</sup>.

In the Mexican population, triple therapy with rabeprazole, amoxicillin and ofloxacin for 14 d achieved significantly superior eradication rates when compared with 7-d therapy (92.3% *vs* 62.2%)<sup>[13]</sup>.

In South America, it is difficult to identify the best regimen, due to the absence of large multicenter controlled trials. Some recommendations have been published based on consensus reports. The last Brazilian consensus, in 2005, recommended a 7-d triple therapy<sup>[14]</sup>.

The aim of *H. pylori* eradication is to stop the chronic inflammatory activity that leads to histological changes in the gastric mucosa. This inflammatory inactivation has also shown to improve patients' symptoms<sup>[15,16]</sup>.

The histopathology associated with *H. pylori* infection includes a specific structural disorganization of the epithelial cells and inflammatory infiltration (neutrophils, lymphocytes)<sup>[17]</sup>. Epithelial nonspecific changes include: presence of microcrypts, reduction of foveolar mucus secretion, atrophy, hyperplasia, metaplasia, atypia, or dysplasia<sup>[17]</sup>.

Many studies have well demonstrated the histological resolution of inflammatory activity after *H. pylori* eradication using PPI associated with two antibiotics (amoxicillin, clarithromycin or metronidazole) for 7 d, 10 d or 14 d<sup>[15,18-20]</sup>. Only two studies, however, correlated bacterial eradication, gastric inflammatory inactivation and duration of treatment, comparing 7-d *vs* 14-d and 10-d *vs* 14-d therapy<sup>[16,21]</sup>.

This is the first study evaluating the best treatment approach for the Ecuadorian population. Our objective was to compare the efficacy of 7-d *vs* 10-d triple therapy regarding *H. pylori* eradication rates, the endoscopic findings and the histological gastric inflammatory inactivation.

## MATERIALS AND METHODS

### Patients

This was a prospective, randomized, open-label study performed at the Ecuadorian Institute of Digestive

Diseases (IECED), in Portoviejo, Ecuador. The study population consisted of patients with dyspepsia who were referred for upper endoscopy. Dyspepsia was defined as pain or discomfort localized in the upper abdomen. Patients were enrolled if they were infected with *H. pylori*.

The exclusion criteria were: (1) Age < 18 years; (2) Use of proton pump inhibitors, antibiotics, H<sub>2</sub>-receptor antagonists or bismuth compounds in the last 4 wk preceding the inclusion of the patient in the study; (3) Chronic use of non-steroidal anti-inflammatory drugs (NSAIDs); (4) Patients with well-known antibiotic allergy; (5) Pregnant women; (6) Patients with chronic hepatic or renal disease; (7) Patients with Zollinger-Ellison syndrome; (8) Previous gastric surgery; (9) Previous failed *H. pylori* therapy.

Written informed consent was obtained from all participants prior to enrollment.

### Endoscopy, histology and assessment of *H. pylori*

During endoscopy, 5 biopsies were obtained for histological assessment in accordance with the updated Sydney System (2 from the antrum, 1 from the incisura angularis and 2 from the corpus)<sup>[22]</sup>. Biopsies were fixed, paraffin embedded and stained with hematoxylin-eosin/Giemsa. A pre-pyloric biopsy was obtained for the rapid urease test. Patients were considered to be infected if histological and rapid urease tests were positive. Eight weeks after treatment, bacterial eradication was checked by using the same endoscopy procedure for histology. At endoscopy, gastritis was defined as the presence of visible alterations of the mucosal appearance, presumably caused by vascular or infiltrative changes (Sydney system definition)<sup>[23]</sup>. Histological diagnosis was coded in two classes according to the Sydney System classification as follows: (1) non-atrophic chronic gastritis, when inflammatory activity of any grade or antral atrophy grade I were present without intestinal metaplasia lesion; and (2) atrophic chronic gastritis, when grade 2 or 3 atrophic mucosa lesions were observed with or without intestinal metaplasia.

The gastric mucosa inflammation activity was also graded in accordance with the updated Sydney System and was considered as the presence of mononuclear cells infiltrate and neutrophilic polymorphonuclear in a background of chronic inflammation<sup>[22]</sup>.

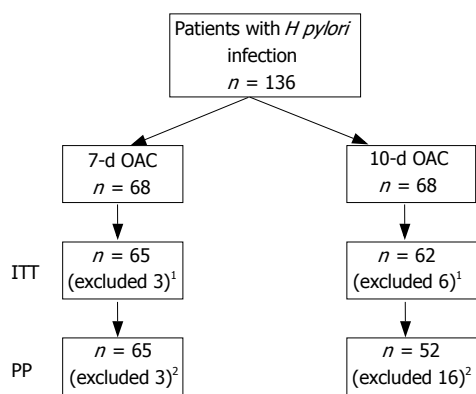
### Treatment

Patients were randomized in a 1:1 ratio to receive omeprazole 20 mg bid, clarithromycin 500 mg bid and amoxicillin 1 g bid for 7 d (group 1) or 10 d (group 2). Treatment compliance was assessed by patient report and pill count at the follow-up visit. Good compliance was defined as consumption of more than 90% of the prescribed drugs. After completing treatment, no patients received PPI or antisecretory drugs during the follow-up period.

### Statistical analysis

Statistical analysis was performed using StatView for Windows (version 5.0). The 95% confidence intervals





**Figure 1** Randomization protocol. <sup>1</sup>Patients were excluded if missing in the follow-up; <sup>2</sup>Patients were excluded if ITT excluded population, non-compliance. ITT: Intention to treat analysis; PP: Per protocol analysis; OAC: Omeprazole, amoxicillin and clarithromycin.

were determined with the chi-square test or Fisher's exact test for small sample sizes, and comparisons were made with Student's test. *P* value of < 0.05 were considered as statistically significant. Per protocol and intention to treat approach was performed for statistical analysis in each group.

### Sample size

As there is no data regarding *H. pylori* eradication rates in the Ecuadorian population, the sample size was calculated a priori, based on available data in the literature. By hypothesizing an 89% eradication rate for the 10-d triple therapy<sup>[30]</sup> and less than 70% (65%) for 7-d triple therapy<sup>[13]</sup>, the estimated sample size was 54 subjects per group, with a power of 0.85 and a significance level of 0.05. Due to the probability of loss from follow-up, the risk of data loss was estimated at around 25%; therefore the final size was considered 68 patients in each group.

## RESULTS

### Baseline demographics

One hundred and thirty-six patients (68 patients by group) were enrolled in the study (Figure 1). At endoscopy, the initial diagnoses were: gastritis in 69/136 (50.7%), duodenal ulcer in 26/136 (19.11%), and gastric ulcer in 22/136 (16.17%). Endoscopy was normal in 19/136 (13.9%).

At histology non-atrophic chronic active gastritis was present in 88.2% of patients for group 1 (60/68) and 82.3% (56/68) for group 2. In group 1, eight patients had atrophic chronic active gastritis (11.7%) in which the presence of intestinal metaplasia was identified in 5/8. In group 2, the presence of atrophic chronic active gastritis was observed in 12 patients (17.6%), all of them with intestinal metaplasia. There were no significant differences between the treatment groups in any of the baseline characteristics. Demographic, endoscopic and histological characteristics are summarized in Table 1.

Three patients (4.4%) in group 1 and six patients (8.8%) in group 2 did not return for follow-up and were excluded from the study. Additionally, ten patients (14.7%) in group 2 were not compliant with the treatment: therapy

**Table 1** Baseline demographic, endoscopic and histological characteristics of patients

	Group 1 (n = 68) OAC 7-d	Group 2 (n = 68) OAC 10-d	P
Age (yr, mean ± SD)	42 ± 11	44 ± 12	NS
Sex (M/F)	23/45	27/41	NS
Endoscopic findings:			
Normal	9	10	NS
Gastritis (IM)	31 (4)	38 (6)	NS
Gastric ulcer (IM)	13 (1)	9 (2)	NS
Duodenal ulcer (IM)	15 (0)	11 (4)	NS
Histological findings:			
Nonatrophic chronic active gastritis (IM)	60	56	NS
Atrophic chronic active gastritis (IM)	8 (5)	12 (12)	

IM: Intestinal metaplasia; M: Male; F: Female; NS: Not significant; OAC: Omeprazole, amoxicillin and clarithromycin.

was stopped at d 8 in 7 patients, at d 3 in 2 patients and at d 4 in 1 patient. In 6 patients (8.8%), the non-compliance was correlated with treatment-adverse events (4 with moderate abdominal pain, 1 with nausea, and 1 with diarrhea).

### Per protocol (PP) analysis

One hundred and seventeen patients underwent a PP analysis (Table 2): 65 patients in group 1 and 52 patients in group 2. Eight weeks after treatment the endoscopy showed a normal gastric mucosa in 56.9% (37/65) of patients in group 1 and in 63.5% (32/52) in group 2. Ulcer healing was observed in 93.7% (45/48) of patients (both groups). In group 1, gastritis was observed in 25 patients (intestinal metaplasia in 5 patients at histology without presence of gastric mucosa inflammation) and an active ulcer in 3 patients (2 with gastric ulcer and 1 with duodenal ulcer). In group 2, gastritis was present in 20 patients (12 patients with intestinal metaplasia at histology and without gastric mucosa inflammation).

*H. pylori* eradication was obtained in 68% (44/65) of patients for group 1 and 88% (46/52) for group 2 (*P* = 0.008; OR = 3.66; 95% CI, 1.4-10).

The total average rate of inflammatory inactivation of gastric mucosa was 80% for both groups. In group 1, 45 patients (69%) showed an inactivation of the inflammation compared with 49 patients (96%) in group 2 (*P* = 0.0002; OR = 7.25; 95% CI, 2-26).

### Intention to treat (ITT) analysis

One hundred and twenty-seven patients underwent an ITT assessment (Table 3): 65 patients in group 1 and 62 patients in group 2. After treatment, the endoscopy showed a normal gastric mucosa in 56.9% (37/65) of patients for group 1 and 61.2% (37/62) in group 2. Gastritis was present in 25 patients in group 2 (12 patients with intestinal metaplasia at histology and without presence of gastric mucosa inflammation).

*H. pylori* eradication was obtained in 68% (44/65) of patients for group 1 and 83.8% (52/62) for group 2 (*P* = 0.03; OR = 2.48; 95% CI, 1.1-5.8).



**Table 2** Per protocol analysis, control results at wk 8 in two groups (%)

	Group 1 (n = 65) OAC 7-d	Group 2 (n = 52) OAC 10-d	P
<i>H. pylori</i> eradication	44/65 (68)	46/52 (88)	0.008
Normal endoscopy	37/65 (56.9)	32/52 (63.5)	NS
Inflammatory inactivation of gastric mucosa	45/65 (69)	49/52 (96)	0.0002

OAC: Omeprazole, amoxicillin and clarithromycin; NS: Not significant.

The total average rate of gastric mucosa inflammatory inactivation was 79.5% for both groups. In group 1, 45/65 patients (69%) showed an inactivation of the inflammation compared with 54/62 patients (88.7%) in group 2 ( $P = 0.007$ ; OR = 3.00; 95% CI, 1.2-7.5).

## DISCUSSION

After the discovery of *H. pylori* many studies were reported for its treatment, including various meta-analyses. Nowadays there is no doubt that triple therapies, that is, PPI & two antibiotics (amoxicillin associated with clarithromycin or metronidazole) are currently the most preferred first-line therapy regimens in clinical practice.

Therapy regimens and their duration could not be standardized because results of clinical data are different between countries. In effect, the results for efficacy of therapy are varied worldwide. This variation of the results could be a consequence of many factors such as bacterial virulence, environmental factors and antibacterial resistance, which are peculiar for each country. In addition, individual factors such as: patient's age less than 60, presence of duodenal ulcer and treatment duration have been also linked to therapy efficacy<sup>[24]</sup>.

Until now, no data or consensus about the duration of treatment for *H. pylori* in Ecuador was available. We therefore evaluated the twice-daily triple therapy of omeprazole 20 mg bid, amoxicillin 1 g bid and clarithromycin 500 mg bid.

Metronidazole resistance in *H. pylori* strains varies geographically, and influences negatively the effectiveness of therapies containing this antibiotic<sup>[25]</sup>. In Latin-American countries, more than 62% of the population is resistant to metronidazole treatment<sup>[3,26]</sup>. This can be explained by the indiscriminate use of these agents for many other diseases (such as parasitosis).

The rates of metronidazole resistance are as high as 81% in Ecuador. In contrast, clarithromycin resistance is still small: < 10%<sup>[27]</sup>. This is the reason for using clarithromycin in our study.

Two clinical trials from South America reported eradication rates of more than 85% with 7-d triple therapy using PPI, amoxicillin and clarithromycin. Coelho *et al.*<sup>[28]</sup>, in the Brazilian population showed 87% of *H. pylori* eradication with a 7-d treatment (using pantoprazole, amoxicillin and clarithromycin) in 71 patients. In Peru, a randomized study with 72 patients showed 86.1% of *H. pylori* eradication with no differences between 7-d *vs* 10-d triple therapy<sup>[29]</sup>.

**Table 3** Intention to treat analysis, control results at wk 8 in two groups (%)

	Group 1 (n = 65) OAC 7-d	Group 2 (n = 62) OAC 10-d	P
<i>H. pylori</i> eradication	44/65 (68)	52/62 (83.8)	0.03
Normal endoscopy	37/65 (56.9)	37/62 (61.2)	NS
Inflammatory inactivation of gastric mucosa	45/65 (69)	54/62 (88.7)	0.007

OAC: Omeprazole, amoxicillin and clarithromycin; NS: Not significant.

In the Ecuadorian population our study showed a 68% and an 86% eradication rate for the 7-d and 10-d triple therapy, respectively, this difference being statistically significant.

Many clinical trials have demonstrated similar eradication rates using 10-d triple therapy with PPI, amoxicillin and clarithromycin<sup>[15,16,30,34]</sup>. A large multicenter double-blind trial in the United States, demonstrated an eradication rate of 84%<sup>[30]</sup>. Fennerty *et al.*, also found comparable results (84% eradication rate) in 284 patients, without difference when compared to 14-d therapy<sup>[16]</sup>. In the Czech Republic, the rate of *H. pylori* eradication obtained was 87%<sup>[15]</sup>.

Moreover, a recent meta-analysis has shown that prolonging triple therapies from 7 to 10 d or 14 d improves treatment cure rates<sup>[31]</sup>. Other meta-analysis suggested that 10-d therapy was associated with less failure than 7-d therapy<sup>[24]</sup>. Our study also indicates that a 10-d regimen was statistically more effective than the 7-d regimen for inactivating the gastric mucosa inflammation.

Acid suppression with PPI in *H. pylori* gastritis only decreases the gastric inflammatory activity, but doesn't produce its inactivation<sup>[32]</sup>. Histological changes after *H. pylori* eradication show a rapid improvement of neutrophils infiltration in the first 2 mo, progressive declination of mononuclear cell infiltration during the first 2 years, and no significant changes in the 12 first mo regarding atrophy or intestinal metaplasia<sup>[17-20,33]</sup>. The presence of an inflammatory activity is seen in fewer cases, even 12 mo after the eradication<sup>[32,33]</sup>.

In our patients, 8 wk after treatment, the endoscopic assessment revealed the persistence of gastric lesions in 43.1% of patients (28/65) in group 1 and 38.5% of patients (20/52) in group 2. However, an inflammatory inactivation was obtained in 69% and 94% of patients in groups 1 and 2 respectively. All cases with intestinal metaplasia had *H. pylori* eradication and gastric inflammatory inactivation. In patients with abnormal control endoscopy, persistence of *H. pylori* was observed in 21/28 patients in group 1 and in 6/20 patients in group 2, being associated with the persistence of an inflammatory activity.

Discordance between endoscopic and histological findings after *H. pylori* eradication with 7-d triple therapy (PPI, amoxicillin, clarithromycin) was previously reported<sup>[16,20]</sup>. Dajani *et al.*, observed a persistence of lesions at the control endoscopy in 46.4% of patients (7.4% of duodenal ulcer and 39% of gastritis lesions), in spite of 93% eradication rates with a remarkable resolution of the

inflammatory activity<sup>[20]</sup>. Another study comparing 10 *vs* 14 d of treatment shows the persistence of an active ulcer in 28% of patients and gastritis in 9% after an eradication rate of 84%<sup>[16]</sup>.

Prolonging therapy duration has been associated with an increased risk of non-compliance due to side-effects<sup>[30,35]</sup>. This was also observed in our study, which shows an increased non-compliance rate in the 10-d therapy group (group 2), being correlated in 6 patients with treatment side-effects.

Finally, although our data points to the superior benefits of the 10-d therapy, we should be careful before establishing definitive recommendations for the Ecuadorian population.

On a day-to-day basis, every medical treatment must consider the cost-benefit analysis, mainly in developing countries, where it is difficult for patients to afford their medications in function of its price. The cost of *H pylori* eradication therapy in South America is very high<sup>[3]</sup>. Some recommended eradication therapies have 7-d duration mainly because of improved compliance and decreased medical cost<sup>[13]</sup>. In our study, 32% of patients in the 7-d *vs* 12% in 10-d therapy did not eradicate *H pylori*. Probably, the re-treatment of this 32% of patients in group 1 with second or third line therapies would be more expensive and less effective than directly treating them for 10 d with the first line approach. This evaluation however was not the objective of our paper, and should be the subject of a future study.

In conclusion, this randomized open study showed that in Ecuador, a 10-d triple therapy using PPI associated with amoxicillin and clarithromycin was significantly more effective than a 7-d triple therapy. The cost-benefit of this treatment in our population should be evaluated in a future study.

## COMMENTS

### Background

The aim of *H pylori* eradication is to stop chronic inflammatory activity that leads to histological changes in the gastric mucosa. A proton pump inhibitor (PPI) associated with two antibiotics is considered the standard therapy for *H pylori* eradication. However, duration of eradication therapy continues to be controversial, due to the variable results from all over the world. There is no data about *H pylori* treatment in the Ecuadorian population and to our knowledge there are no studies correlating the bacterial eradication, gastric inflammatory inactivation and duration of treatment comparing 7-d *vs* 10-d.

### Research frontiers

This study determined the best valid *H pylori* eradication treatment in a population with higher rates of infection correlating the bacterial eradication rate, the endoscopic and histological results with the treatment duration.

### Innovations and breakthroughs

Many studies have well demonstrated the histological resolution of inflammatory activity after *H pylori* eradication using PPI associated with two antibiotics (amoxicillin, clarithromycin or metronidazole) for 7 d, 10 d or 14 d. Only two studies, however, correlated bacterial eradication, gastric inflammatory inactivation and duration of treatment, comparing 7-d *vs* 14-d and 10-d *vs* 14-d therapy. This is the first study evaluating the best treatment approach for the Ecuadorian population. We compared the efficacy of 7-d *vs* 10-d triple therapy regarding *H pylori* eradication rates, the endoscopic findings and the histological gastric inflammatory inactivation.

## Applications

In this Ecuadorian population, 10-d triple therapy using PPI associated with amoxicillin and clarithromycin was significantly more effective than 7-d triple therapy. The cost-benefit of this treatment in our population should be evaluated in a future study.

## Peer review

This is a reasonably well done clinical trial comparing 7-d and 10-d PPI-based triple therapy for *H pylori* infection. Ten-day treatment was significantly superior. The trial is well described.

## REFERENCES

- 1 Gisbert JP, Gonzalez L, Calvet X. Systematic review and meta-analysis: proton pump inhibitor vs. ranitidine bismuth citrate plus two antibiotics in *Helicobacter pylori* eradication. *Helicobacter* 2005; **10**: 157-171
- 2 Pounder RE, Ng D. The prevalence of *Helicobacter pylori* infection in different countries. *Aliment Pharmacol Ther* 1995; **9** Suppl 2: 33-39
- 3 Castro Lde P, Coelho LG. *Helicobacter pylori* in South America. *Can J Gastroenterol* 1998; **12**: 509-512
- 4 Gomez NA, Alvarez LR, Zapatier JA, Vargas PE. [Efficacy of stool antigen and serologic tests in the diagnosis of *Helicobacter pylori* in Ecuadorian population] *Rev Gastroenterol Mex* 2005; **70**: 146-150
- 5 Malfertheiner P, Megraud F, O'Morain C, Hungin AP, Jones R, Axon A, Graham DY, Tytgat G. Current concepts in the management of *Helicobacter pylori* infection--the Maastricht 2-2000 Consensus Report. *Aliment Pharmacol Ther* 2002; **16**: 167-180
- 6 Bytzer P, O'Morain C. Treatment of *Helicobacter pylori*. *Helicobacter* 2005; **10** Suppl 1: 40-46
- 7 Malfertheiner P, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, Hunt R, Rokkas T, Vakil N, Kuipers EJ. Current concepts in the management of *Helicobacter pylori* infection: the Maastricht III Consensus Report. *Gut* 2007; **56**: 772-781
- 8 Chey WD, Wong BC. American College of Gastroenterology guideline on the management of *Helicobacter pylori* infection. *Am J Gastroenterol* 2007; **102**: 1808-1825
- 9 Chaudhary A, Ahuja V, Bal CS, Das B, Pandey RM, Sharma MP. Rank order of success favors longer duration of imidazole-based therapy for *Helicobacter pylori* in duodenal ulcer disease: a randomized pilot study. *Helicobacter* 2004; **9**: 124-129
- 10 Kim BG, Lee DH, Ye BD, Lee KH, Kim BW, Kim SG, Kim SW, Kim SK, Kim JJ, Kim HY, Park JJ, Park CY, Baik GH, Lee YC, Lee JH, Lee JH, Chun HJ, Hahm KB, Hong SJ, Lee SW, Jung HC. Comparison of 7-day and 14-day proton pump inhibitor-containing triple therapy for *Helicobacter pylori* eradication: neither treatment duration provides acceptable eradication rate in Korea. *Helicobacter* 2007; **12**: 31-35
- 11 Sivri B, Simsek I, Hulagu S, Kadayifci A, Tozun N, Akarsu M, Uraz S, Savas MC, Koruk M, Bozbas A. The efficacy, safety and tolerability of pantoprazole-based one-week triple therapy in *H. pylori* eradication and duodenal ulcer healing. *Curr Med Res Opin* 2004; **20**: 1301-1307
- 12 Altintas E, Sezgin O, Ulu O, Aydin O, Camdeviren H. Maastricht II treatment scheme and efficacy of different proton pump inhibitors in eradicating *Helicobacter pylori*. *World J Gastroenterol* 2004; **10**: 1656-1658
- 13 Bosques-Padilla FJ, Garza-Gonzalez E, Calderon-Lozano IE, Reed-SanRoman G, de Arino Suarez M, Valdovinos-Diaz MA, Orozco-Gamiz A, Blancas-Valencia JM, Tamayo-de la Cuesta JL. Open, randomized multicenter comparative trial of rabeprazole, ofloxacin and amoxicillin therapy for *Helicobacter pylori* eradication: 7 *vs*. 14 day treatment. *Helicobacter* 2004; **9**: 417-421
- 14 Coelho LG, Zaterka S. [Second Brazilian Consensus Conference on *Helicobacter pylori* infection] *Arq Gastroenterol* 2005; **42**: 128-132
- 15 Kyzekeve J, Arlt J, Arltová M. Is there any relationship

- between functional dyspepsia and chronic gastritis associated with *Helicobacter pylori* infection? *Hepatogastroenterology* 2001; **48**: 594-602
- 16 **Fennerty MB**, Kovacs TO, Krause R, Haber M, Weissfeld A, Siepmann N, Rose P. A comparison of 10 and 14 days of lansoprazole triple therapy for eradication of *Helicobacter pylori*. *Arch Intern Med* 1998; **158**: 1651-1656
  - 17 **Warren JR**. Gastric pathology associated with *Helicobacter pylori*. *Gastroenterol Clin North Am* 2000; **29**: 705-751
  - 18 **Abdul Aal GM**, Dajani AI, Nounou M, Awad S, Abdul Rasheed Z, Gautam S, Ukabam S, Nayal S. Resolution of gastritis induced by *Helicobacter pylori* 4-5 weeks after successful eradication of infection using a triple therapy regimen of pantoprazole, amoxycillin and clarithromycin for one week. *Digestion* 1999; **60**: 286-297
  - 19 **Jakic-Razumovic J**, Tentor D, Kusec V, Cuzic S, Brkic T. Histopathological features of gastritis before and after treatment for *Helicobacter pylori*. *Croat Med J* 2000; **41**: 159-162
  - 20 **Dajani AI**, Awad S, Ukabam S, Nounou MA, Abdul Rasheed Z, Gautam S, Abdul Aal G, Nayal S. One-week triple regime therapy consisting of pantoprazole, amoxicillin and clarithromycin for cure of *Helicobacter pylori*-associated upper gastrointestinal diseases. *Digestion* 1999; **60**: 298-304
  - 21 **Paoluzi P**, Iacopini F, Crispino P, Nardi F, Bella A, Rivera M, Rossi P, Gurnari M, Caracciolo F, Zippi M, Pica R. 2-week triple therapy for *Helicobacter pylori* infection is better than 1-week in clinical practice: a large prospective single-center randomized study. *Helicobacter* 2006; **11**: 562-568
  - 22 **Dixon MF**, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. *Am J Surg Pathol* 1996; **20**: 1161-1181
  - 23 **Tytgat GN**. The Sydney System: endoscopic division. Endoscopic appearances in gastritis/duodenitis. *J Gastroenterol Hepatol* 1991; **6**: 223-234
  - 24 **Broutet N**, Tchamgoue S, Pereira E, Lamouliatte H, Salamon R, Megraud F. Risk factors for failure of *Helicobacter pylori* therapy--results of an individual data analysis of 2751 patients. *Aliment Pharmacol Ther* 2003; **17**: 99-109
  - 25 **Chey WD**. *Helicobacter Pylori*. *Curr Treat Options Gastroenterol* 1999; **2**: 171-182
  - 26 **NA**. World Gastroenterology Organisation (WGO). WGO Practice Guideline *Helicobacter Pylori* in developing countries. November 2006. Accessed Feb 14, 2007. Available at URL address: <http://www.omge.org/globalguidelines/guide15/guideline15.htm>
  - 27 **Debets-Ossenkopp YJ**, Reyes G, Mulder J, aan de Stegge BM, Peters JT, Savelkoul PH, Tanca J, Pena AS, Vandenbroucke-Grauls CM. Characteristics of clinical *Helicobacter pylori* strains from Ecuador. *J Antimicrob Chemother* 2003; **51**: 141-145
  - 28 **Coelho LG**, Mattos AA, Francisconi CF, Castro Lde P, Andre SB. [Efficacy of the dosing regimen of pantoprazole 40 mg, amoxicillin 1000 mg and clarithromycin 500 mg, twice daily for 7 days, in the eradication of *Helicobacter pylori* in patients with peptic ulcer] *Arq Gastroenterol* 2004; **41**: 71-76
  - 29 **Rodriguez W**, Pareja Cruz A, Yushimito L, Ramirez Ramos A, Gilman RH, Watanabe Yamamoto J, Rodriguez Ulloa C, Mendoza Requena D, Guerra Valencia J, Leey Casella J, Chinga Alayo E, Velapatioño B, Valencia T. Omeprazole, amoxicillin and clarithromycin in the treatment of *Helicobacter pylori*, in 7 and 10-day regimens. *Rev Gastroenterol Peru* 2003; **23**: 177-183
  - 30 **Laine L**, Suchower L, Frantz J, Connors A, Neil G. Twice-daily, 10-day triple therapy with omeprazole, amoxicillin, and clarithromycin for *Helicobacter pylori* eradication in duodenal ulcer disease: results of three multicenter, double-blind, United States trials. *Am J Gastroenterol* 1998; **93**: 2106-2112
  - 31 **Calvet X**, Garcia N, Lopez T, Gisbert JP, Gene E, Roque M. A meta-analysis of short versus long therapy with a proton pump inhibitor, clarithromycin and either metronidazole or amoxicillin for treating *Helicobacter pylori* infection. *Aliment Pharmacol Ther* 2000; **14**: 603-609
  - 32 **Moayyedi P**, Wason C, Peacock R, Walan A, Bardhan K, Axon AT, Dixon MF. Changing patterns of *Helicobacter pylori* gastritis in long-standing acid suppression. *Helicobacter* 2000; **5**: 206-214
  - 33 **Salih BA**, Abasiyanik MF, Saribasak H, Hutten O, Sander E. A follow-up study on the effect of *Helicobacter pylori* eradication on the severity of gastric histology. *Dig Dis Sci* 2005; **50**: 1517-1522
  - 34 **Laine L**, Estrada R, Trujillo M, Fukunaga K, Neil G. Randomized comparison of differing periods of twice-a-day triple therapy for the eradication of *Helicobacter pylori*. *Aliment Pharmacol Ther* 1996; **10**: 1029-1033
  - 35 **Vakil N**, Connor J. *Helicobacter pylori* eradication: equivalence trials and the optimal duration of therapy. *Am J Gastroenterol* 2005; **100**: 1702-1703

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RAPID COMMUNICATION

## Laparo-endoscopic “Rendezvous” to treat cholecysto-choledocolithiasis: Effective, safe and simplifies the endoscopist’s work

Gaetano La Greca, Francesco Barbagallo, Michele Di Blasi, Andrea Chisari, Rosario Lombardo, Rosario Bonaccorso, Saverio Latteri, Andrea Di Stefano, Domenico Russello

Gaetano La Greca, Francesco Barbagallo, Andrea Chisari, Rosario Lombardo, Rosario Bonaccorso, Saverio Latteri, Andrea Di Stefano, Domenico Russello, Department of Surgical Sciences, Transplantation and Advanced Technologies, University of Catania, Cannizzaro Hospital, Via Messina 829, Catania 95126, Italy

Michele Di Blasi, Endoscopic Unit, Cannizzaro Hospital, Via Messina 829, Catania 95126, Italy

**Author contributions:** La Greca G designed the study, performed the research and wrote the paper; Barbagallo F, chief investigator and guided the research work; Di Blasi M, performed endoscopic procedures; Lombardo R, Bonaccorso R, Di Stefano A, Russello D provided the patient material and other clinical data; Chisari A, Latteri S, tables and drafting.

**Correspondence to:** Gaetano La Greca, MD, PhD, Via Messina 354; Catania 95126, Italy. [glaireca@unict.it](mailto:glaireca@unict.it)

Telephone: +39-095-7263586 Fax: +39-095-7122221

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effectiveness and safety at least comparable to those reported for other options. The endoscopist is very often satisfied with this approach because of the minimization of some steps of the endoscopic procedure and avoidance of relevant iatrogenic risk factors. If the mandatory collaboration between surgeons and endoscopists is guaranteed, this approach can often be preferable for the patient, the surgeon, the endoscopist and the hospital.

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**Key words:** Gallstones; Common bile duct; Endoscopic retrograde cholangio-pancreatography; Endoscopic sphincterotomy; Rendezvous; Intra-operative cholangiography; Laparoscopic cholecystectomy

**Peer reviewer:** Yasuji Arase, MD, Department of Gastroenterology, Toranomon Hospital, 2-2-2 Toranomonminato-ku, Tokyo 105-8470, Japan

### Abstract

**AIM:** To investigate our clinical experience with combined laparo-endoscopic Rendezvous (RV) for the treatment of patients affected by gallstones and common bile duct (CBD) stones and especially to study the never evaluated opinion of the endoscopist concerning the difficulty of the intraoperative endoscopic procedure during the RV in comparison with standard endoscopic retrograde cholangio-pancreatography (ERCP).

**METHODS:** Eighty consecutive patients affected by cholecystolithiasis and diagnosed or suspected CBD stones were treated with a standardized “tailored” RV. The relevant technical features, the feasibility, the effectiveness in stone clearance, the safety but also the simple evaluation of difficulty and agreement of the endoscopist were analyzed with a questionnaire.

**RESULTS:** The feasibility was 97.5% and the effectiveness 100% concerning CBD clearance and solution of coexisting problems at the papilla. Minor morbidity was 3.3%, the operating time was prolonged by a mean of 14 min, the mean hospital stay was 3.8 d and only one stone’s recurrence occurred. The endoscopist evaluated the procedure to be simpler than standard ERCP-ES in 81.2% of the cases.

**CONCLUSION:** Simultaneous RV carries high

La Greca G, Barbagallo F, Di Blasi M, Chisari A, Lombardo R, Bonaccorso R, Latteri S, Di Stefano A, Russello D. Laparo-endoscopic “Rendezvous” to treat cholecysto-choledocolithiasis: Effective, safe and simplifies the endoscopist’s work. *World J Gastroenterol* 2008; 14(18): 2844-2850 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2844.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2844>

### INTRODUCTION

Cholecystolithiasis is one of the most common diseases in which treatment involves general practitioners, gastroenterologists, endoscopists and general surgeons. The management of patients affected by gallstones complicated by common bile duct (CBD) stones and/or problems at the papilla of Vater is anyway a challenge as there are many available options for treatment, all being effective but with the best practice is still unknown. The development of minimally invasive surgery has been associated with development and diffusion of totally laparoscopic exploration of the CBD which is considered the best approach by the pioneers of minimally invasive surgery. This convention contrasts with the fact that the sequential approach, combining the endoscopic retrograde cholangio-pancreatography (ERCP) with endoscopic



sphincterotomy (ES) prior or after cholecystectomy, was for a long time considered to be the preferable choice and also today many gastroenterologists, endoscopists and surgeons still prefer it in clinical practice. The third option, namely the laparo-endoscopic “Rendezvous” (RV) combines in one procedure laparoscopic cholecystectomy (LC), intra-operative cholangiography (IOC) and endoscopic CBD clearance being an actual alternative to the other two. This laparo-endoscopic approach is for the surgeon technically simpler than total laparoscopy, carries interesting advantages but is more complex to organize and is perhaps also considered to reduce the role of the laparoscopic surgeon. Also for the endoscopist this procedure could carry many advantages. Believing in its usefulness we used this approach in 80 consecutive patients affected by gallstones and CBD stones and here we report the results concerning effectiveness, safety and for the first time in the literature we particularly analysed the agreement, evaluation and opinion of the endoscopist normally used to perform ERCP-ES.

## MATERIALS AND METHODS

Eighty patients affected by cholecystolithiasis and CBD stones or suspected stones at IOC were treated simultaneously during the same operation by patient tailored combined laparo-endoscopic “RV”. Our definition of the term “RV” consists of the combined laparo-endoscopic simultaneous approach involving the endoscopist and surgeon in the operating room during one single administration of anesthesia to clear CBD stones or solve associated problems related to sludge or other problems at the papilla of Vater. All the records of the treated patients were analyzed concerning main clinical data, history, diagnosis, surgical or endoscopic technical particularities, duration of the procedures, feasibility, failure, conversion to open surgery, effectiveness in stone clearance, post-operative complications, duration of the hospital stay, late complications and recurrence of stones. At the end of every procedure we submitted a questionnaire to the endoscopist to analyze his satisfaction concerning the intraoperative endoscopic procedure. The questionnaire elicited a simple immediate opinion of the endoscopist concerning his evaluation of the endoscopic difficulty of the procedure graded as: (1) simpler; (2) comparable; (3) more difficult, if compared to a standard ERCP-ES.

## RESULTS

### *Clinical data*

From 2002 to 2006, 80 consecutive patients were submitted to the laparo-endoscopic RV. The mean patient age was 58 years (9-88 years), 29 patients were male and 51 female. The youngest patient was a 9-year-old female patient with a BMI of 27, with gallstones and recurrence of acute biliary pancreatitis due to common bile duct sludge and sphincter of Oddi dysfunction. The oldest patient was a female patient with increasing jaundice, severe cholangitis, and CBD stones impacted in the papilla and coming out of two failed attempts to treat the jaundice and the

stones with ERCP-ES. The diagnosis of CBD stones was given preoperatively in 47 patients (58.7%), in 33 patients (41.2%) the diagnosis was not available preoperatively. 23 patients (28.7%) had a recent history of mild acute pancreatitis or abnormal increase of amylase or lipase levels associated with biliary pain. In 49 patients (61.2%) there was an abnormal increase of bilirubin levels. In 53 patients (66.2%) suspicion or the diagnosis of CBD stones was obtained by ultrasonography. In 46 patients (57.5%) a cholangio-MRI was also performed preoperatively and in 38 of them (82.6%) this confirmed the diagnosis. In 24 patients (30%) with CBD stones a CT scan was also performed during diagnostic work-up but this confirmed the stones in only 9 patients (37.5%). In fact, in 23 of the 33 patients without preoperative diagnosis (69.6%) there was preoperatively a high clinical suspicion of CBD stones but in 10 (31.1%) the diagnosis could be obtained only during surgery by IOC performed because of the surgeon's own decision based on the above mentioned criteria. All 80 patients therefore underwent antretrograde transcystic IOC. The criteria used to perform IOC were the diagnosis of CBD stones or the positiveness of at least one of the following risk factors: history of acute pancreatitis, pre-operative abnormal increase of direct bilirubinemia, ALP or gamma-GT, abnormally dilated CBD at ultrasonography, multiple small stones or sludge in the gallbladder, intraoperative evidence of a large cystic duct or an enlarged CBD. The feasibility of the RV was 97.5%.

### *Technical data*

None of these 80 patients underwent surgical open or laparoscopic CBD exploration. Only in one patient a transcystic biliary drain (Pedinelli) was left at the end of the procedure because the inexperienced endoscopist inflated too much air in the bowel during the endoscopic procedure preventing the surgeon from having a good view of the cystic duct to clip it safely. Conversion to open surgery occurred in 2 cases (2.5%) because of surgical problems related to adhesences due to previous abdominal surgery that rendered the identification of the biliary anatomy difficult. In these two patients the clearance of the CBD was performed by the endoscopist anyway and therefore the procedure remained a RV with open cholecystectomy. Intraoperative cholangiography confirmed CBD stones in 68 patients (85%) and these could be extracted in all cases with stone clearance obtained in 100% of the cases. In 15 patients (18.7%) a delayed contrast medium passage ( $> 30$  min from the injection) was also observed during IOC and resolved by ES. In 12 of the patients (15%) cholangiography did not clearly show stones but ES was performed anyway because of a pre-operative diagnosis of stones, clinical history of recurrent pancreatitis, hyperbilirubinemia or because of CBD dilatation and delay in contrast medium discharge in the duodenum. In 5 of these patients with abnormal delay, the ES could not identify stones in the sudden relevant bile flow after sphincterotomy but in 3, biliary sludge was evident. In 2 of these the ES did not show stones and the images were interpreted as false positives due to air bubbles in the CBD. In only 19 patients (23.7%)

**Table 1** Main differences for the endoscopist between the main steps of endoscopic procedures at the papilla of Vater for CBD exploration comparing RV with standard ERCP-ES

Factors of difference of the endoscopic procedure	RV	ERCP-ES
(1) Position of the patient	Supine Rarely more difficult endoscopy	Lateral Preferred because of habit
(2) Cholangiography	Antegrade transcystic Positive (time reduction)	Retrograde Negative
(3) Wirsung injection (chemical damage)	Absent Positive (risk reduction)	Possible Negative
(4) Ductal hyperpression (physical damage)	Absent Positive (risk reduction)	Frequent Negative
(5) Guide wire help for papilla cannulation	Transcystic Positive (time reduction)	Retrograde Negative
(6) Flushing of the CBD	Antegrade during basket retrieval (synergic) Positive (time reduction)	Retrograde Not synergic
(7) Papilla manipulation causing oedema or Oddi's spasm	Limited or absent Positive (risk reduction)	Frequent Negative
(8) Precut of the Papilla	Absent Positive (risk reduction)	Possible Negative
(9) Reduction of the steps of endoscopic procedure	Possible Positive (time reduction)	Uncommon Negative
Total of positive factors	8/9	1/9

there was the need to pass a guide wire transcystically to help the endoscopist to cannulate the papilla while in the other 61 patients the endoscopist cannulated the papilla without need of surgical help. In the only pediatric case a SOD (sphincter of Oddi dysfunction) was associated with cholecystolithiasis and was successfully managed with pneumatic papilla dilatation. In 5 cases (6.2%) the endoscopist used a mechanical lithotripter to shatter larger stones to facilitate their extraction. In one case with suspected CBD stones at IOC the ES and CBD exploration resulted negative for stones, and this was the only endoscopic procedure considered retrospectively to be an over treatment but unfortunately it was supported by a positive preoperative cholangio-MRI together with multiple risk factors of CBD stones.

### Complications

Only one case of intraoperative complications occurred (1.2%) with self limiting bleeding of the papilla after sphincterotomy. In 7 patients (8.7%) there was an increase of amylase levels after the RV but only in 3 (3.7%) it was a real pathologic increase (3X n.v). Of these 7 patients with an increase of amylase levels 5 (71.4%) had had a retrograde transpapillary injection of the contrast medium. This occurred because they were all patients in the initial group (up to the 19th case) in which the endoscopist was used to injecting in retrograde because of the convention and in one later case the retrograde injection was needed because of accidental intra-operative mobilization of the cholangiography catheter out of the cystic duct. The mean duration of the whole RV was 114 min (49-221 min) whereas the mean duration of the intraoperative endoscopic procedures was 14 min (range 6-33 min). The mean postoperative hospital stay of the patients was 3.8 d, thus 1 d longer than our standard simple LC. All these patients were followed for almost 6 mo and all were symptom free up to the last follow-up. All patients had a bile duct in the normal range at the 6 mo US

control and all without clinical evidence of recurrence of stones or cholangitis. Only in one patient (1.2%) an asymptomatic recurrence of a common bile duct stone was diagnosed 13 mo after RV. This was a patient with a known incomplete ES because of intraoperative bleeding, which led us to suspend the endoscopic procedure. No patient developed jaundice or hyperbilirubinemia (0%) and no patient developed symptoms related to ERCP or acute pancreatitis. Oral feeding started in all patients within 36 h. No patient had to be treated post-operatively for incomplete clearance of the CBD with a post-operative ERCP or ES. We collected the data regarding the endoscopist's opinion concerning the intraoperative endoscopic procedure.

### Endoscopist's opinion

In 65 cases (81.2%) the endoscopist considered the endoscopic procedure simpler, in 12 cases (15%) indifferent if compared to a normal ERCP-ES and in only 3 cases (3.7%) he considered the procedure more difficult, especially because of the problems in papilla cannulation due to the different positions related to the supine position of the patient on the operating table. The main factors that were considered important to facilitate the endoscopic procedure are summarized in Table 1. When considering all the factors, avoidance of some of the steps of standard endoscopic procedures can lead to an overall reduction of time and main risk factors in iatrogenic damage.

## DISCUSSION

In all other papers concerning the laparo-endoscopic RV mostly published by surgeons<sup>[1-8]</sup>, there never was an analysis of the problems related to the main technical factors of the endoscopic procedure, nor were the compliance and explanations from the point of view of the endoscopist considered. This is in our opinion a relevant lacking in the analysis of this particular procedure,

**Table 2** Main indications for the laparo-endoscopic RV with evaluation of the factors that suggest its preferability instead of the other treatment's options

Main indications for the laparo-endoscopic RV	RV preferable vs laparoscopic CBD exploration	RV preferable vs sequential ERCP-ES
(1) Common bile duct stones not easily extractable through the cystic duct Positive factor -> (time reduction)	(A) Need of higher surgical skill (B) Longer operation time (C) Need of biliary drain	(a) Risk of synchronization (b) Risk of unnecessary ERCP (c) Risk of difficult retrograde cannulation
(2) Multiple small CBD stones and large friable stones Positive factor -> (reduction of risk of recurrence)	A, B, C + (D) High risk of residual fragments and recurrence	a, b, c
(3) Any type of CBD stones with delayed passage of the contrast medium during IOC or T-tube-IOC after laparoscopic CBD exploration Positive factor -> (reduction of risk of recurrence)	A, B, C, D + (E) high risk of undertreatment of chronic papillitis and of maintenance of underlying causes	a, b, c
(4) CBD stones with previous cholangitis Positive factor -> (reduction of risk of recurrence)	A, B, C, D + (E) high risk of maintenance of underlying causes at the papilla	a, b, c + (d) Avoidance of contrast medium injection with risk of recurrence of cholangitis
(5) CBD stones after recurrent acute biliary pancreatitis or hyperbilirubinemia Positive factor -> (iatrogenic risk reduction)	A, B, C, D, E	a, b, c, d + (e) risk of recurrence of ERCP related acute pancreatitis
(6) Known or unsuspected Sphincter of Oddi Dysfunction, cholecysto-lithiasis with or without CBD stones Positive factor -> (iatrogenic risk reduction)	A, B, C, D, E	a, b, c, d, e
(7) CBD stones and/or abovementioned problems in patients with Billroth II during open cholecystectomy Positive factor -> (iatrogenic risk reduction)	A, B, C, D, E + (F) Manual drive of the endoscope by the surgeon in the afferent jejunal loop	a, b, c, d, e + (f) more difficult ERCP
(8) CBD stones, SOD, acute pancreatitis in children/CBD stones in patients with normal or thin CBD Positive factor -> (iatrogenic risk reduction)	A, B, C, D, E + (G) difficult laparoscopic CBD exploration and risk of stenosis of the suture	a, b, c, d, e, f + (h) avoidance of sphincterotomy in children
(9) CBD stones and/or SOD after failure of preoperative ERCP-ES or recurrence of acute biliary pancreatitis Positive factor -> (iatrogenic risk reduction)	A, B, C, D, E	a, b, c, d, e, f
(10) Inexperienced surgeon for laparoscopic CBD exploration Positive factor -> (iatrogenic risk reduction)	A, B, C, D, E, G	a, b, c, d, e, f

for which we tried to make aware to both the surgeon and endoscopist concerning its utility as mandatory for its immediate outcome and for its development. On the other hand, it was impossible to plan a prospective randomized study as every case is different and the endoscopist could never treat the same patient with RV and also ERCP-ES so that the evaluation could be based exclusively on the endoscopist's subjective opinion. We would like to underline that our definition is somewhat different because we define a laparo-endoscopic RV also when the guide wire is not passed, in contrast to others that consider a RV only when the surgeon passes a guide wire through the cystic duct to help the endoscopist<sup>[1-3]</sup>. We do not believe that the guide wire has to be passed in every case but, to avoid unnecessary iatrogenic risks, only if needed especially when the endoscopist cannot easily cannulate the papilla. Certainly this combined laparo-endoscopic approach has its main negative factor as the need for synchronized collaboration between surgeon and endoscopist and this is still, not only in our opinion, the main factor that limits its diffusion<sup>[4,5]</sup>. Cholecystolithiasis is worldwide a very common illness that involves general practitioners, gastroenterologists, and surgeons but also frequently the endoscopist particularly if the presence or the simple suspicion of stones in the CBD becomes the main problem. All the cases where the CBD stones are

easily extractable through the cystic duct by the surgeon during LC should be excluded in this discussion but unfortunately this evaluation is never possible prior to surgery so that a rational plan of all treatment options is mandatory. Especially when transcystic extraction of stones is not possible, as happens in about 30%-40% of the patients with CBD stones, the combined laparo-endoscopic approach should be considered often in our opinion, as the preferable option for many simple technical and clinical reasons. These reasons are summarized in Table 2 that shows our main indications explaining the points of preferability of the RV compared to other options. Some other general considerations have to be met as well. First, the treatment of CBD stones in cholecystectomized patients today remains the exclusive work of the endoscopist who often performs a salvage procedure for the patient and helps the surgeon as well<sup>[9-11]</sup>. This endoscopic approach is highly effective<sup>[12]</sup> with sporadic mortality and minimal early and late morbidity, all anyway lower when compared to surgical interventions. The surgical approach is therefore never proposed today as a first option in cholecystectomized patients and moreover the endoscopic treatment with ERCP-ES is never considered wrong or dangerous for these patients. If cholecystectomy still has to be performed, with the combined approach RV, the surgeon can help the

endoscopist or otherwise the endoscopist can help the surgeon to clear the CBD, it only depends on whose point of view is considered. The questionable risk of an “avoidable ERCP-ES” curiously appears only if LC has to be performed. According to our results, the endoscopist’s opinion and literature results, ERCP-ES with help seems to be easier for the endoscopist so that radiologic-endoscopic rendezvous was also used<sup>[13,14]</sup>. On the other hand, CBD clearance during LC with help of the endoscopist also seems easier for the surgeon as all the surgeons using the RV were always satisfied, never reporting results or aspects that lead them to abandon it<sup>[1-8,15,16]</sup>. These patients are all treated in an in-patient hospital setting so normally both surgeon and endoscopist are available and the other mandatory factors to gain the organization of a RV are functioning clocks and telephones to coordinate them. Sometimes the surgeon is also able to perform an endoscopy and in this case he can himself complete the auto-RV. The RV solves both cholecystolithiasis and CBD stones but it can especially avoid the main negative technical aspects of both laparoscopic CBD exploration and of the standard sequential ERCP-ES. Close to the technical reasons shown in our original analysis there are also several clinical reasons and evidences in the literature to use it. The development of minimally invasive surgery with the diffusion of totally laparoscopic exploration of CBD leads many authors to consider it the best option<sup>[17-19]</sup>. This concept is somewhat strained as it is certain that the sequential approach was considered for a long time to be the preferable one<sup>[20]</sup> and even today gastroenterologists, endoscopists and many surgeons still continue to prefer it in the clinical practice<sup>[21]</sup>. In fact, a recent NIH state of the science statement on ERCP showed that both ERCP and laparoscopic CBD clearance are safe and reliable to clear stones<sup>[22,23]</sup>. This statement includes the concept that the simultaneous combination of laparoscopy and ERCP-ES should be considered equally safe and reliable. If this statement concerning ERCP is true, the sequential approach is also reliable and effective so that the RV should theoretically be better, especially for the patient, because for the sequential approach there is the well known old problem of optimal timing between ERCP and LC, to also be considered “The bilateral interface...” between the two procedures<sup>[24]</sup> is eliminated in the simultaneous RV. The RV especially avoids the risks of ineffectiveness of both the pathways of the sequential approach related especially to no synchronization between diagnosis of CBD stones and its treatment. This concept of no synchronization is very important as only during RV the IOC shows the real-time situation of the CBD and moreover other relevant and often unsuspected underlying problems at the papilla. With the sequential approach it could be a renewed passage of stones in the CBD if the ERCP-ES is performed prior to cholecystectomy but moreover ERCP-ES could be unnecessary if performed after LC as stones can often pass spontaneously<sup>[25]</sup>. These risks are confirmed by a recent analysis of management of suspected CBD stones in children<sup>[26]</sup> that clearly shows the poor results and pitfalls of the two common sequential pathways of ERCP-ES. If performed prior to LC a total

of 71% of ERCP were unnecessary and 7% failed. If performed after LC 50% of ERCP were unnecessary because stones were no longer found. Therefore, a large number of unnecessary invasive procedures, all potentially related to morbidity and mortality, are performed but often the majority are ineffective especially because they are out of synch with the evolving pathophysiology of gallstone disease<sup>[25]</sup>. Comparing the RV to totally laparoscopic CBD exploration, it is clear that the RV also solves problems at the papilla of Vater that certainly can not always be solved by laparoscopic CBD exploration and which are the main causes of retained stones and recurrence. Another important problem of the choice of the best option appears in those cases with an unclear IOC with uncertain images of stones or persistence or delayed contrast medium passage in the duodenum. In these unclear situations both transcystic laparoscopic CBD exploration and sequential ERCP-ES are questionable as often related to dense biliary sludge associated with stenosis of the papilla in which ES is certainly the safest solution for the patient. The laparoscopic CBD exploration normally consists of major biliary surgery and needs higher laparoscopic skills and prolongs the duration of the entire operation also increasing the overall risk for the patient<sup>[18,19,27]</sup>. The mean prolongation of the time of RV is normally shorter than those reported for totally laparoscopic CBD exploration<sup>[27,28]</sup>. This difference becomes more evident for those patients with multiple large stones and sludge and problems at the papilla. The laparoscopic CBD exploration in a recent review carries a conversion rate of 2%-8%, a failure of 3.1%. These unlucky patients have to be treated by the endoscopist in a sequential manner with a risk of ineffectiveness<sup>[28]</sup>. Retained stones after laparoscopic CBD exploration in very experienced hands are also up to 8% and a biliary drainage after surgical transverse choledochotomy is needed in up to 94% of the patients so that there is a need for repeated controls, prolonged hospitalization and readmittances<sup>[29]</sup>. The success rate of laparoscopic duct exploration in a review of 28 papers from 1993 to 2000 was between 81% and 98% (mean of 88.4%) with an incidence of retained stones between 0% and 19% and a conversion rate up to 10%. On the contrary in a large review concerning the patients free of biliary symptoms after ERCP-ES this was up to 90% after 14.2 years<sup>[28]</sup>. The randomized multicenter EAES trial showed a therapeutic success of 84% for the ERCP-ES and a success rate of 83% for the laparoscopic exploration<sup>[10]</sup>. The patients matched for simple cholecystectomy reported significantly lower morbidity compared to laparoscopic CBD treatment and biliary complications are up to 16% of the patients, mostly due to the need of biliary drains<sup>[30]</sup>. The reduction and facilitation of the steps of the endoscopic procedure due to the surgeon’s help of the endoscopist finally brings a relevant reduction of the time of the endoscopic procedure. Moreover the post-procedural hyperamylasemia and acute pancreatitis are strongly reduced or absent after RV if compared to standard ERCP<sup>[6,8,16]</sup> and this is principally related to avoidance of the risk factors reported in Table 1. These factors are those clearly related to the incidence and mechanism of post-ERCP pancreatitis in



different analyses<sup>[31-34]</sup>. It is also remarkable that after laparoscopic CBD exploration, acute pancreatitis can be comparably high to sequential ERCP-ES (7.3% *vs* 8.8%)<sup>[12]</sup>. Certainly standard ERCP whenever possible should be limited because, as suggested by experienced endoscopists the only sure way to avoid post-ERCP complications is to avoid ERCP itself<sup>[35]</sup>. The unique prospective randomized comparison of ERCP-ES with laparo-endoscopic RV by Morino<sup>[8]</sup> showed that the risk of incomplete duct clearance with ERCP-ES is 20% and in 77.7% of the cases the cause is the inability to cannulate the papilla. This confirms the main advantage of the RV for the endoscopist, namely the surgeon's help passing the guide wire transcystically. The same study shows that 88% of these ineffective ERCP-ES were brought to an effective intraoperative ERCP-ES during laparoscopic RV, and this happened also in one patient in our series. Simplifying the concept the endoscopist alone was not able to solve the problem but on the contrary, together with the surgeon was effective, confirming the value of the RV.

Our analysis concerning endoscopist's compliance and opinion concerning the RV procedure shows clearly, the very good results concerning feasibility, effectiveness and safety and shows also that in the majority of the cases he was happy and satisfied to perform the endoscopic procedure intra-operatively. This is very simple to understand because the endoscopist is involved only if absolutely needed reducing in this manner both the risk of endoscopic over-treatment and also the risk of failure. Moreover the endoscopist avoids many steps of the procedure that are to his mind the main risk of iatrogenic damage, he is facilitated to easily understand his position inside the CBD, the surgeon can help him with the guide wire or immediately converting to laparoscopic CBD exploration or to open surgery in case of major problems like impacted stone or blockage of the basket inside the CBD. These positive factors for the endoscopist are also positive for the patient, reducing the risk of repeated or unnecessary procedures, the risk of ineffectiveness altogether, and reducing in our opinion the "cumulated iatrogenic risk" if compared to both other options. For all these reasons our positive clinical experience from the point of view of the surgeon and especially of the endoscopist suggests that this combined laparo-endoscopic simultaneous rendezvous approach, despite some, in our opinion questionable, organizational problems can often result in being the preferable treatment option for the patient with cholecystolithiasis and CBD stones.

## COMMENTS

### Background

Cholecystolithiasis is one of the most common diseases in which treatment involves general practitioners, gastroenterologists, endoscopists and general surgeons but the management of patients affected by gallstones complicated by common bile duct (CBD) stones and/or problems at the papilla of Vater is anyway a challenge as there are many available options for treatment, all being effective. A third alternative to the two main treatment's option, sequential or totally laparoscopic, consists of the simultaneous laparoendoscopic "Rendezvous (RV)". This study analyzed the results concerning 80 consecutive patients treated with a laparoendoscopic rendezvous procedure and especially analyzed the previously unanalyzed endoscopist's opinion which is also a very important point for this combined approach.

### Research frontiers

The gold standard for the treatment of cholecystolithiasis combined with CBD stones is still not available. Large prospective randomized studies could help to clarify the issue but the literature shows that the cooperation between surgeons and endoscopists is usually scarce and that each specialist prefers what he believes is better.

### Innovations and breakthroughs

Using the laparoendoscopic rendezvous approach for the treatment of gallstones and CBD stones both feasibility (97.5%) and effectiveness (100%) in stone clearance are very high while morbidity and stone recurrence are low. These results are comparable or even better than those reported for both the other two treatment's options. Moreover the work of the endoscopists results is simpler and safer than standard ERCP-ES in 4/5 of the cases, because of the reduction of the steps of the endoscopic procedure and the avoidance of relevant iatrogenic risk factors.

### Applications

This study shows that the simultaneous laparoendoscopic rendezvous approach is often preferable for the patient, the surgeon, the endoscopist and the hospital. This approach reduces hospital stay and the iatrogenic risk compared to the sequential approach and reduces the need of higher surgical skills and biliary drainage compared to the total laparoscopic treatment. The main problems that still limit the diffusion of this procedure are the problems in organizing the mandatory cooperation between endoscopist and surgeon.

### Terminology

Sequential treatments combine ERCP clearance of the CBD stones with previous or successive laparoscopic cholecystectomy. The laparoendoscopic treatment consists of laparoscopic cholecystectomy (LC) combined with endoscopic treatment of stones or underlying problems at the papilla of Vater during a unique anesthesia. The term "RV" means the meeting of a guide wire (the surgeon passes the guide wire antegradely through the cystic duct) with the endoscope inside the duodenum; the presence of the guide wire and the meeting of the two instruments facilitate the cannulation of the Vater's papilla by the endoscopist himself and so also the clearance of the CBD from the stones. The term RV underlines the concept of "reciprocal implementation" of both surgeon's and endoscopist's work. The laparoendoscopic treatment does not automatically include a rendezvous procedure itself and this brings confusion in the evaluation of the results of the published papers.

### Peer review

The authors described excellent results of the laparoendoscopic rendezvous, without the use of a statistical method.

## REFERENCES

- 1 **Basso N**, Pizzuto G, Surgo D, Materia A, Silecchia G, Fantini A, Fiocca F, Trentino P. Laparoscopic cholecystectomy and intraoperative endoscopic sphincterotomy in the treatment of cholecysto-choledocholithiasis. *Gastrointest Endosc* 1999; **50**: 532-535
- 2 **Cemachovic I**, Letard JC, Begin GF, Rousseau D, Nivet JM. Intraoperative endoscopic sphincterotomy is a reasonable option for complete single-stage minimally invasive biliary stones treatment: short-term experience with 57 patients. *Endoscopy* 2000; **32**: 956-962
- 3 **Iodice G**, Giardiello C, Francica G, Sarrantonio G, Angelone G, Cristiano S, Finelli R, Tramontano G. Single-step treatment of gallbladder and bile duct stones: a combined endoscopic-laparoscopic technique. *Gastrointest Endosc* 2001; **53**: 336-338
- 4 **Wright BE**, Freeman ML, Cumming JK, Quickel RR, Mandal AK. Current management of common bile duct stones: is there a role for laparoscopic cholecystectomy and intraoperative endoscopic retrograde cholangiopancreatography as a single-stage procedure? *Surgery* 2002; **132**: 729-735; discussion 735-737
- 5 **Meyer C**, Le JV, Rohr S, Duclos B, Reimund JM, Baumann R. Management of common bile duct stones in a single operation combining laparoscopic cholecystectomy and peroperative

- endoscopic sphincterotomy. *J Hepatobiliary Pancreat Surg* 2002; **9**: 196-200
- 6 **Lella F**, Bagnolo F, Rebuffat C, Scalambra M, Bonassi U, Colombo E. Use of the laparoscopic-endoscopic approach, the so-called "rendezvous" technique, in cholecystocholedocholithiasis: a valid method in cases with patient-related risk factors for post-ERCP pancreatitis. *Surg Endosc* 2006; **20**: 419-423
  - 7 **Enochsson L**, Lindberg B, Swahn F, Arnelo U. Intraoperative endoscopic retrograde cholangiopancreatography (ERCP) to remove common bile duct stones during routine laparoscopic cholecystectomy does not prolong hospitalization: a 2-year experience. *Surg Endosc* 2004; **18**: 367-371
  - 8 **Morino M**, Baracchi F, Miglietta C, Furlan N, Ragona R, Garbarini A. Preoperative endoscopic sphincterotomy versus laparoendoscopic rendezvous in patients with gallbladder and bile duct stones. *Ann Surg* 2006; **244**: 889-893; discussion 893-896
  - 9 **Park AE**, Mastrangelo MJ Jr. Endoscopic retrograde cholangiopancreatography in the management of choledocholithiasis. *Surg Endosc* 2000; **14**: 219-226
  - 10 **Cuschieri A**, Lezoche E, Morino M, Croce E, Lacy A, Toouli J, Faggioni A, Ribeiro VM, Jakimowicz J, Visa J, Hanna GB. E.A.E.S. multicenter prospective randomized trial comparing two-stage vs single-stage management of patients with gallstone disease and ductal calculi. *Surg Endosc* 1999; **13**: 952-957
  - 11 **Borie F**, Fingerhut A, Millat B. Acute biliary pancreatitis, endoscopy, and laparoscopy. *Surg Endosc* 2003; **17**: 1175-1180
  - 12 **Nathanson LK**, O'Rourke NA, Martin IJ, Fielding GA, Cowen AE, Roberts RK, Kendall BJ, Kerlin P, Devereux BM. Postoperative ERCP versus laparoscopic choledochotomy for clearance of selected bile duct calculi: a randomized trial. *Ann Surg* 2005; **242**: 188-192
  - 13 **Soehendra N. H.** Joachim Burhenne Lecture. Common areas of interest between interventional biliary radiology and endoscopy. *AJR Am J Roentgenol* 1995; **164**: 547-551
  - 14 **Maetani I**, Hoshi H, Ohashi S, Yoshioka H, Sakai Y. Cholangioscopic extraction of intrahepatic stones associated with biliary strictures using a rendezvous technique. *Endoscopy* 1993; **25**: 303-306
  - 15 **Cavina E**, Franceschi M, Sidoti F, Goletti O, Bucciatti P, Chiarugi M. Laparo-endoscopic "rendezvous": a new technique in the choledocholithiasis treatment. *Hepatogastroenterology* 1998; **45**: 1430-1435
  - 16 **La Greca G**, Barbagallo F, Di Blasi M, Di Stefano M, Castello G, Gagliardo S, Latteri S, Russello D. Rendezvous technique versus endoscopic retrograde cholangiopancreatography to treat bile duct stones reduces endoscopic time and pancreatic damage. *J Laparoendosc Adv Surg Tech A* 2007; **17**: 167-171
  - 17 **Lauter DM**, Froines EJ. Laparoscopic common duct exploration in the management of choledocholithiasis. *Am J Surg* 2000; **179**: 372-374
  - 18 **Lezoche E**, Paganini AM. Technical considerations and laparoscopic bile duct exploration: transcystic and choledochotomy. *Semin Laparosc Surg* 2000; **7**: 262-278
  - 19 **Paganini AM**, Guerrieri M, Sarnari J, De Sanctis A, D'Ambrosio G, Lezoche G, Lezoche E. Long-term results after laparoscopic transverse choledochotomy for common bile duct stones. *Surg Endosc* 2005; **19**: 705-709
  - 20 **Chan AC**, Chung SC, Wyman A, Kwong KH, Ng EK, Lau JY, Lau WY, Lai CW, Sung JJ, Li AK. Selective use of preoperative endoscopic retrograde cholangiopancreatography in laparoscopic cholecystectomy. *Gastrointest Endosc* 1996; **43**: 212-215
  - 21 **Costi R**, DiMauro D, Mazzeo A, Boselli AS, Contini S, Violi V, Roncoroni L, Sarli L. Routine laparoscopic cholecystectomy after endoscopic sphincterotomy for choledocholithiasis in octogenarians: is it worth the risk? *Surg Endosc* 2007; **21**: 41-47
  - 22 **NIH state-of-the-science statement on endoscopic retrograde cholangiopancreatography (ERCP) for diagnosis and therapy.** *NIH Consens State Sci Statements* 2002; **19**: 1-26
  - 23 **Martin DJ**, Vernon DR, Toouli J. Surgical versus endoscopic treatment of bile duct stones. *Cochrane Database Syst Rev* 2006; CD003327
  - 24 **Esber EJ**, Sherman S. The interface of endoscopic retrograde cholangiopancreatography and laparoscopic cholecystectomy. *Gastrointest Endosc Clin N Am* 1996; **6**: 57-80
  - 25 **Gigot JF**. [Current diagnostic and therapeutic approach to common bile duct calculi: a rapidly evolving field] *Ann Chir* 1998; **52**: 161-165
  - 26 **Mah D**, Wales P, Njere I, Kortan P, Masiakos P, Kim PC. Management of suspected common bile duct stones in children: role of selective intraoperative cholangiogram and endoscopic retrograde cholangiopancreatography. *J Pediatr Surg* 2004; **39**: 808-812; discussion 808-812
  - 27 **Tokumura H**, Umezawa A, Cao H, Sakamoto N, Imaoka Y, Ouchi A, Yamamoto K. Laparoscopic management of common bile duct stones: transcystic approach and choledochotomy. *J Hepatobiliary Pancreat Surg* 2002; **9**: 206-212
  - 28 **Thompson MH**, Tranter SE. All-comers policy for laparoscopic exploration of the common bile duct. *Br J Surg* 2002; **89**: 1608-1612
  - 29 **Paganini AM**, Guerrieri M, Sarnari J, De Sanctis A, D'Ambrosio G, Lezoche G, Perretta S, Lezoche E. Thirteen years' experience with laparoscopic transcystic common bile duct exploration for stones. Effectiveness and long-term results. *Surg Endosc* 2007; **21**: 34-40
  - 30 **Tranter SE**, Thompson MH. Comparison of endoscopic sphincterotomy and laparoscopic exploration of the common bile duct. *Br J Surg* 2002; **89**: 1495-1504
  - 31 **Testoni PA**. Why the incidence of post-ERCP pancreatitis varies considerably? Factors affecting the diagnosis and the incidence of this complication. *JOP* 2002; **3**: 195-201
  - 32 **Pezzilli R**, Romboli E, Campana D, Corinaldesi R. Mechanisms involved in the onset of post-ERCP pancreatitis. *JOP* 2002; **3**: 162-168
  - 33 **Vandervoort J**, Soetikno RM, Tham TC, Wong RC, Ferrari AP Jr, Montes H, Roston AD, Slivka A, Lichtenstein DR, Ruymann FW, Van Dam J, Hughes M, Carr-Locke DL. Risk factors for complications after performance of ERCP. *Gastrointest Endosc* 2002; **56**: 652-656
  - 34 **Masci E**, Mariani A, Curioni S, Testoni PA. Risk factors for pancreatitis following endoscopic retrograde cholangiopancreatography: a meta-analysis. *Endoscopy* 2003; **35**: 830-834
  - 35 **Fogel EL**. Endoscopic retrograde cholangiopancreatography topics. *Endoscopy* 2003; **35**: 913-919

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## N-acetyl-L-cysteine combined with mesalamine in the treatment of ulcerative colitis: Randomized, placebo-controlled pilot study

Luis G Guijarro, Jose Mate, Javier P Gisbert, Jose Luis Perez-Calle, Ignacio Marín-Jimenez, Encarna Arriaza, Tomás Olleros, Mario Delgado, Maria S Castillejo, David Prieto-Merino, Venancio Gonzalez Lara, Amado Salvador Peña

Luis G Guijarro, Mario Delgado, Maria S Castillejo, David Prieto-Merino, Department of Biochemistry and Molecular Biology, CIBERehd, Alcalá University, Alcalá de Henares, Madrid, Spain

Jose Mate, Javier P Gisbert, Gastroenterology Unit, CIBERehd La Princesa University Hospital, Autonomous University, Madrid, Spain

Jose Luis Perez-Calle, Ignacio Marín-Jimenez, Venancio Gonzalez Lara, Gastroenterology Unit, Gregorio Marañón University Hospital, Complutense University, Madrid, Spain

Encarna Arriaza, Tomás Olleros, Group Farmasierra SL, Ctra N-II Km 26.200, San Sebastián de los Reyes, Madrid, Spain

Amado Salvador Peña, Laboratory of Immunogenetics, Department of Pathology, VU University Medical Center, Amsterdam, The Netherlands

**Author contributions:** Guijarro LG designed the study, carried out the statistical analysis and wrote the first draft of the manuscript; Mate J compiled the data patients; Gisbert JP helped write and correct the paper; Perez-Calle JL compiled the data patients; Marín-Jimenez I compiled the data patients; Arriaza E organized patient data; Olleros T organized patient data; Delgado M did ELISA assays; Castillejo MS did ELISA assays and GSH measurement; Prieto-Merino D carried out statistical data; Gonzalez Lara V compiled the data patients; Peña AS helped write, corrected and supervised the organization process.

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**Correspondence to:** Luis G Guijarro, PhD, Unidad de Toxicología Molecular Hepática, Department de Bioquímica & Biología Molecular, Universidad de Alcalá, E-28871, Alcalá de Henares, Spain. [luis.gonzalez@uah.es](mailto:luis.gonzalez@uah.es)

Telephone: +34-918854865 Fax: +34-918854585

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### Abstract

**AIM:** To evaluate the effectiveness and safety of oral N-acetyl-L-cysteine (NAC) co-administration with mesalamine in ulcerative colitis (UC) patients.

**METHODS:** Thirty seven patients with mild to moderate UC were randomized to receive a four-wk course of oral mesalamine (2.4 g/d) plus N-acetyl-L-cysteine (0.8 g/d) (group A) or mesalamine plus placebo (group B). Patients were monitored using the Modified Truelove-

Witts Severity Index (MTWSI). The primary endpoint was clinical remission ( $MTWSI \leq 2$ ) at 4 wk. Secondary endpoints were clinical response (defined as a reduction from baseline in the MTWSI of  $\geq 2$  points) and drug safety. The serum TNF- $\alpha$ , interleukin-6, interleukin-8 and MCP-1 were evaluated at baseline and at 4 wk of treatment.

**RESULTS:** Analysis per-protocol criteria showed clinical remission rates of 63% and 50% after 4 wk treatment with mesalamine plus N-acetyl-L-cysteine (group A) and mesalamine plus placebo (group B) respectively (OR = 1.71; 95% CI: 0.46 to 6.36;  $P = 0.19$ ; NNT = 7.7). Analysis of variance (ANOVA) of data indicated a significant reduction of MTWSI in group A ( $P = 0.046$ ) with respect to basal condition without significant changes in the group B ( $P = 0.735$ ) during treatment. Clinical responses were 66% (group A) vs 44% (group B) after 4 wk of treatment (OR = 2.5; 95% CI: 0.64 to 9.65;  $P = 0.11$ ; NNT = 4.5). Clinical improvement in group A correlated with a decrease of IL-8 and MCP-1. Rates of adverse events did not differ significantly between both groups.

**CONCLUSION:** In group A (oral NAC combined with mesalamine) contrarily to group B (mesalamine alone), the clinical improvement correlates with a decrease of chemokines such as MCP-1 and IL-8. NAC addition not produced any side effects.

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**Key words:** Ulcerative colitis; Interleukin; Mesalamine; N-acetyl-L-cysteine

**Peer reviewers:** Jay Pravda, MD, Inflammatory Disease Research Center, Gainesville, Florida, 32614-2181, United States; Navneet K Ahluwalia, MD, FRCP, MHSM, PhD, Stepping Hill Hospital, Poplar Grove, stockport Sk2 7JE, United Kingdom; Limas Kupcinskis, Professor, Dr, Gastroenterology of Kaunas University of Medicine, Mickeviciaus 9, Kaunas LT 44307, Lithuania

Guijarro LG, Mate J, Gisbert JP, Perez-Calle JL, Marín-Jimenez I, Arriaza E, Olleros T, Delgado M, Castillejo MS, Prieto-Merino D, Gonzalez Lara V, Peña AS. N-acetyl-L-cysteine combined with mesalamine in the treatment of ulcerative colitis: Randomized, placebo-controlled pilot study. *World J Gastroenterol* 2008;

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## INTRODUCTION

Ulcerative colitis (UC) is a chronic idiopathic inflammatory disease of the colon characterized by rectal bleeding, diarrhea, abdominal pain, weight loss and fever<sup>[1]</sup>. Histological examination of biopsy specimens reveals the presence of infiltrated white blood cells such as neutrophils, monocytes and lymphocytes in the colonic interstitium<sup>[2]</sup>. In the recruitment and activation of white blood cells, participate cytokines [interleukin (IL)-1 $\beta$ , IL-2, IL-6, IL-12 and tumour necrosis factor- $\alpha$ ]<sup>[3]</sup>, chemokines [IL-8, monocyte chemoattractant protein (MCP)-1 and MCP-3]<sup>[4]</sup>, cell adhesion molecules<sup>[5]</sup> as well as the inducible isoforms of nitric oxide synthase<sup>[6]</sup> and cyclooxygenase<sup>[7]</sup>. Moreover, the infiltration of phagocytes results in the stimulation of the latent plasma membrane associated NADPH oxidase, which release large amounts of superoxide (O<sub>2</sub><sup>-</sup>) and H<sub>2</sub>O<sub>2</sub>, producing hydroxyl radicals<sup>[8]</sup>.

Recently, a new hypothesis termed "Radical Induction Theory" has been proposed to explain the etiology of UC<sup>[9]</sup>. The excess of un-neutralized hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), produced within colonic epithelial cells as a result of aberrant cellular metabolism, diffuses through cell membranes to the extracellular space, where it is converted to the highly damaging hydroxyl radical (OH) resulting in oxidative damage to structures comprising the colonic epithelial barrier. Once damaged, the barrier is unable to exclude highly immunogenic fecal bacteria invading the normally sterile submucosa. This antigen exposure provokes an initial immune response of white blood cell infiltration into colonic mucosa. The injurious "reactive oxygen species" would be inactivated by protective enzymes (superoxide dismutase, catalase and glutathione peroxidase) and by non-enzymatic antioxidants (glutathione, ascorbate and  $\alpha$ -tocopherol)<sup>[10,11]</sup>. A common feature of patients with inflammatory bowel disease is a depletion of endogenous oxidant defenses such as ascorbate,  $\beta$ -carotene,  $\alpha$ -tocopherol and glutathione<sup>[12,13]</sup>. This last compound is a naturally occurring tripeptide ( $\gamma$ -Glu-Cys-Gly) found in high concentrations within tissues. In experimental acute colitis, cellular glutathione levels decreased significantly and the administration of the antioxidant N-acetyl-L-cysteine (NAC) restored glutathione level and decreased colonic inflammation<sup>[14]</sup>. More recently it has been showed that the combination of NAC and mesalamine accelerates mucosal healing in a rodent model of colitis<sup>[15]</sup>. Taken together, these results suggest an imbalance and inefficient endogenous antioxidant response in the intestinal mucosa of UC patients, which may contribute to the etiology, the pathogenesis and the perpetuation of the inflammatory processes<sup>[10]</sup>. The aim of the present pilot study was to evaluate the possible effectiveness and safety of NAC associated with mesalamine in mild to moderate UC.

## MATERIALS AND METHODS

### Patient's selection criteria and study protocol

Thirty seven patients with mild to moderate UC according to the Modified Truelove and Witts Severity Index (MTWSI)<sup>[16]</sup> were randomized to receive a 4-wk course of oral mesalamine (2.4 g/d) plus N-acetyl-L-cysteine (NAC) (0.8 g/d) (group A) or mesalamine plus placebo (group B). Exclusion criteria were age over 70 or less 18 years, pregnancy, serious underlying systemic diseases and MTWSI > 10. Treatment with oral or topical steroids, topical mesalamine, immunosuppressors (azathioprine, 6-mercaptopurine, *etc.*) or antioxidants was discontinued at least three weeks before commencement of the trial. The protocol was approved by the Ethical Investigation Committee of the corresponding Institutions, and written informed consent was obtained from all the patients. Diagnosis of UC was established by standard clinical, radiological, histological, and endoscopic criteria<sup>[11]</sup>. The patient's characteristics are described in Table 1. Patients were allocated to one of two treatment groups according to a centrally computer-assisted randomization list and received oral mesalamine at a dose of 2.4 g/d combined with NAC at a dose of 0.8 g/d (group A) or oral mesalamine at a dose of 2.4 g/d and placebo (group B) during four wk. NAC was provided as 200 mg sacks of water-soluble powder identical in taste, bulk and appearance to those of placebo. Mesalamine was provided as an 800 mg tablets coated with 1:2 copolymer of metacrylic acid and methylmethacrylate (Eudragit-S) used for colonic delivery of mesalamine<sup>[17]</sup>.

### Measurement of disease activity

Clinical and biochemical findings were assessed by the gastroenterologist at intervals of two and four weeks respectively. All patients were asked to record stool frequency (number of daily stools) and consistency, nocturnal stools, visible blood in stool, fecal incontinence, abdominal pain, abdominal tenderness, need for antidiarrheals and a patient self-rating evaluation based upon the impact of symptoms on normal life activities. For stool frequency and abdominal pain, a scale from 0 (normal) to 4 (markedly abnormal) was used. For use of antidiarrheal medication, a scale from 0 (no) to 1 (yes) was used. For the other parameters, the scale ranged from 0 (normal) to 3 (markedly abnormal). The Modified Truelove-Witts Severity Index, which has been considered useful in therapeutic trials<sup>[16]</sup>, was calculated from these data. The primary endpoint was clinical remission (MTWSI  $\leq$  2) at 4 wk. Secondary endpoints were clinical response (defined as a reduction from baseline in the MTWSI of  $\geq$  2 points) and drug safety.

### Assessment of safety

The hematological and biochemical studies were performed at regular intervals by the analytical laboratory services of the corresponding hospitals: complete blood count, hepatic enzymes, bilirubin, erythrocyte sedimentation rate and C-reactive protein were measured between other biochemical parameters.



Table 1 Basal and demographic data

Data	Group A Mesalamine + NAC (n = 19)	Group B Mesalamine + placebo (n = 18)	Total (n = 37)
Age (mean $\pm$ SD)	51.4 $\pm$ 14	42.2 $\pm$ 13	46.9 $\pm$ 14
White race, n (%)	11 (57.8)	13 (72.2)	24 (64.8)
Smoker, n (%)	2 (10.5)	3 (16.7)	5 (13.5)
Male, n (%)	8 (42.1)	5 (27.8)	13 (35.1)
Basal modified Truelove-Witts severity index (mean $\pm$ SD)	5.95 $\pm$ 2.22	4.61 $\pm$ 2.09	5.30 $\pm$ 2.20

NAC: N-acetyl-L-cysteine.

### Evaluation of reduced glutathione, TNF- $\alpha$ , IL-6, IL-8 and MCP-1 circulating levels

Blood samples were obtained by venipuncture and placed into tubes containing lithium heparin as anticoagulant. For the measurement of GSH in whole-blood samples, 0.5 mL of blood was treated immediately with 0.25 mL of trichloroacetic acid (12%) on ice. After 5 min tubes were centrifuged at 13 000 g during 10 min at 4°C and the acidic supernatants were immediately used for GSH measure. GSH determinations were performed as described previously<sup>[18]</sup> with some modifications. Briefly, the amount of lactoyl-glutathione formed between methylglyoxal (110 mmol/L) and GSH in presence of glyoxalase-I (lactoyl-glutathione lyase) at pH 7.0 buffered with 0.1mmol/L sodium phosphate was measured spectro-photometrically at 240 nm.

The concentration of IL-8, MCP-1, TNF- $\alpha$  and IL-6 present in plasma was determined by using specific sandwich ELISA following manufacturer protocol. Briefly plates (Costar) were coated overnight at 4°C with specific mouse anti-human monoclonal antibody (Becton Dickinson) in 0.1 mol/L Na<sub>2</sub>HPO<sub>4</sub> (pH 9) (dilution 1:200). After washing with PBS containing 0.5% Tween 20 unspecific sites were blocked with PBS containing 3% BSA. Plasma was added to each well and incubated for 12 h at 4°C. Unbound material was discarded and biotinylated mouse anti-human monoclonal antibody (Becton Dickinson) was incubated during 1 h at room temperature. After washing bound antibodies were detected by incubation with avidin-peroxidase (Sigma) for 30 min in presence of the 2,2 azinobis (3-ethybenzthiazolinesulfonic acid) (ABTS of Sigma) as substrate. Absorbance was measured at 405 nm. A Standard curve was constructed for each cytokine or/and chemokines by using recombinant human molecules (Becton Dickinson) in PBS containing 3% BSA.

### Statistical analysis

For quantitative variables, mean and standard deviation were calculated. Statistical analysis was performed with the SAS program. For the comparison of treatment effect on Modified Truelove-Witts Severity Index between both groups of patients, the analysis of covariance (ANCOVA) was used after adjusting for baseline values. To study the evolution of disease score for each group, the analysis of variance (ANOVA) with Bonferroni corrected *post hoc*

Table 2 Changes in disease activity assessed using modified Truelove-Witts Severity Index (mean  $\pm$  SD) in subjects who received the drugs for 2 wk and 4 wk

Week of treatment	0	2	4	ANOVA statistical test (number of patients)
Group A mesalamine plus NAC	5.95 $\pm$ 2.22	4.68 $\pm$ 3.40	3.32 $\pm$ 3.71 <sup>a</sup>	P = 0.046 (n = 19)
Group B mesalamine plus placebo	4.61 $\pm$ 2.09	4.17 $\pm$ 3.85	3.72 $\pm$ 3.91	P = 0.735 (n = 18)

<sup>a</sup>P < 0.05 represents the significance levels compared with the corresponding basal values using the ANOVA statistical test.

comparisons was used to determine significant treatment effects. P < 0.05 was considered statistically significant.

## RESULTS

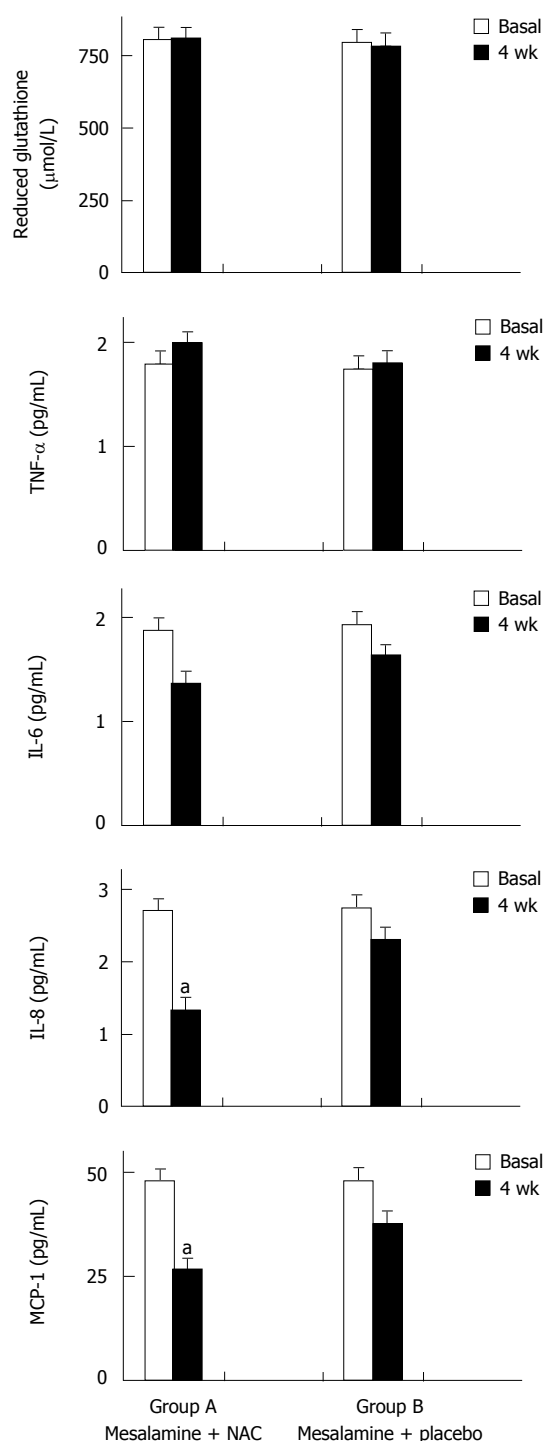
Thirty seven patients were included in the trial. All patients had mild to moderate UC according to the criteria of Modified Truelove-Witts Severity Index<sup>[16]</sup>. Treatment groups were comparable at randomization for baseline demographic characteristics (Table 1). All patients completed the one month study (19 treated with mesalamine plus NAC and 18 treated with mesalamine plus placebo) following the correct protocol.

### Treatment effect on Modified Truelove-Witts Severity Index

The disease score was calculated by “per-protocol” criteria, the data of all randomized patients at 0, 2 and 4 wk of treatment were included. Twelve of 19 patients (63%) in the combination treatment group (A) underwent remission (score  $\leq$  2) at 4 wk of treatment. At the same period of time nine of 18 patients (50%) in the mesalamine plus placebo group (B) underwent clinical remission (score  $\leq$  2). However, statistically not-significant differences were observed between both groups in this clinical parameter (OR = 1.71; 95% CI: 0.46 to 6.36; P = 0.19; NNT = 7.7). During 4 wk of treatment, clinical response measured by MTWSI improved in group A compared with the corresponding baseline values (P = 0.046, ANOVA test) (Table 2). Whereas in the group B no statistical improvement were reached (P = 0.735, ANOVA test) at the same period of time. Although a better favorable trend was observed with the combined therapy (group A) as compared to that observed in the mesalamine plus placebo arm (group B), the significance threshold was not reached (ANCOVA test). The disease score decreased in both groups of patients at 4 wk of treatment with respect to the corresponding baseline values, however, the group B was not statistically significant; the MTWSI change was 2.63  $\pm$  0.82 (P = 0.004) and 0.89  $\pm$  0.9 (P = 0.33) (paired student’ t-test) at 4 wk of treatment for A and B groups, respectively.

### Effect of treatment on reduced glutathione, TNF- $\alpha$ , IL-6, IL-8 and MCP-1 circulating levels

Measurement of biochemical parameters was performed



**Figure 1** Reduced glutathione levels in whole blood and tumor necrosis factor alpha (TNF- $\alpha$ ), interleukin IL-6, IL-8, and monocyte chemoattractant protein (MCP)-1 levels in plasma of subjects after 4 wk of treatment with mesalamine plus NAC (Group A) or mesalamine plus placebo (Group B). mean  $\pm$  SD. <sup>a</sup> $P < 0.05$  represent the significance level compared with basal values using the Student's *t*-test.

at the beginning and at 4 wk of treatment (Figure 1). Blood levels of reduced glutathione (GSH) did not change significantly at 4 wk of treatment in group A (NAC plus mesalamine) with respect to control group (placebo plus mesalamine). TNF- $\alpha$  plasma levels remained constant and did not change along the period studied in either group A or B. IL-6 plasma levels decreased slightly, although

not significantly, in group A, without changes in the group B. By contrast, circulating levels of IL-8 decreased significantly ( $P < 0.05$ ), with respect to baseline values, at 4 wk of treatment in group A, without changes in the group B. The decrease in IL-8 coincided with the induction of remission of the disease in group A. Finally, the profile of MCP-1 circulating levels was similar to that found for IL-8. Thus, at 4 wk of treatment with NAC plus mesalamine (Group A), a significant ( $P < 0.05$ ) decrease in MCP-1 with respect to the corresponding baseline values was demonstrated, while statistically non-significant changes were observed in group B.

### Safety

No serious adverse effects, clinical or analytical, were observed in either treatment group. The hematological and biochemical results at baseline and 4 wk are summarized in Tables 3 and 4 respectively.

### DISCUSSION

The idea that NAC treatment could be useful in UC emerged from previous studies in animal models of that disease<sup>[14]</sup>. Very recently it has been showed that antioxidant therapy with NAC plus mesalamine accelerates mucosal healing in rodent model of colitis<sup>[15]</sup>. Mucosal biopsies taken from active gut inflammation sites from UC patients produced significantly more reactive oxygen species (H<sub>2</sub>O<sub>2</sub> and superoxide) compared to either uninvolved or healthy tissue<sup>[10,19]</sup>. Glutathione and glutathione peroxidase are the most important system for elimination of H<sub>2</sub>O<sub>2</sub>. Consequently, the reduced glutathione levels in the inflamed mucosa from patients with UC could be decreased<sup>[12,13]</sup>. The oral treatment with NAC (a precursor of glutathione) of UC patients could restore the circulating and local levels of reduced glutathione to respond to the excess of reactive oxygen species. Recently, the effectiveness of antioxidant therapy in pediatric Crohn's disease has been reported in an open-label pilot study<sup>[20]</sup>.

Monitoring of standard laboratory data of our patients demonstrated no worsening of the values with respect to baseline data during treatment (with or without NAC). Moreover, no significant differences in standard laboratory data were observed between the values of the two treatment groups (Tables 3 and 4). Finally, no significant adverse events related with the NAC treatment occurred in any of the patients.

Treatment with mesalamine was maintained constant throughout the study for all patients, with improvement of the disease measured by MTWSI. However in the group receiving a supplement of NAC (0.8 g/d), a more favorable trend was seen with respect to mesalamine treatment alone. The mesalamine plus NAC group underwent a significant clinical response with respect to basal state measured by MTWSI at 4 wk of treatment. However, in the control group (mesalamine plus placebo) no significant improvement was observed in the course of the disease at that time. These results suggest that NAC could accelerate the healing process in UC. However, the conclusion could be affected as consequence of non-homogeneity of both

Table 3 Hematological parameters

	Group A		Group B	
	Basal	4 wk	Basal	4 wk
Haemoglobin (g/100 mL)	14.28	13.98	13.81	13.79
Haematocrit (g/100 mL)	42.55	42.01	40.50	40.61
Erythrocytes ( $\times 10^6/\text{mm}^3$ )	4.81	4.69	4.60	4.58
Platelet count ( $\times 10^3/\text{mm}^3$ )	232.95	249.84	263.22	260.50
VSG (mm/h)	17.13	15.00	19.60	17.38
Leukocytes ( $\times 10^3/\text{mm}^3$ )	7.14	7.06	7.86	7.90
Neutrophils (%)	62.63	61.45	63.75	58.61
Eosinophils (%)	2.56	3.34	3.81	3.22
Basophils (%)	0.32	0.55	0.44	0.22
Lymphocytes (%)	25.89	28.03	27.82	27.74
Monocytes (%)	6.97	7.20	7.14	7.88

Group A: Mesalamine + NAC; Group B: Mesalamine + placebo.

groups according to the age; a definite limitation of this pilot trial is the small size of the groups. This fact must be kept in mind for planning further studies (with higher doses of NAC, route of administration or larger sample size).

The beneficial effect of NAC at 4 wk did not correlate with changes in reduced glutathione in blood, which suggests that the mechanism of action of NAC is local, and not systemic, at that time. Similar pharmacokinetic results were observed in the treatment with 0.6 g/d of NAC of HIV infected patients, resulting in a glutathione increase at 8 wk of treatment<sup>[21]</sup>. Although oral doses of 1.8 g/d of NAC increased the percentage of CD4 lymphocyte counts in HIV infected patients, it did not produce changes in plasma glutathione levels<sup>[22]</sup>, only high oral doses (4-8 g/d of NAC) of this antioxidant could replenished glutathione levels and improved survival rates of HIV infected patients<sup>[23]</sup>. Treatment with comparables doses (100 mg/kg NAC) of acetic acid-induced experimental colitis substantially reduced the degree of colonic injury. Lower doses (20 mg/kg) had no protective effect<sup>[24]</sup>. Moreover, clinical studies suggest liver absorption of NAC when it is administered orally because hepatic clearance of NAC decreased significantly during cirrhosis<sup>[25]</sup> and its metabolism improves hepatosplanchnic flow and liver function in septic shock patients<sup>[26]</sup>. In experimental colitis the beneficial effects of NAC were observed by intrarectal and intraperitoneal administration<sup>[27]</sup>. For all these reasons, clinical trials have used higher doses of NAC bypassing digestive tract without serious adverse events<sup>[28,29]</sup>.

Our study also suggests that the beneficial effect of NAC in UC is related to the down-regulation of chemokines such as MCP-1 and IL-8. At 4 wk of treatment, both molecules decreased in plasma of patients treated with NAC plus mesalamine, whereas in the mesalamine plus placebo group this change was not statistically significant. The induction of the remission in the group A coincided with the decrease in IL-8 and MCP-1 plasma levels, suggesting that the effect of NAC is related to the decrease of molecules that activates the recruitment and activation of neutrophils and monocytes to the inflamed mucosa. Our data are in concordance with previous studies performed in alcoholic hepatitis, where MCP-1 production stimulated by lipopolysaccharides decreased with NAC treatment,

Table 4 Biochemical parameters

	Group A		Group B	
	Basal	4 wk	Basal	4 wk
SGOT (units/L)	16.89	16.47	20.47	25.06
SGPT (units/L)	15.58	17.53	36.94	43.59
Phosphatase alkaline (units/L)	93.56	86.61	93.59	86.71
Bilirubin (mg/100 mL)	0.68	0.67	0.50	0.51
LDH (units/L)	258.22	265.45	307.63	299.30
Serum creatinine (mg/100 mL)	0.95	0.95	0.90	0.93
Urea/BUN (mg/100 mL)	35.23	34.64	35.90	33.75
Total Proteins (g/100 mL)	7.17	7.06	7.18	7.08
Albumin (g/100 mL)	4.16	4.11	4.13	4.12
Na <sup>+</sup> (mmol/L)	138.10	139.20	138.11	139.10
K <sup>+</sup> (mmol/L)	4.17	4.24	4.34	4.28
Glucose (mg/100 mL)	91.53	89.54	83.53	85.88
Iron ( $\mu\text{g}/\text{dL}$ )	70.27	69.00	78.56	84.82
Orosomucoid (mg/100 mL)	85.19	90.98	98.06	89.98
CRP (mg/L)	5.37	4.27	5.20	4.85

Group A: Mesalamine + NAC; Group B: Mesalamine + placebo.

suggesting that the antioxidant act directly on target cells<sup>[30]</sup>. Previously, it was observed that the percentage of cells expressing IL-8 and MCP-1 was significantly enhanced in UC samples as compared to controls<sup>[31]</sup>. Furthermore, the local overexpression of MCP-1 and IL-8 was associated with inflammation in UC<sup>[32]</sup>. A close correlation has been demonstrated between mucosa IL-8 mRNA expression and the colonic inflammation in UC patients, and a decrease of local IL-8 expression has been reported during UC improvement after granulocytapheresis<sup>[33]</sup>. Local changes in IL-8 expression correlates with urinary IL-8, and this chemokine increases around ten fold in the urine during active UC compared with control subjects<sup>[34]</sup>, suggesting that circulating IL-8 is a good indirect marker for the assessment of active inflammation in the colonic mucosa. Other pro-inflammatory cytokines such as TNF- $\alpha$  and IL-6, previously implicated in inflammatory bowel diseases, did not change significantly in plasma with the treatment prescribed in our study. However we can not rule out the hypothesis that down-regulation of TNF- $\alpha$  and IL-6 in the mucosa could explain the improvement observed.

In conclusion, the results of the present pilot study suggest that combined therapy (NAC and mesalamine) produces a clinical improvement of UC patients which correlates with a decrease of MCP-1 and IL8. However, the difference in clinical effect with respect to the control group (mesalamine alone) is not conclusive. NAC is safe and well tolerated.

## COMMENTS

### Background

A new hypothesis termed "Radical Induction Theory" has been proposed to explain the etiology of ulcerative colitis (UC) (J Pravda. *World J Gastroenterol* 2005; 11: 2371). The excess of hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) could damage the colonic epithelial barrier. In experimental acute colitis, cellular glutathione levels decreased and the administration of their precursor (N-acetyl-L-cysteine) (NAC) restore the endogenous level and decreased colonic inflammation. Recently, it has been showed that the combination of NAC and mesalamine accelerates mucosal healing in a rodent model of colitis (Siddiqui *et al*, *Dig Dis Sci* 2006; 51: 698).



## Innovations and breakthroughs

Local overexpression of MCP-1 and IL-8 was associated with inflammation in UC and a decrease of colonic IL-8 has been reported during active UC. Our results show that antioxidant therapy with NAC decreases the levels of IL-8 that activates the recruitment and activation of neutrophils to the inflamed mucosa. It is suggested that higher dose or targeted NAC can alleviate colonic inflammation and hopefully become a novel agent for the treatment of UC.

## Peer review

This is an excellent research paper which highlights the dual role antioxidants play in the pathogenesis of ulcerative colitis. And this is the first paper to cite radical induction theory which logically explains why the addition of NAC to conventional 5-ASA therapy significantly increases the remission rate observed in ulcerative colitis. The findings are potentially important for planning of further studies (e.g. with higher dose of N-acetyl-L-cysteine; more homogenous groups of comparison, larger sample size, et cetera).

## REFERENCES

- 1 Stange EF, Travis SP, Vermeire S, Beglinger C, Kupcinkas L, Geboes K, Barakauskiene A, Villanacci V, Von Herbay A, Warren BF, Gasche C, Tilg H, Schreiber SW, Scholmerich J, Reinisch W; European Crohn's and Colitis Organisation. European evidence based consensus on the diagnosis and management of Crohn's disease: definitions and diagnosis. *Gut* 2006; **55** Suppl 1: i1-i15
- 2 Zhong YQ, Huang HR, Zhu ZH, Chen QK, Zhan J, Xing LC. Effects of sulfasalazine on biopsy mucosal pathologies and histological grading of patients with active ulcerative colitis. *World J Gastroenterol* 2005; **11**: 4435-4438
- 3 Dotan I, Rachmilewitz D. Probiotics in inflammatory bowel disease: possible mechanisms of action. *Curr Opin Gastroenterol* 2005; **21**: 426-430
- 4 Gijssbers K, Geboes K, Van Damme J. Chemokines in gastrointestinal disorders. *Curr Drug Targets* 2006; **7**: 47-64
- 5 Van Assche G, Rutgeerts P. Physiological basis for novel drug therapies used to treat the inflammatory bowel diseases. I. Immunology and therapeutic potential of antiadhesion molecule therapy in inflammatory bowel disease. *Am J Physiol Gastrointest Liver Physiol* 2005; **288**: G169-G174
- 6 Palatka K, Serfozo Z, Vereb Z, Hargitay Z, Lontay B, Erdodi F, Banfalvi G, Nemes Z, Udvardy M, Altorjay I. Changes in the expression and distribution of the inducible and endothelial nitric oxide synthase in mucosal biopsy specimens of inflammatory bowel disease. *Scand J Gastroenterol* 2005; **40**: 670-680
- 7 Singer II, Kawka DW, Schloemann S, Tessner T, Riehl T, Stenson WF. Cyclooxygenase 2 is induced in colonic epithelial cells in inflammatory bowel disease. *Gastroenterology* 1998; **115**: 297-306
- 8 Szanto I, Rubbia-Brandt L, Kiss P, Steger K, Banfi B, Kovari E, Herrmann F, Hadengue A, Krause KH. Expression of NOX1, a superoxide-generating NADPH oxidase, in colon cancer and inflammatory bowel disease. *J Pathol* 2005; **207**: 164-176
- 9 Pravda J. Radical induction theory of ulcerative colitis. *World J Gastroenterol* 2005; **11**: 2371-2384
- 10 Pavlick KP, Laroux FS, Fuseler J, Wolf RE, Gray L, Hoffman J, Grisham MB. Role of reactive metabolites of oxygen and nitrogen in inflammatory bowel disease. *Free Radic Biol Med* 2002; **33**: 311-322
- 11 Menor C, Fernandez-Moreno MD, Fueyo JA, Escribano O, Olleros T, Arriaza E, Cara C, Lorusso M, Di Paola M, Roman ID, Guíjarro LG. Azathioprine acts upon rat hepatocyte mitochondria and stress-activated protein kinases leading to necrosis: protective role of N-acetyl-L-cysteine. *J Pharmacol Exp Ther* 2004; **311**: 668-676
- 12 Sido B, Hack V, Hochlehnert A, Lipps H, Herfarth C, Droge W. Impairment of intestinal glutathione synthesis in patients with inflammatory bowel disease. *Gut* 1998; **42**: 485-492
- 13 Buffinton GD, Doe WF. Depleted mucosal antioxidant defences in inflammatory bowel disease. *Free Radic Biol Med* 1995; **19**: 911-918
- 14 Ardite E, Sans M, Panés J, Romero FJ, Piqué JM, Fernández-Checa JC. Replenishment of glutathione levels improves mucosal function in experimental acute colitis. *Lab Invest* 2000; **80**: 735-744
- 15 Siddiqui A, Ancha H, Tedesco D, Lightfoot S, Stewart CA, Harty RF. Antioxidant therapy with N-acetylcysteine plus mesalamine accelerates mucosal healing in a rodent model of colitis. *Dig Dis Sci* 2006; **51**: 698-705
- 16 Lichtiger S, Present DH. Preliminary report: cyclosporin in treatment of severe active ulcerative colitis. *Lancet* 1990; **336**: 16-19
- 17 Prantera C, Cottone M, Pallone F, Annese V, Franzè A, Cerutti R, Bianchi Porro G. Mesalamine in the treatment of mild to moderate active Crohn's ileitis: results of a randomized, multicenter trial. *Gastroenterology* 1999; **116**: 521-526
- 18 Martins AM, Cordeiro C, Freire AP. Glyoxalase II in *Saccharomyces cerevisiae*: in situ kinetics using the 5,5'-dithiobis(2-nitrobenzoic acid) assay. *Arch Biochem Biophys* 1999; **366**: 15-20
- 19 Kruidenier L, Kuiper I, Lamers CB, Verspaget HW. Intestinal oxidative damage in inflammatory bowel disease: semi-quantification, localization, and association with mucosal antioxidants. *J Pathol* 2003; **201**: 28-36
- 20 Phylactos AC, Fasoula IN, Arnaud-Battandier F, Walker-Smith JA, Fell JM. Effect of enteral nutrition on antioxidant enzyme systems and inflammation in paediatric Crohn's disease. *Acta Paediatr* 2001; **90**: 883-888
- 21 Treitinger A, Spada C, Masokawa IY, Verdi JC, Van Der Sander Silveira M, Luis MC, Reis M, Ferreira SI, Abdalla DS. Effect of N-acetyl-L-cysteine on lymphocyte apoptosis, lymphocyte viability, TNF-alpha and IL-8 in HIV-infected patients undergoing anti-retroviral treatment. *Braz J Infect Dis* 2004; **8**: 363-371
- 22 Look MP, Rockstroh JK, Rao GS, Barton S, Lemoch H, Kaiser R, Kupfer B, Sudhop T, Spengler U, Sauerbruch T. Sodium selenite and N-acetylcysteine in antiretroviral-naïve HIV-1-infected patients: a randomized, controlled pilot study. *Eur J Clin Invest* 1998; **28**: 389-397
- 23 De Rosa SC, Zaretsky MD, Dubs JG, Roederer M, Anderson M, Green A, Mitra D, Watanabe N, Nakamura H, Tjioe I, Deresinski SC, Moore WA, Ela SW, Parks D, Herzenberg LA, Herzenberg LA. N-acetylcysteine replenishes glutathione in HIV infection. *Eur J Clin Invest* 2000; **30**: 915-929
- 24 Akgun E, Caliskan C, Celik HA, Ozutemiz AO, Tuncyurek M, Aydin HH. Effects of N-acetylcysteine treatment on oxidative stress in acetic acid-induced experimental colitis in rats. *J Int Med Res* 2005; **33**: 196-206
- 25 Jones AL, Jarvie DR, Simpson D, Hayes PC, Prescott LF. Pharmacokinetics of N-acetylcysteine are altered in patients with chronic liver disease. *Aliment Pharmacol Ther* 1997; **11**: 787-791
- 26 Rank N, Michel C, Haertel C, Lenhart A, Welte M, Meier-Hellmann A, Spies C. N-acetylcysteine increases liver blood flow and improves liver function in septic shock patients: results of a prospective, randomized, double-blind study. *Crit Care Med* 2000; **28**: 3799-3807
- 27 Cetinkaya A, Bulbuloglu E, Kurutas EB, Ciralik H, Kantarceken B, Buyukbese MA. Beneficial effects of N-acetylcysteine on acetic acid-induced colitis in rats. *Tohoku J Exp Med* 2005; **206**: 131-139
- 28 Medved I, Brown MJ, Bjorksten AR, Murphy KT, Petersen AC, Sostaric S, Gong X, McKenna MJ. N-acetylcysteine enhances muscle cysteine and glutathione availability and attenuates fatigue during prolonged exercise in endurance-trained individuals. *J Appl Physiol* 2004; **97**: 1477-1485
- 29 Soldini D, Zwahlen H, Gabutti L, Marzo A, Marone C. Pharmacokinetics of N-acetylcysteine following repeated intravenous infusion in haemodialysed patients. *Eur J Clin Pharmacol* 2005; **60**: 859-864
- 30 Devalaraja MN, McClain CJ, Barve S, Vaddi K, Hill DB. Increased monocyte MCP-1 production in acute alcoholic hepatitis. *Cytokine* 1999; **11**: 875-881



- 31 **Banks C**, Bateman A, Payne R, Johnson P, Sheron N. Chemokine expression in IBD. Mucosal chemokine expression is unselectively increased in both ulcerative colitis and Crohn's disease. *J Pathol* 2003; **199**: 28-35
- 32 **McCormack G**, Moriarty D, O'Donoghue DP, McCormick PA, Sheahan K, Baird AW. Tissue cytokine and chemokine expression in inflammatory bowel disease. *Inflamm Res* 2001; **50**: 491-495
- 33 **Tsukada Y**, Nakamura T, Iimura M, Iizuka BE, Hayashi N. Cytokine profile in colonic mucosa of ulcerative colitis correlates with disease activity and response to granulocytapheresis. *Am J Gastroenterol* 2002; **97**: 2820-2828
- 34 **Taha AS**, Grant V, Kelly RW. Urinalysis for interleukin-8 in the non-invasive diagnosis of acute and chronic inflammatory diseases. *Postgrad Med J* 2003; **79**: 159-163

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RAPID COMMUNICATION

## Etiology and portal vein thrombosis in Budd-Chiari syndrome

Oguz Uskudar, Meral Akdogan, Nurgul Sasmaz, Sevinc Yilmaz, Muharrem Tola, Burhan Sahin

Oguz Uskudar, Meral Akdogan, Nurgul Sasmaz, Burhan Sahin, Türkiye Yüksek İhtisas Hospital, clinics of gastroenterology, Ankara 06010, Turkey  
Sevinc Yilmaz, Türkiye Yüksek İhtisas Hospital, hematology unit, Ankara 06010, Turkey

Muharrem Tola, Türkiye Yüksek İhtisas Hospital clinics of radiology, Ankara 06010, Turkey

**Author contributions:** Uskudar O and Akdogan M contributed equally to this work; Uskudar O and Akdogan M designed research; Uskudar O, Akdogan M, Sasmaz N, Yilmaz S, Tola M, Sahin B performed research; Uskudar O and Akdogan M wrote the paper.

**Correspondence to:** Oguz Uskudar, Dr, Yayla Mah. 1. cadde no: 123/3, Kecioren, Ankara 06010, Turkey. [ouskudar@hotmail.com](mailto:ouskudar@hotmail.com)  
Telephone: +90-312-3788638 Fax: +90-312-3124120

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vein thrombosis while patients having thrombophilic factors more than one are prone to develop portal vein thrombosis with worse clinical outcome.

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**Key words:** Budd-Chiari syndrome; Etiology; Web; Behcet's disease; Portal thrombosis

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### Abstract

**AIM:** To research the etiology, portal vein thrombosis and other features of Budd-Chiari syndrome (BCS) patients prospectively.

**METHODS:** A total of 75 patients (40 female, 35 male) who were diagnosed between January 2002 and July 2004 as having BCS were studied prospectively. Findings from on physical examination, ultrasonography, duplex ultrasonography and venography were analyzed. Hemogram and blood chemistry were studied at the time of diagnosis and on each hospital visit. Bone marrow examination and immune phenotyping were performed by a hematologist when necessary. Protein C, S, antithrombin III, activated protein C resistance, and anticardiolipin antibodies, antinuclear antibodies, and anti ds-DNA were studied twice. The presence of ascite, esophageal varices, and portal thrombosis were evaluated at admission and on every visit.

**RESULTS:** At least one etiological factor was determined in 54 (72%) of the patients. The etiology could not be defined in 21 (28%) patients. One etiological factor was found in 39, 2 factors in 14 and 3 factors in 1 patient. The most common cause was the web (16%), the second was Hydatid disease (11%), the third was Behcet's disease (9%). Portal vein thrombosis was present in 11 patients and at least one etiology was identified in 9 of them (82%).

**CONCLUSION:** Behcet's disease and hydatid disease are more prominent etiological factors in Turkey than in other countries. Patients with web have an excellent response to treatment without signs of portal

### INTRODUCTION

Budd-Chiari syndrome (BCS) is a rare heterogeneous group of disorders characterized by hepatic venous outflow obstruction at the level of hepatic venules, the large hepatic veins, the inferior vena cava, or the right atrium<sup>[1]</sup>. It has been increasingly recognized that a combination of several thrombogenic conditions and triggering factors are necessary for the development of venous thrombosis in general and hepatic vein or cava thrombosis in particular.

The etiology of Budd-Chiari syndrome changes according to geography. In most of the western cases of BCS an etiologic factor has been defined, whereas the causes of BCS in many of patients from India and Japan are not defined<sup>[2-6]</sup>. While thromboses are more prominent in the west, webs are prominent in the east and in Japan<sup>[7-9]</sup>.

Turkey is a country acting as a bridge in between east and west. To date there is insufficient data about the etiology and features of BCS in Turkey. The relation between the etiologic factor and presence of portal vein thrombosis and yet outcome is not well established to date. In this study we prospectively searched the etiology, characteristics and other features of BCS patients in Turkey.

### MATERIALS AND METHODS

#### Patients

Türkiye Yüksek İhtisas Hospital is a tertiary referral center



**Figure 1** A patient with prominent abdominal wall collateral veins as a result of inferior vena cava thrombosis at the hepatic portion.

in gastroenterology in Turkey. A total of 75 patients (40 female, 35 male) who were diagnosed between January 2002 and July 2004 in Türkiye Yüksek İhtisas Hospital as having BCS were studied prospectively.

The diagnosis of BCS was established using ultrasonography, color Doppler ultrasonography (CDU) and venography. A detailed patient history including onset of symptoms, medications, previous disease, pregnancy and use of oral contraceptives for women, oral and genital ulcers, previous surgery, and trauma were especially noted. The onset of symptoms was classified, whether acute, sub acute or chronic. The diagnosis and date of last hospital visit were noted.

Patients were evaluated with a multidisciplinary approach (involving gastroenterology, gastroenterology surgery, hematology, radiology and other clinics when necessary). Findings on physical examination were noted. Ultrasonography was performed by experienced gastroenterologists and CDU and venography were performed by experienced radiologists. The sites of lesions were classified as hepatic vein, inferior vena cava or combined and note was also taken as to whether the obstruction was complete or partial.

Hemogram and blood chemistry were studied at the time of diagnosis and on each visit. All patients were evaluated by a hematologist for myeloproliferative disorders and bone marrow examination and immune phenotyping were performed when necessary. Protein C, S, antithrombin III, factor V Leiden mutation, and anticardiolipin antibodies were studied twice in suitable conditions. Antinuclear antibody and anti ds-DNA were studied when systemic lupus erythematosus was suspected.

The presence of ascite, esophageal varices, and portal vein thrombosis was evaluated at admission and on every visit. Treatments were classified as conservative management, anticoagulation, angioplasty, TIPS and surgery. Complications that occurred during the follow up were recorded.

### Statistical analysis

Variables between groups were compared using the Mann-Whitney *U* test, correlations between parameters were tested by Spearman's correlation analysis and the "Statistical Package for Social Sciences 10.0" program was used for statistical analysis.  $P < 0.05$  was considered as statistically significant.

**Table 1** Etiology

Etiological factors	Patients (%)	Etiological factors	Patients (%)
Web	12 (16)	Anticardiolipin antibodies	3 (4)
Hydatid disease	8 (11)	Oral contraceptive use	3 (4)
Behçet' disease	7 (9)	Tumor	3 (4)
Protein C def.	7 (9)	Anti-thrombin 3 def.	2 (3)
Myeloproliferative disorder	6 (8)	Lupus erythematosus	1 (1)
Protein S def.	5 (7)	Ulcerative colitis	1 (1)
Pregnancy	5 (7)	Paroxysmal nocturnal hemoglobinuria	1 (1)
Activated protein C resistance	3 (4)	Acute pancreatitis	1 (1)

**Table 2** Laboratory parameters

	First			Last		
	min	max	mean	min	max	mean
AST (IU/L)	1300	119700	7520	1000	82900	7044
ALT (IU/L)	700	90700	6118	1700	157200	9477
GGT (IU/L)	1600	78100	13930	2900	64200	14044
Albumin (g/dL)	2100	5100	3460	2400	4800	3781
Bilirubin (mg/dL)	100	1300	286	100	3200	326
Urea (mg/dL)	1300	10600	3478	1100	6100	3078
Prothrombin time (s)	1100	4400	1731	1100	3000	1708

## RESULTS

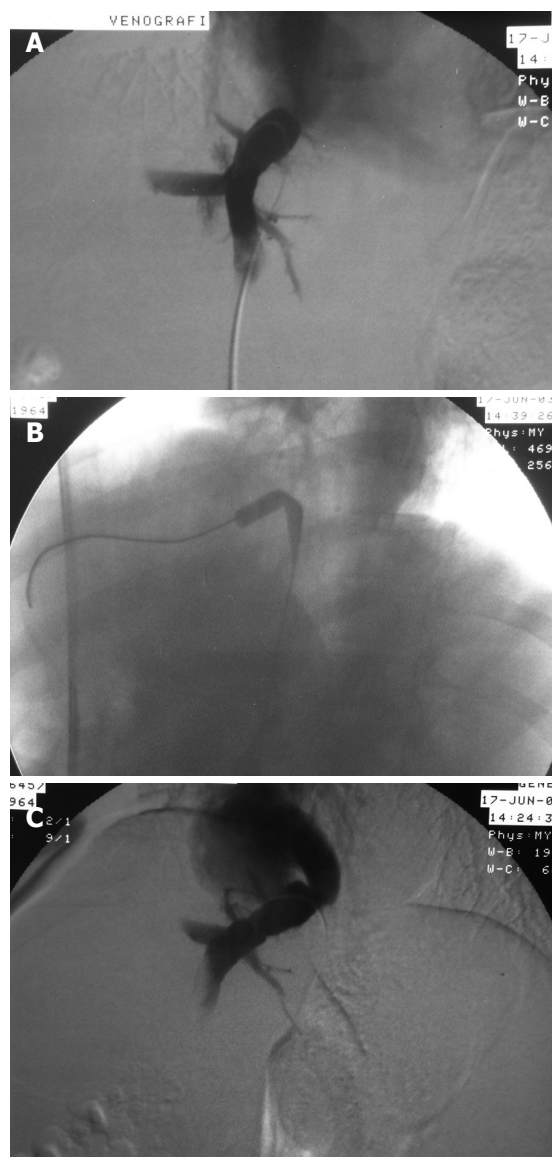
There were 40 female and 35 male patients. Mean age at first visit was  $33.49 \pm 13.88$  (14-72) years. Most of the patients were diagnosed during the chronic phase of the disease (53.3%). Other patients were diagnosed during acute (26.7%) and sub acute (16%) periods. The site of obstruction was in hepatic veins in 47%, in IVC+ hepatic vein in 30% and IVC in 23% of the patients (Figure 1). A complete obstruction was observed in 54% of the patients. Venography was available for 80% of the patients. Esophageal varices and ascites were observed in 79% and 84% of the patients at admission, and 54% and 35% at last visit, respectively. The patients were followed-up for 1-30 mo (mean, 18 mo). During the follow-up seven patients died (9%).

At least one etiological factor was determined in 54 (72%) of the patients. The etiology could not be defined in 21 (28%) patients. One etiological factor was found in 39, 2 factors in 14 and 3 factors in 1 patient. The most common cause was the web and this was determined in 12 (16%) patients (Table 1). The second most common cause was Hydatid disease and this was found in 8 (11%) of the patients. Behçet's disease was the third most common cause (7 patients, 9%). Etiological factors are presented in Table 1.

Laboratory parameters at admission and at the last hospital visit are presented in Table 2.

Significant positive correlations between first AST-ALT and first prothrombin time ( $P < 0.01$ ), and between last GGT and last urea levels ( $P < 0.05$ ) were found. A significant negative correlation was found between the first prothrombin time and first albumin levels ( $P < 0.01$ ).

No differences were observed when patients were compared according to sex or age over or less than 35 years old.



**Figure 2** A patient with web in the right hepatic vein before angioplasty (A), during angioplasty (B) and after dilatation of the web (C).

Patients who presented with ascites initially had lower albumin and higher Prothrombin times compared to those who did not. Patients who had ascites at their last visit had significantly lower albumin and AST levels than others.

Portal veins were patent in 85% and thrombosed in 15% of the patients. At least one etiology was identified in 9 of the 11 patients (82%) with portal vein thrombosis and BCS. Five patients had 1, 3 patients had 2 and 1 patient had 3 etiological factors. One of the striking results was that none of the patients with web or Behcet's disease as a cause of BCS had portal vein thrombosis. Hydatid disease was observed in 4, protein C deficiency in 3, protein S deficiency in 2, mass lesion in 2, and ulcerative colitis, pregnancy and antithrombin 3 deficiencies in 1 patient. Last bilirubin values were significantly higher ( $16.5/1.7$ ,  $P = 0.002$ ) and last albumin levels were significantly lower ( $32/39$ ,  $P = 0.022$ ) in patients with portal vein thrombosis. Nine patients with portal vein thrombosis were in the chronic, and 2 in a subacute but none in acute phase of BCS.

When compared by means of the extent of the obstruction, patients with complete obstruction had lower initial albumin levels ( $3.2/3.7$ ,  $P = 0.007$ ) and higher prothrombin times ( $19\text{ s}/14\text{ s}$ ,  $P = 0.001$ ). No other significant difference was found between other parameters.

Patients were treated conservatively (53%), with balloon angioplasty (24%, Figure 2), oral anticoagulation (17%), TIPS (4%) or surgery (7%). Three patients experienced warfarin related complications (2 intracranial bleeding, one of whom died, 1 intra abdominal bleeding). Three of the patients who underwent surgery died because of liver failure. The balloon angioplasty procedure was performed in 18 patients and failed in 6 of them. When we examined the patients where it was unsuccessful it was found that none of them were webs but, rather, long segment caval thrombosis or hepatic vein thromboses. Two of the 12 successful web dilatation patients later developed thrombosis and one underwent surgery, the other was treated with TIPS procedure.

## DISCUSSION

The prevalence of underlying thrombophilias is markedly increased in patients with BCS. Factors that confer a predisposition to the development of BCS including hereditary and acquired hypercoagulable states and a variety of other causes can be identified in about 75 percent of patients<sup>[1]</sup>. In our study we identified at least one etiological factor in 54 (72%) patients. The presence of multiple causes in the same patient is increasingly recognized<sup>[3]</sup>. We identified 2 etiological factors in 14 and 3 factors in 1 patient. These results were similar with reports in the literature.

There were slightly more female patients than males ( $40\text{ vs }35$ ) but the imbalance was not as marked as in the literature ( $2/1$ )<sup>[10]</sup>. This may be due to the less frequent use of oral contraceptives in Turkey. Mean age at the time of diagnosis was 33.

The site of the lesion varies according to geography. In our patients hepatic veins were involved in 77% and the inferior vena cava was involved in 53% of the patients. While inferior vena cava involvement is more prominent in Nepal, South Africa, China, India and Japan, hepatic vein involvement is more prominent in the western countries<sup>[7,11-13]</sup>. Our results lay in between these, but were closer to the western countries.

When we look to the etiology, we see that myeloproliferative disorders are found to be the leading cause of BCS in western countries, with a range between 20%-53%<sup>[3,4]</sup>. In this study we identified myeloproliferative disease in 8% of the patients. We confirmed these disorders by bone marrow aspiration and yet only overt forms of disease were diagnosed. We could not look for JAC2 V617f mutation<sup>[14-16]</sup>. So we might have missed some of these patients or myeloproliferative disorders may be less frequent in Turkish population.

Webs were the most common etiology and the most easily treated lesions in our patients (16%). This ratio is not as high as in eastern countries but higher than in western ones. It may be another indicator of Turkey as a bridge



between east and west. These patients rapidly responded to balloon dilatation and ascites dramatically disappeared after successful dilatation. None of these patients had portal vein thrombosis. The chances of a patient with BCS may depend on a web etiology. The etiology of BCS is the most important factor for the prognosis.

The second most frequent etiology in our study was Hydatid disease, at 12%, and this is the highest ratio in the literature. Hydatid disease is a common health problem and deserves more interest in Turkey. Half of the patients with BCS and Hydatid disease also had portal vein thrombosis.

Another frequently observed etiology was Behcet's disease with a ratio of 9%. This ratio is also the highest ratio in the literature. Every patient with BCS must be closely questioned for any history of Behcet's disease. The difficulty in managing these patients was that they usually had long segment thrombosis and thrombosis of other vascular structures. While Bayraktar has reported portal vein thrombosis in these patients, another interesting observation is that none of our patients with Behcet's disease and BCS had portal vein thrombosis<sup>[17]</sup>. This might be due to Bayraktar's long term follow up period. Other etiological factors were similar with the literature.

Portal vein thrombosis in patients with BCS is a poor prognostic factor<sup>[18,19]</sup>. In this study we observed that patients with portal vein thrombosis when compared with those not thrombosed, had lower albumin and higher bilirubin level at their last check. Another difference in patients with portal vein thrombosis was the etiology. Since none of the patients with webs or Behcet's disease had or developed portal vein thrombosis during follow up, this deserves more attention. In our patients one patient had 3 etiological factors and he had also portal vein thrombosis. Since nine patients with portal vein thrombosis were in the chronic, and 2 in a subacute phase of BCS, but none were in an acute phase, we can state that prevention of portal vein thrombosis might be considered in these patients.

Ascites was present in 84% of the patients at admission which means that most of the patients were diagnosed when symptomatic. This ratio lies between the ratio of before and after the 1990 results of Hadengue, indicating that we should diagnose more asymptomatic patients<sup>[10]</sup>. With an increased knowledge about BCS, patients can be diagnosed earlier and further development of new thrombosis and deterioration of liver functions can be prevented. In patients with poor liver functions surgery is unsuccessful, and this may be due to lack of experience as well as to a very enlarged liver that causes difficulty during surgery.

In conclusion, the etiology of BCS changes according to geographic region. Webs, Behcet's disease and Hydatid disease are more prominent etiological factors in Turkey which sets it apart from other countries. Complete obstruction of hepatic veins or inferior vena cava and presence of portal vein thrombosis affects the course of the disease. Presence of more than one prethrombotic factor increases the risk of portal vein thrombosis. Patients with web have excellent treatment response and prognosis.

## COMMENTS

## COMMENTS

The etiology of Budd-Chiari syndrome (BCS) changes according to geography. There is insufficient data about the etiology and portal vein thrombosis relation BCS. The aim of the present study was to evaluate the etiology, presentation, portal vein thrombosis and other features of BCS patients.

### Research frontiers

Several studies have identified that portal vein thrombosis in patients with BCS is a poor prognostic factor. However there are few prospective studies regarding the etiology and portal vein thrombosis relation. We studied the relationship between the etiology, portal vein thrombosis and find that some etiological factors are not associated with portal vein thrombosis.

### Innovations and breakthroughs

The present research studied cases with BCS, but the follow up period was not long enough and some etiological factors such as JAC2 V617f mutation was missing.

### Applications

The results of this study suggest that patients with etiology other than web or Behcet's disease are prone to develop portal vein thrombosis and since portal vein thrombosis mostly were in the chronic, some in a subacute but none in acute phase of BCS, early diagnosis and anticoagulation may be important.

### Peer review

This paper clearly defines that initially the extent of thrombosis and etiology and lately portal vein thrombosis are important factors for BCS. The results are valuable.

## REFERENCES

- 1 Menon KV, Shah V, Kamath PS. The Budd-Chiari syndrome. *N Engl J Med* 2004; **350**: 578-585
- 2 Janssen HL, Meinardi JR, Vleggaar FP, van Uum SH, Haagsma EB, van Der Meer FJ, van Hattum J, Chamuleau RA, Adang RP, Vandenbroucke JP, van Hoek B, Rosendaal FR. Factor V Leiden mutation, prothrombin gene mutation, and deficiencies in coagulation inhibitors associated with Budd-Chiari syndrome and portal vein thrombosis: results of a case-control study. *Blood* 2000; **96**: 2364-2368
- 3 Denninger MH, Chait Y, Casadevall N, Hillaire S, Guillin MC, Bezeaud A, Erlinger S, Briere J, Valla D. Cause of portal or hepatic venous thrombosis in adults: the role of multiple concurrent factors. *Hepatology* 2000; **31**: 587-591
- 4 Hirshberg B, Shouval D, Fibach E, Friedman G, Ben-Yehuda D. Flow cytometric analysis of autonomous growth of erythroid precursors in liquid culture detects occult polycythemia vera in the Budd-Chiari syndrome. *J Hepatol* 2000; **32**: 574-578
- 5 Deltenre P, Denninger MH, Hillaire S, Guillin MC, Casadevall N, Briere J, Erlinger S, Valla DC. Factor V Leiden related Budd-Chiari syndrome. *Gut* 2001; **48**: 264-268
- 6 Bismuth E, Hadengue A, Hammel P, Benhamou JP. Hepatic vein thrombosis in Behcet's disease. *Hepatology* 1990; **11**: 969-974
- 7 Shrestha SM, Okuda K, Uchida T, Maharjan KG, Shrestha S, Joshi BL, Larsson S, Vaidya Y. Endemicity and clinical picture of liver disease due to obstruction of the hepatic portion of the inferior vena cava in Nepal. *J Gastroenterol Hepatol* 1996; **11**: 170-179
- 8 Mohanty D, Shetty S, Ghosh K, Pawar A, Abraham P. Hereditary thrombophilia as a cause of Budd-Chiari syndrome: a study from Western India. *Hepatology* 2001; **34**: 666-670
- 9 Okuda H, Yamagata H, Obata H, Iwata H, Sasaki R, Imai F, Okudaira M, Ohbu M, Okuda K. Epidemiological and clinical features of Budd-Chiari syndrome in Japan. *J Hepatol* 1995; **22**: 1-9
- 10 Hadengue A, Poliquin M, Vilgrain V, Belghiti J, Degott C, Erlinger S, Benhamou JP. The changing scene of hepatic vein thrombosis: recognition of asymptomatic cases.

- Gastroenterology* 1994; **106**: 1042-1047
- 11 **Valla DC**. Hepatic vein thrombosis (Budd-Chiari syndrome). *Semin Liver Dis* 2002; **22**: 5-14
- 12 **Okuda K**, Kage M, Shrestha SM. Proposal of a new nomenclature for Budd-Chiari syndrome: hepatic vein thrombosis versus thrombosis of the inferior vena cava at its hepatic portion. *Hepatology* 1998; **28**: 1191-1198
- 13 **Victor S**, Jayanthi V, Panchanadam M, Chitra S, Vijayalakshmi CS, Madanagopalan N. Budd Chiari syndrome and pericaval filariasis. *Trop Gastroenterol* 1994; **15**: 161-168
- 14 **Patel RK**, Lea NC, Heneghan MA, Westwood NB, Milojkovic D, Thanigaikumar M, Yallop D, Arya R, Pagliuca A, Gaken J, Wendon J, Heaton ND, Mufti GJ. Prevalence of the activating JAK2 tyrosine kinase mutation V617F in the Budd-Chiari syndrome. *Gastroenterology* 2006; **130**: 2031-2038
- 15 **Smalberg JH**, Murad SD, Braakman E, Valk PJ, Janssen HL, Leebeek FW. Myeloproliferative disease in the pathogenesis and survival of Budd-Chiari syndrome. *Haematologica* 2006; **91**: 1712-1713
- 16 **Plume G**, Vaya A, Ferrando F, Mira Y, Orbis F. JAK2V617F mutation as a marker of a latent myeloproliferative disorder in a patient with Budd-Chiari syndrome and factor V Leiden mutation. *Thromb Haemost* 2007; **98**: 681-682
- 17 **Bayraktar Y**, Balkanci F, Bayraktar M, Calguneri M. Budd-Chiari syndrome: a common complication of Behcet's disease. *Am J Gastroenterol* 1997; **92**: 858-862
- 18 **Mahmoud AE**, Helmy AS, Billingham L, Elias E. Poor prognosis and limited therapeutic options in patients with Budd-Chiari syndrome and portal venous system thrombosis. *Eur J Gastroenterol Hepatol* 1997; **9**: 485-489
- 19 **Murad SD**, Valla DC, de Groen PC, Zeitoun G, Haagsma EB, Kuipers EJ, Janssen HL. Pathogenesis and treatment of Budd-Chiari syndrome combined with portal vein thrombosis. *Am J Gastroenterol* 2006; **101**: 83-90
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# Clinical usefulness of transpapillary removal of common bile duct stones by frequency doubled double pulse Nd:YAG laser

Tae Hyeon Kim, Hyo Jeong Oh, Chang-Soo Choi, Dong Han Yeom, Suck Chei Choi

Tae Hyeon Kim, Hyo Jeong Oh, Chang-Soo Choi, Dong Han Yeom, Suck Chei Choi, Department of Internal Medicine and Digestive Disease Research Institute, Wonkwang University School of Medicine, Iksan 570180, Korea

Author contributions: Kim TH designed research plan and wrote the paper; Oh HJ and Choi CS performed research; Yeom DH and Choi SC analyzed data.

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Correspondence to: Hyo-Jeong Oh, MD, Department of Internal Medicine, Wonkwang University School of Medicine, Sinyong Dong 344-2, Iksan 570-180, Korea. [kth@wonkwang.ac.kr](mailto:kth@wonkwang.ac.kr)

Telephone: +82-63-8592670 Fax: +82-63-8552025

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## Abstract

**AIM:** To study the efficacy and the safety of laser lithotripsy without direct visual control by using a balloon catheter in patients with bile duct stones that could not be extracted by standard technique.

**METHODS:** The seventeen patients (7 male and 10 female; mean age 67.8 years) with difficult common bile duct (CBD) stones were not amenable for conventional endoscopic maneuvers such as sphincterotomy and mechanical lithotripsy were included in this study. Laser wavelengths of 532 nm and 1064 nm as a double pulse were applied with pulse energy of 120 mJ. The laser fiber was advanced under fluoroscopic control through the ERCP balloon catheter. Laser lithotripsy was continued until the fragment size seemed to be less than 10 mm. Endoscopic extraction of the stones and fragments was performed with the use of the Dormia basket and balloon catheter.

**RESULTS:** Bile duct clearance was achieved in 15 of 17 patients (88%). The mean number of treatment sessions was  $1.7 \pm 0.6$ . Endoscopic stone removal could not be achieved in 2 patients (7%). Adverse effects were noted in three patients (hemobilia, pancreatitis, and cholangitis).

**CONCLUSION:** The Frequency Doubled Double Pulse Nd:YAG (FREDDY) laser may be an effective and safe technique in treatment of difficult bile duct stones.

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**Key words:** Bile duct stones; Frequency doubled double pulse Nd:YAG laser; Transpapillary removal; Mechanical lithotripsy; Balloon catheter

**Peer reviewer:** Seyed A Taghavi, Associate Professor, Department of Internal Medicine, Nemazee Hospital, No.23, 59th Alley, Ghasrodasht St., Shiraz 71838-95453, Iran

Kim TH, Oh HJ, Choi CS, Yeom DH, Choi SC. Clinical usefulness of transpapillary removal of common bile duct stones by frequency doubled double pulsed Nd:YAG laser. *World J Gastroenterol* 2008; 14(18): 2863-2866 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2863.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2863>

## INTRODUCTION

About 90% of all patients with common bile duct stones are currently treated non-surgically using endoscopic sphincterotomy and stone extraction. However, standard endoscopic procedure and mechanical lithotripsy for removal of stones from the bile duct fail in 5% to 10% of patients, because the stones are too large or impacted<sup>[1]</sup>. In such cases, additional methods such as extracorporeal shockwave lithotripsy (ESWL), laser shockwave lithotripsy (LSWL) or electrohydraulic lithotripsy (EHL) are required<sup>[2]</sup>. EHL and LSWL need visual control *via* a mother-baby endoscope system or cholangioscopy for safety because of the potential for accidental damage to the bile duct wall. Moreover, a peroral cholangioscope is expensive, fragile and difficult to use. Laser lithotripsy is expensive due to the cost of the cumbersome endoscopic equipment. Although EHL is inexpensive, it uses high-pulse energy to disintegrate stones<sup>[3-5]</sup>.

The Frequency Doubled Double Pulse Nd:YAG (FREDDY) laser (World of Medicine, Berlin, Germany) is a newly developed economical, short-pulse, double frequency, solid-state laser with wavelengths of 532 nm and 1064 nm that cause less tissue damage. It was specifically designed to fragment urinary and biliary calculi<sup>[6]</sup>. We used a balloon catheter for FREDDY laser lithotripsy (FREDDY LL) in an effort to improve fluoroscopic targeting and to prevent damage to the bile duct. The purpose of this study was to investigate the safety and the effectiveness of using a FREDDY laser to treat bile duct stones that were not amenable to conventional endoscopic therapy.

## MATERIALS AND METHODS

### Patients

Seventeen patients (7 male and 10 female; mean age 67.8 years, range 55-82 years) with extra-hepatic bile duct

stones were included in this study. The inclusion criterion for this study was extrahepatic bile duct stones that were not amenable to standard endoscopic procedure including endoscopic sphincterotomy and mechanical lithotripsy. Stone removal by using conventional methods failed because the stones were not captured in the basket for mechanical lithotripsy or endoscopic sphincterotomy inadequately was done for large perampullary diverticulum with large CBD stones ( $> 1.5$  cm). The main reasons were the following; large stone ( $n = 7$ ), impacted stone ( $n = 3$ ), and large stones with perampullary diverticulum ( $n = 7$ ).

All patients were diagnosed as having bile duct stones by endoscopic retrograde cholangiography and/or MRCP at our hospital. The data regarding stone size, number, and location was based on the endoscopic retrograde cholangiogram (by using the diameter of the distal end of standard duodenoscopy as a reference). The properties of the CBD stones were as follows: 1 stone ( $n = 5$ ), 2 stones ( $n = 7$ ) and more than 2 stones ( $n = 5$ ); diameter of large stone ( $18.5 \pm 3.2$  mm) with 10-20 mm ( $n = 8$ ) and with 21-30 mm ( $n = 9$ ). Large stones with perampullary diverticulum were found in 11 of 17 patients. The study was approved by our institutional review board. Written informed consent was obtained from each patient for laser therapy.

### Methods

We used the FREDDY laser (frequency doubled double pulse Nd:YAG laser, Laser 100, World of Medicine). Laser wavelengths of 532 nm and 1064 nm as a double pulse were applied with pulse energy of 120 mJ. The laser fiber with a 250 nm core diameter was inserted in a 6.8 Fr standard extraction balloon catheter (Wilson-Cook Medical Inc., Winston-Salem, N.C.) with an 18-mm-diameter balloon and this was then passed through the papilla *via* the working channel of a standard duodenoscope (JF-240; Olympus Optical Co., Ltd. Tokyo, Japan).

As the fiber itself is not detectable by fluoroscopy, the radio-opaque tip of the balloon catheter was positioned near the stone, and the balloon was expanded to fix the position of the catheter and the fiber. The tip of the fiber was advanced a few millimeters beyond the radio-opaque tip of the balloon catheter, under fluoroscopic guidance, to ensure that the fiber was positioned on the stone surface. Only one fluoroscopic plane was used to target the stone and to observe fragmentation. The treatment energy level was 120 mJ per pulse at a repetition rate of 8 to 10 Hz. During the laser treatment, we tried to listen to the fragmentation sound of stone by using stethoscope and the fragmentation effect was monitored by fluoroscopy after instillation of contrast media into the bile duct. During laser lithotripsy, the bile duct was continuously irrigated with a mixture of contrast media and saline solution because fluid is required for the generation of shock waves.

After stone disintegration, endoscopic extraction of stones and fragments was done with a Dormia basket and balloon catheter. Laser lithotripsy was limited to a maximum duration of 60 min per session. If, at that time, the ductal clearance was incomplete, then a nasobiliary catheter was inserted. Failure of laser lithotripsy was defined as the inability to achieve complete bile duct

clearance after a maximum of three lithotripsy sessions.

All patients were followed for at least two days after the FREDDY LL. Follow-up included simple radiographs and laboratory tests (hemoglobin, white blood cell count, bilirubin, alanine aminotransferase, aspartate aminotransferase, creatinine, serum urea nitrogen, amylase and lipase levels). The occurrence of any complication within one month of the FREDDY LL procedure was assessed by a patient visit or telephone interview.

## RESULTS

### Lithotripsy

Complete stone fragmentation using FREDDY LL was achieved in 15 of 17 patients without direct visual control (Figure 1). The number of endoscopic retrograde cholangiography sessions per patient that was necessary to totally clear the bile ducts was  $1.7 \pm 0.6$ . After FREDDY LL, 3 patients underwent mechanical lithotripsy to achieve complete clearance of the bile duct.

The stone fragmentation failed in two patients, in whom the positioning of the laser fiber on the stone was inadequate due to a huge impacted stone in the tortuous common bile duct and the biliary stricture. One patient was sent to the surgical department to clear the bile duct by laparoscopic surgery. The other patient was treated by percutaneous cholangioscopy with electro-hydraulic lithotripsy.

### Complications

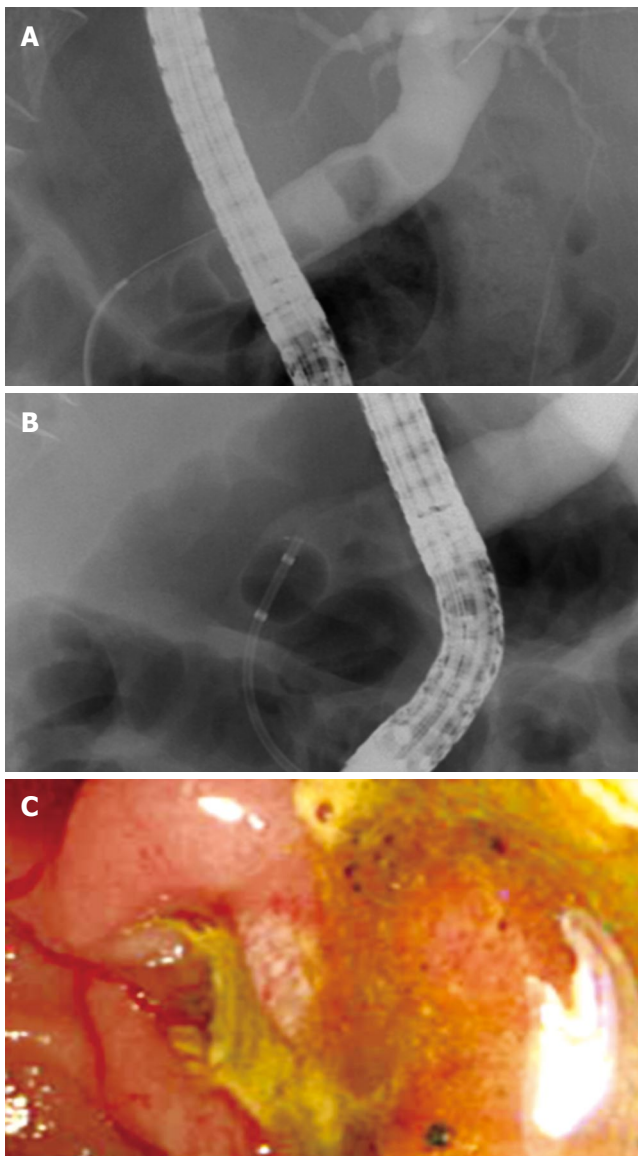
The side effects and complications of FREDDY LL were mostly mild. Although acute pancreatitis occurred in one patient, it was treated by conservative management, and there was no pseudocyst. Transient hemobilia was observed in two patients. However, the bleeding stopped spontaneously without any change of the hemoglobin level. The hemobilia may have been caused by mucosal damage from insertion of the guiding catheter or the balloon catheter. A patient with acute cholangitis was treated by administration of antibiotics and nasobiliary tube irrigation. This complication may have been caused by transient obstruction of the nasobiliary tube.

## DISCUSSION

Endoscopic retrograde cholangiopancreatography (ERCP) and sphincterotomy have been the standard treatments for choledocholithiasis, yet large stones present a major challenge for the endoscopist. Extending a sphincterotomy increases the risk of bleeding and perforation, and mechanical lithotripters are generally expensive, cumbersome to use, and fragile, and they fail to grasp the stones in a significant number of cases. For treating these patients, the complementary techniques such as extracorporeal shock wave, intracorporeal electrohydraulic, or laser induced lithotripsy are required<sup>[2]</sup>.

Electrohydraulic intracorporeal lithotripsy (EHL) represents an effective treatment option for the endoscopic treatment of difficult bile duct stones. However, EHL requires continuous visual control of the fragmentation procedure because of the high pulse energies that are used.





**Figure 1** Complete stone fragmentation using FREDDY without direct visual control. **A:** Cholangiogram showing stone impacted in the common bile duct; **B:** Cholangiogram made during FREDDY laser lithotripsy, showing multiple stone fragments; **C:** Endoscopic findings showing the fragmented stones from the bile duct.

The probe contacting with the bile duct wall may result in perforation or bleeding. Cholangioscopy with continuous saline solution irrigation is routinely performed during EHL. Transpapillary cholangioscopy with a mother-baby endoscope requires two experienced endoscopists. In addition, this type of endoscope is so fragile, and its cost is high. Also, EHL is associated with a high level of exposure to ionizing radiation for both the patient and the endoscopist<sup>[4,5]</sup>. Data on laser lithotripsy for complicated bile duct stones have been reported by several centers. Stone fragmentation rates of about 80%-90% have been reported for the coumarin green and Nd:YAG laser under direct cholangioscopic control. When performing laser lithotripsy under fluoroscopic guidance, the method failed in up to 80% of patients, because positioning of the glass fiber on the stone was difficult. But in 1993, a flash-lamp pulsed laser with a tissue-stone recognition system was introduced that could identify gallstones by

analyzing the back-scattered light and the energy pulse was interrupted in case of tissue contact. The visual control of placing the tip of the probe to the surface of the stone is facilitated by a helium-aiming beam. This system allows the treatment to be performed under fluoroscopic control with excellent safety and fragmentation success rates of up to 90%. Fluoroscopic control using a rhodamine 6G laser was effective in clearing the bile ducts in about 80% of patients<sup>[2,8]</sup>.

The FREDDY laser has been developed for endoscopic lithotripsy to disintegrate urinary stones. We applied this laser on the biliary stone that was not amenable to standard endoscopic procedure including endoscopic sphincterotomy and mechanical lithotripsy through the transpapillary route. In our study, stone disintegration through the transpapillary route was achieved in 15 of 17 patients (88%). The FREDDY laser is a short pulsed Q-switched frequency-doubled, double pulse Nd:YAG solid-state laser that allows the emission of long pulses. This laser is capable of producing very high pulse intensity because of the partial frequency doubling of the infrared ray into the green range that works synergistically. Laser light at 532 nm (the green spectrum) initiates plasma formation at the stone surface, while light at a wavelength of 1064 nm heats the preformed plasma, to cause expansion and contraction, which fragments calculi, and the pulse duration is 0.5-1.5  $\mu$ s<sup>[9]</sup>. This laser generates very short impulses that are poorly absorbed by soft tissue, and so the tissue is exposed to virtually no thermal effect. *In vitro* experiments show that FREDDY laser is very suitable for performing lithotripsy, and animal model studies have shown little to no effect on normal tissues<sup>[10]</sup>.

Also, in comparison with the holmium laser, the FREDDY laser produces no thermal effect<sup>[11]</sup>. This laser showed a high degree of fragmentation efficiency (95%) on the urinary stone, but yet any studies of its effect on biliary stones are rare. There was an 88% fragmentation rate in our study, although the number of patient was small. The FREDDY laser system combines the advantage of solid-state and pulse-dye lasers such as lower cost, good reliability, and excellent effectiveness.

The number of ERCP sessions necessary for duct clearance was higher in our study than reported for other study groups. When laser therapy was done as the first line method for difficult cases, additional ERCP sessions were necessary to achieve complete bile duct clearance<sup>[12]</sup>. A disadvantage of the FREDDY laser system is the high x-ray exposure to patients and the endoscopist. Also, it is difficult and time consuming to target the stone by fluoroscopy because of the positioning of the laser fiber that is inserted through a balloon catheter<sup>[13]</sup>. A certain level of endoscopic technique is required to contact the balloon catheter with laser fiber on the surface of a stone. Positioning of the balloon may be unsatisfactory in a tortuous angulated bile duct like our cases that laser lithotripsy failed. To ensure that the fiber would target the stone exactly and avoid contact with the bile duct, we monitored stone movement in the bile duct, fragmentation sound by applying stethoscope and hemobilia during this laser treatment.

In conclusion, our results suggest that laser lithotripsy using FREDDY without direct visual control was an effective and safe technique for patients with difficult CBD stones that could not be removed by mechanical lithotripsy. In the future, comparative studies with EHL, ESWL, or other laser systems will be required to assess the utility and efficacy of FREDDY laser.

## COMMENTS

### Background

Endoscopic papillotomy is successful in more than 90% of the cases of choledocholithiasis. For patients with difficult bile duct stones not responding to mechanical lithotripsy, different methods for stone fragmentation such as laser lithotripsy or electrohydraulic lithotripsy (EHL) with direct visual control have been developed. A major problem in using this device is the requirement of cholangioscopic guidance or the "mother and baby" endoscope.

### Research frontiers

Recently, a new laser, the Frequency Doubled Double Pulse Nd:YAG (FREDDY), was developed for endoscopic lithotripsy for urinary stones, and has less tissue damage. The efficacy and safety of laser lithotripsy without cholangioscopy by using the balloon catheter were evaluated in patients with bile duct stones that could not be extracted by standard technique.

### Innovation and breakthroughs

The FREDDY laser is a newly developed economical, short-pulse, double frequency, solid-state laser that results in less tissue damage. Fluoroscopically guided laser lithotripsy with a balloon catheter through a peroral duodenoscope appears to be effective treatment for bile duct stones that cannot be extracted by using conventional techniques such as mechanical lithotripsy.

### Applications

For the fragmentation of the biliary stone, cholangioscopy guidance is expensive due to the cost of the cumbersome endoscopic equipment and the need for two experienced endoscopists operating the "mother and baby" endoscope. When direct visual control is not available or limited, laser lithotripsy with a balloon catheter may be an alternative. To target the laser fiber accurately on the stone in the bile duct, a new and easier method is required.

### Peer review

It is a well-written and interesting paper. The FREDDY laser may be a useful technique in treatment of difficult bile duct stones.

## REFERENCES

- 1 **Classen M**, Hagenmuller F, Knyrim K, Frimberger E. Giant bile duct stones--non-surgical treatment. *Endoscopy* 1988; **20**: 21-26
- 2 **Sauerbruch T**, Feussner H, Frimberger E, Hasegawa H, Ihse I, Riemann JF, Yasuda H. Treatment of common bile duct stones. A consensus report. *Hepatogastroenterology* 1994; **41**: 513-515
- 3 **Neuhaus H**, Hoffmann W, Zillinger C, Classen M. Laser lithotripsy of difficult bile duct stones under direct visual control. *Gut* 1993; **34**: 415-421
- 4 **Binmoeller KF**, Bruckner M, Thonke F, Soehendra N. Treatment of difficult bile duct stones using mechanical, electrohydraulic and extracorporeal shock wave lithotripsy. *Endoscopy* 1993; **25**: 201-206
- 5 **Hochberger J**, Bayer J, May A, Muhldorfer S, Maiss J, Hahn EG, Ell C. Laser lithotripsy of difficult bile duct stones: results in 60 patients using a rhodamine 6G dye laser with optical stone tissue detection system. *Gut* 1998; **43**: 823-829
- 6 **Delvecchio FC**, Auge BK, Brizuela RM, Weizer AZ, Zhong P, Preminger GM. In vitro analysis of stone fragmentation ability of the FREDDY laser. *J Endourol* 2003; **17**: 177-179
- 7 **Neuhaus H**, Hoffmann W, Gottlieb K, Classen M. Endoscopic lithotripsy of bile duct stones using a new laser with automatic stone recognition. *Gastrointest Endosc* 1994; **40**: 708-715
- 8 **Ell C**, Hochberger J, May A, Fleig WE, Bauer R, Mendez L, Hahn EG. Laser lithotripsy of difficult bile duct stones by means of a rhodamine-6G laser and an integrated automatic stone-tissue detection system. *Gastrointest Endosc* 1993; **39**: 755-762
- 9 **Helfmann J**, Muller G. Laser lithotripsy: process and overview. *Med Laser Appl* 2001; **16**: 30-37
- 10 **Zorcher T**, Hochberger J, Schrott KM, Kuhn R, Schafhauser W. In vitro study concerning the efficiency of the frequency-doubled double-pulse Neodymium:YAG laser (FREDDY) for lithotripsy of calculi in the urinary tract. *Lasers Surg Med* 1999; **25**: 38-42
- 11 **Delvecchio FC**, Preminger GM. Endoscopic management of urologic disease with the holmium laser. *Curr Opin Urol* 2000; **10**: 233-237
- 12 **Jakobs R**, Adamek HE, Maier M, Kromer M, Benz C, Martin WR, Riemann JF. Fluoroscopically guided laser lithotripsy versus extracorporeal shock wave lithotripsy for retained bile duct stones: a prospective randomised study. *Gut* 1997; **40**: 678-682
- 13 **Ponchon T**, Gagnon P, Valette PJ, Henry L, Chavaillon A, Thieulin F. Pulsed dye laser lithotripsy of bile duct stones. *Gastroenterology* 1991; **100**: 1730-1736

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## Persistent alanine aminotransferase elevation among the general Iranian population: Prevalence and causes

Raika Jamali, Mahmoodreza Khonsari, Shahin Merat, Masoud Khoshnia, Elham Jafari, Alireza Bahram Kalhori, Hassan Abolghasemi, Sedighe Amini, Mahtab Maghsoudlu, Mohammad Reza Deyhim, Houri Rezvan, Akram Pourshams

Raika Jamali, Masoud Khoshnia, Shahin Merat, Elham Jafari, Akram Pourshams, Digestive Disease Research Center, Shariati Hospital, Medical Sciences/University of Tehran, Tehran 14117, Iran  
Mahmoodreza Khonsari, Department of Internal Medicine Hamadan, Hamadan University of Medical Sciences, Iran  
Alireza Bahram Kalhori, Department of Radiology Gonbad, Social Insurance Organization Khatam Hospital, Golestan, Iran  
Hassan Abolghasemi, Sedighe Amini, Mahtab Maghsoudlu, Mohammad Reza Deyhim, Houri Rezvan, Iranian Blood Transfusion Research Center, Tehran 14117, Iran  
Author contributions: Pourshams A designed the research; Merat S, Jamali R, Khoshnia M, Jafari E and Khonsari M performed the research and wrote the paper; Bahram Kalhori A, Abolghasemi H, Amini S, Maghsoudlu M, Deyhim MR and Rezvan H analyzed the data.

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Correspondence to: Dr. Akram Pourshams, Digestive Disease Research Center, Medical Sciences/University of Tehran, Shariati Hospital, North Kargar Avenue, Tehran 14117, Iran. [purshams@ams.ac.ir](mailto:purshams@ams.ac.ir)

Telephone: +98-21-2415140 Fax: +98-21-2415400

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### Abstract

**AIM:** To determine the prevalence and causes of persistently elevated alanine aminotransferase (ALT) levels among the general population in northern Iran.

**METHODS:** A total of 2292 (1376 female, aged 18-75 year), were selected by systematic clustered random sampling from the cities and villages of Gonbad and Kalaleh in Golestan Province and invited to participate in the study. A comprehensive history regarding alcohol drinking and medication was taken. Body mass index (BMI), viral markers and ALT levels were measured. If ALT level was  $\geq 40$  U/L, it was rechecked twice within 6 mo. Those with  $\geq 2$  times elevation of ALT were considered as having persistently elevated ALT level. Non-alcoholic fatty liver disease (NAFLD) was diagnosed based on evidence of fatty liver upon sonography and excluding other etiology.

**RESULTS:** A total of 2049 (1351 female) patients participated in the study, 162 (7.9%) had elevated ALT level at the first measurement. Persistently elevated ALT level was detected in 64 (3.1%) participants, with

51 (79.6%) with no obvious etiology, six (9.3%) with Hepatitis B, four (6.2%) with Hepatitis C virus (HCV) infection and three (4.6%) with alcoholic hepatitis. The prevalence of NAFLD and alcoholic hepatitis was 2.04% (42 patients) and 0.1% (three), respectively. There was correlation between NAFLD and male gender, overweight, diabetes and living in an urban area [odds ratio = 3.03 (95% CI: 1.6-5.72), 4.21 (95% CI: 1.83-9.68), 2.86 (95% CI: 1.05-7.79) and 2.04 (95% CI: 1.00-4.16) respectively].

**CONCLUSION:** NAFLD is the most common cause of persistently elevated serum ALT level among the general population of Iran.

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**Key words:** Alanine aminotransferase; Iran; Non-alcoholic fatty liver disease; Viral hepatitis

**Peer reviewers:** Edoardo G Giannini, Assistant Professor, Department of Internal Medicine, Gastroenterology Unit, Viale Benedetto XV, No. 6, Genoa, 16132, Italy; Michael Kremer, MD, Skipper Bowles Center for Alcohol Studies, CB# 7178, 3011 Thurston-Bowles Building, University of North Carolina, Chapel Hill, NC 27599, United States

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### INTRODUCTION

End-stage liver disease is among the top ten common causes of morbidity and mortality in adults older than 15 years, worldwide<sup>[1]</sup>. The World Health Organization (WHO) estimates that in 2002, cirrhosis caused 382000 deaths worldwide<sup>[1]</sup>. According to the 2002 mortality report of the Ministry of Health of Iran, chronic liver diseases accounted for 1% of mortality of inhabitants aged > 15 years across the whole country<sup>[2]</sup>. Liver cirrhosis was the third and first leading cause of mortality and



hospitalization, respectively, in an Iranian gastrointestinal and hepatology ward<sup>[3]</sup>. Recognition of the relative contribution of various etiologies to a disease burden is important for setting public health priorities and prevention guidelines. Iran, a Middle East developing country, has an average prevalence of Hepatitis B virus (HBV) and Hepatitis C virus (HCV) infection, 3%-5% and 0.5% respectively<sup>[4-9]</sup>. HBV has been reported as the most common cause of chronic liver disease in the last decade in Iran<sup>[10,11]</sup>. The etiology of chronic liver disease changes by health concerns, national prevention programs, and people's lifestyle. The prevalence of HBV infection among healthy Iranian blood donors has declined; this may be due to better donor selection programs and an increasing number of repeat donors<sup>[12]</sup>. Rapidly increasing metabolic syndrome manifestations, including non-alcoholic fatty liver disease (NAFLD), are reported in Iran<sup>[13]</sup>. Information regarding the causes of chronic liver disease in the general population is rare in Middle-east countries. Alanine aminotransferase (ALT) is a sensitive indicator of liver cell injury and has been used to identify patients with liver disease for almost 50 years<sup>[14]</sup>. Currently, determination of serum ALT level constitutes the most frequently applied test for the identification of patients suffering from liver disease. The aim of this study was to determine the causes of chronic liver disease in the general population in Iran, using ALT as a surrogate marker of liver disease.

## MATERIALS AND METHODS

Our study was carried out according to the ethical standards for human experimentation and was approved by the ethics committee of the Digestive Disease Research Center, Tehran University of Medical Sciences. After explaining the aim of the study and the possible need for further blood tests and follow up, written informed consent was obtained.

### Subjects

Golestan province is located in North East Iran and has 1 614 376 inhabitants. Gonbad and Kalaleh are located in the North east of Golestan province. Gonbad has a population of 43 960, Kalaleh has a population of 154 349. In 2006, a total of 2292 (1376 females) 18-79 year old inhabitants of the villages and cities of Gonbad and Kalaleh were selected by systematic clustering random sampling, according to the data from family registry in health care centers, and were invited to take part in this study. Among the invited subjects, 698 of 916 males and 1351 of 1376 females participated in the study (participation rate: 76.2% and 98.1% for men and women, respectively). General parameters including age, gender, weight, height, drug history, especially during the past 3 mo, alcohol consumption (the number and type of drinks per day), hypertension and diabetes mellitus were ascertained and recorded by a trained general physician. An alcohol drinker was defined as a subject with an alcohol intake of > 20 g/d. Diabetes mellitus and hypertension were diagnosed according to previous diagnosis of these diseases by a physician or the use of drugs to control them. BMI of 25-30 kg/m<sup>2</sup> is defined as overweight and

BMI  $\geq$  30 kg/m<sup>2</sup> is obese<sup>[15]</sup>. Morning serum was obtained for measuring ALT and detection of Hepatitis B surface antigen (HBsAg) and HCV antibody (HCV Ab). HCV Ab was detected using ELISA. HCV recombinant immunoblot assay III was carried out on positive samples of HCV by ELISA. Those with a positive test result were considered positive for anti-HCV and exposed to HCV.

The normal range for the kit, as recommended by the manufacturer, for serum ALT level was 0-40 U/L. ALT levels  $\geq$  40 U/L were considered elevated. In step 2 of the study, all participants with serum ALT level  $\geq$  40 U/L in the first blood sampling were invited again and reassessed for alcohol consumption and medication. In step 3 of the study, participants with elevated serum ALT in the second step were considered as having persistently elevated serum ALT level, and were excluded. Those with normal ALT value in the second step were checked again in 3 mo, and if elevated, were considered to have persistently elevated serum ALT. Participants with persistently elevated ALT levels were examined by liver sonography to detect fatty liver. Participants with persistently elevated ALT levels and a negative test result for viral Hepatitis B and C, non-alcohol drinkers, and a negative history of medication intake, along with evidence of fatty infiltration of the liver upon sonography, were presumed to have NAFLD.

### Laboratory assessments

Serum was tested for ALT level using Hitachi autoanalyser 704 (Roche, Switzerland) with Pars Azmoon reagents kit (Tehran, Iran). HBsAg was measured by Enzygnost HBs Ag 5.0 kit (Dade Behring, Germany). HCV Ab was checked by ELISA using the Anti-HCV-EIA-Avicenna kit (Moscow, Russia), and confirmed with recombinant immunoblot assay III using INNOLIA<sup>TM</sup> HCV-Score kit (Innogenetics, Ghent, Belgium). All sonography was carried out by an expert sonographer using a 3.5-MHz probe (Logiq 200 PRO, Tokyo, Japan). Fatty infiltration of the liver was graded from 1 to 3, based on the echogenicity of the liver. In grade 1, echogenicity was slightly increased, with normal visualization of the diaphragm and the intrahepatic vessel borders. In grade 2, echogenicity was moderately increased, with slightly impaired visualization of the diaphragm or intrahepatic vessels. In grade 3, echogenicity was markedly increased, with poor or no visualization of the diaphragm, intrahepatic vessels, and posterior portion of the right lobe. Liver size was also assessed. If the liver was > 15 cm in length in the mid-clavicular line in the sagittal view, a diagnosis of hepatomegaly was made<sup>[16]</sup>.

### Statistical analysis

Statistical analysis was performed using SPSS, version 10.1 (SPSS, Chicago, IL, USA). Continuous variables were analyzed with the *t* test and categorical variables with  $\chi^2$ . *P* < 0.05 was considered statistically significant. Logistic regression analysis (multivariate analysis) was performed to identify factors independently associated with NAFLD.

## RESULTS

A total of 2049 participants (1351 females and 1073



**Table 1** Relative frequency of the participants according to demographic characteristics, laboratory test results, BMI and gender *n* (%)

Characteristic	Male (698)	Female (1351)	<i>P</i> -value	Total (2049)
Mean age $\pm$ SD (yr)	43.91 $\pm$ 15.52	38.88 $\pm$ 13.94	0.000	40.59 $\pm$ 14.69
Mean BMI $\pm$ SD (kg/m <sup>2</sup> )	25.02 $\pm$ 4.29	26.70 $\pm$ 5.34	0.000	26.13 $\pm$ 5.07
Median BMI (kg/m <sup>2</sup> )	24.91	25.94		25.56
BMI (kg/m <sup>2</sup> )				
< 18.5	40 (5.9)	43 (3.3)		83 (4.2)
18.5-24.9	308 (45.3)	509 (38.8)		817 (41)
25-29.9	240 (35.3)	418 (31.8)		650 (33)
30-34.9	82 (12.1)	248 (18.9)		330 (16.6)
35-39.9	9 (1.3)	73 (5.6)		82 (4.1)
$\geq$ 40	1 (0.1)	22 (1.7)		23 (1.2)
Mean ALT $\pm$ SD (U/L)	24.01 $\pm$ 15.67	19.38 $\pm$ 12.56	0.000	20.96 $\pm$ 13.87
HBsAg positive	38 (5.4)	64 (4.7)	0.340	102 (5)
HCV Ab positive	8 (1.1)	12 (0.8)	0.360	20 (1)
Diabetes mellitus	28 (4)	59 (4.3)	0.400	87 (4.2)
Hypertension	64 (9.1)	155 (11.4)	0.060	219 (10.6)

urban) were included in the study. Relative frequency of the participants according to demographic characteristics, laboratory tests, BMI and sex is shown in Table 1.

A total of 162 participants (7.9%) had elevated serum ALT level at the first measurement. Among the 147 of 162 participants who were invited for further evaluation, 64 were considered to have persistently elevated ALT level. In this group 51 (79.6%) had no obvious etiology, six (9.3%) had Hepatitis B, four (6.2%) had Hepatitis C, and three (4.6%) had alcoholic hepatitis. Drug history was unremarkable. Liver sonography was performed for 51 participants with persistently elevated ALT level with no obvious cause, in which 42 (2.04%) showed fatty liver and were presumed to have NAFLD. Demographic characteristics, laboratory test results, and sonography findings in participants with persistently elevated ALT level and fatty liver are shown in Table 2.

The mean BMI of NAFLD participants was  $30.47 \pm 5.07$  kg/m<sup>2</sup> compared with  $26.04 \pm 5.02$  kg/m<sup>2</sup> of those without NAFLD ( $P = 0.001$ ). In participants with NAFLD, there were 34 who were overweight and obese, and seven who were normal weight ( $P = 0.001$ ). In this study, 34/1095 (3.1%) of the overweight and 22/435 (5%) of the obese participants had NAFLD.

The mean BMI in urban participants ( $26.52 \pm 5.32$  kg/m<sup>2</sup>) was greater than that in their rural counterparts ( $25.69 \pm 4.74$  kg/m<sup>2</sup>) ( $P = 0.035$ ). The prevalence of overweight and obese participants was greater in urban (626) than rural (469) participants ( $P = 0.001$ ). NAFLD was more common in urban (31) than rural (11) participants, and also in men (24) than in women (18) ( $P = 0.005$  and  $0.001$ , respectively). There was a correlation between NAFLD and male gender, overweight and obese, diabetes and living in an urban area [odds ratio = 3.03 (95% CI: 1.6-5.72), 4.21 (95% CI: 1.83-9.68), 2.86 (95% CI: 1.05-7.79) and 2.04 (95% CI: 1.00-4.16), respectively].

## DISCUSSION

The prevalence of once-only elevated ALT levels, after excluding individuals with viral hepatitis, alcohol and

**Table 2** Demographic characteristics, laboratory test results, and sonography findings of presumed non-alcoholic steatohepatitis subjects *n* (%)

Characteristics	Male (24)	Female (18)	Total (42)
Mean age $\pm$ SD (yr)	41.04 $\pm$ 12.32	44.33 $\pm$ 9.12	42.45 $\pm$ 11.06
Mean BMI $\pm$ SD (kg/m <sup>2</sup> )	29.65 $\pm$ 4.07	31.62 $\pm$ 7.41	30.47 $\pm$ 5.7
BMI (kg/m <sup>2</sup> )			
< 18.5	0 (0)	0 (0)	0 (0)
18.5-24.9	3 (12.5)	4 (23.5)	7 (17.1)
25-29.9	9 (37.5)	3 (17.6)	12 (29.3)
30-34.9	9 (37.5)	5 (29.4)	14 (34.1)
35-39.9	3 (12.5)	2 (11.8)	5 (12.2)
$\geq$ 40	0 (0)	3 (17.6)	3 (7.3)
First mean ALT $\pm$ SD (U/L)	62.58 $\pm$ 21.51	61.11 $\pm$ 24.20	61.95 $\pm$ 22.42
Second mean ALT $\pm$ SD (U/L)	51.42 $\pm$ 15.16	45.18 $\pm$ 12.28	48.83 $\pm$ 14.22
Third mean ALT $\pm$ SD (U/L)	62.50 $\pm$ 20.46	54.00 $\pm$ 27.99	56.83 $\pm$ 25.10
Liver texture at sonography			
Grade 1	17 (70.8)	11 (61.1)	28 (66.7)
Grade 2	7 (29.2)	7 (38.9)	14 (33.3)
Grade 3	0 (0)	0 (0)	0 (0)
Liver span > 15 cm in sonography	0 (0)	0 (0)	0 (0)
Diabetes mellitus	1 (4.1)	4 (22)	5 (11.9)
Hypertension	1 (4.1)	2 (11)	3 (7.1)

drug etiology, was 2.1%, which was nearly half of the previously reported prevalence of 5.7% in healthy blood donors in Iran<sup>[7]</sup>. This may have been due to the greater prevalence of overweight and obese participants and male predominance (70.9% and 74.7%, respectively) in the previous study compared with the present study (54.8% and 34%, respectively). It has already been shown that serum ALT level is associated with male gender and obesity<sup>[17-19]</sup>. This value is near the prevalence reported in the Third National Health and Nutrition study in the United States (2.8%)<sup>[18]</sup>.

The prevalence of persistently elevated serum ALT levels (3.1%) in our study is comparable with that in previous studies from Western countries and the study of healthy blood donors in Iran<sup>[7,20]</sup>. The prevalence of HBsAg positivity was 5% in the present study, which is comparable to previous studies in the general population of Hamadan (2.49%), Nahavand (2.3%) and Golestan (9.7%)<sup>[4-6]</sup>. There are no published data about HCV prevalence in the general population in Iran for comparison. According to previous studies performed in healthy blood donors in Tehran, the prevalence of HBsAg positivity has declined sharply during the past 20 years, which is mainly due to better donor selection and an increasing number of repeat donors<sup>[12]</sup>.

NAFLD is a diagnosis of exclusion when clinical and laboratory examination fail to reveal a cause of liver disease. It is suggested when an imaging study shows fatty liver. Liver sonography is non-invasive, safe, widely available and highly sensitive for the detection of fatty liver. In one study that compared the sensitivity of MRI, CT and liver sonography to detect fatty liver, sonography and CT had sensitivity of 100% and 93%, respectively, in detecting fatty changes that involved > 33% of the liver, with a positive predictive value of 62% and 76%, respectively<sup>[21]</sup>. We did not perform liver biopsy for diagnosis of NAFLD, and we used criteria similar to those

of previous epidemiological studies of NAFLD<sup>[18,22]</sup>. Large epidemiological studies on the prevalence of NAFLD in the general population are lacking, but prevalence of 2.8%-24% has been reported<sup>[18,23,24]</sup>. In the present study, the prevalence of NAFLD was 2.04%, which is comparable to that reported previously in healthy blood donors (2.35%) and autopsies (2.1%) in Iran<sup>[7,25]</sup>. Although the participation rate in men was less than in women, mainly due to work constraints in men, NAFLD was three times more prevalent in men, which accords with previous studies in Asia, including the study of healthy blood donors in Iran<sup>[7,26,27]</sup>. Previously, it was thought NAFLD is more prevalent in women, but recent data suggest there is no significant difference between the sexes. Women are overrepresented in studies of NAFLD, but it is unclear if gender is an independent risk factor for the disease<sup>[22]</sup>. The frequency of overweight (54.9%) and obesity (21.9%) in the present study was close to the frequencies reported previously in northern Iran, which showed 62.2% were overweight and 28% were obese<sup>[28]</sup>. The frequency of NAFLD reaches 80% in obese persons; NASH is reported in 9%-30% of obese adults<sup>[29,30]</sup>. In the present study, 34/1095 (3.1%) overweight and 22/435 (5%) obese participants had NAFLD. There is a correlation between BMI and steatosis in several studies<sup>[22]</sup>. We showed that overweight and obesity were associated with a 4.21-fold increased risk in NAFLD participants, and mean BMI was also greater in NAFLD than non-NAFLD participants.

This study showed that living in an urban area is associated with a 2.04-fold increase in NAFLD, and this may be due to daily physical activity, lifestyle and greater prevalence of overweight and obesity in urban participants. NAFLD is associated with type 2 diabetes mellitus and glucose intolerance with or without superimposed obesity. Type 2 diabetes has been described in 20%-75% of NAFLD patients, and may increase the risk of NAFLD more than two-fold compared with that in non-diabetics. In the present study, prevalence of diabetes was 11.9% in NAFLD, and diabetes was associated with a 2.86-fold increase in NAFLD compared with non-diabetics<sup>[30]</sup>. NAFLD is now recognized as the hepatic manifestation of the metabolic syndrome, which includes hyperlipidemia, hypertension, glucose intolerance and obesity. The risk and severity of NAFLD increase with the number of components of the metabolic syndrome<sup>[31]</sup>. According to previous studies in the general population of Iran, metabolic syndrome has a prevalence of 33%, which is more common than the rate of 22% reported by the Third National Health and Nutrition Examination Survey for the USA<sup>[13,32]</sup>. We did not test for uncommon liver diseases such as Wilson's disease,  $\alpha$ 1-antitrypsin deficiency, primary biliary cirrhosis and primary sclerosing cholangitis, which can also be responsible for elevated ALT levels; as they are too infrequent to have had a major impact on our results. There are two studies that show that self-reported use of alcohol and opium are reliable and valid in this population<sup>[27,33]</sup>.

NAFLD was the most common cause of persistently elevated serum ALT levels in our study. The importance of NAFLD and ALT elevation is their impact on mortality as a component of the metabolic syndrome. NAFLD is

not a western disease, and is the most frequent cause of persistently elevated serum ALT level in the Asia-Pacific region<sup>[26]</sup>. Identifying its risk factors and therapeutic strategies to control this public health problem seems reasonable.

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## COMMENTS

### Background

End-stage liver disease is among the top ten common causes of mortality in adults worldwide. Recognition of the risk factors for chronic liver disease is important for establishing prevention guidelines.

### Research frontiers

Non-alcoholic fatty liver disease (NAFLD) has been recognized as the main cause of chronic liver injury in both western and developing countries.

### Innovations and breakthroughs

Prevalence of NAFLD in Iranian populations is comparable to that in western countries. Obesity and other manifestations of the metabolic syndrome, in addition to urban lifestyle, are the main risk factors for NAFLD.

### Applications

National planning to increase knowledge regarding NAFLD and control of its risk factors are recommended.

### Terminology

NAFLD is the hepatic manifestation of the metabolic syndrome that includes insulin resistance, obesity, hyperlipidemia and hypertension. NAFLD is recognized by fat infiltration of hepatocytes and inflammatory reaction in the liver.

### Peer review

This is a good and interesting study. It is a very descriptive report about persistently elevated ALT levels in the Iranian population. The authors screened almost 3200 patients in a north eastern part of Iran for elevated ALT.

## REFERENCES

- 1 **World Health Organization.** The World Health Report: Shaping the future. Geneva: World Health Organization, 2003; 17
- 2 **Naghavi M.** Etiology of death in 18 provinces of Iran in year 2001. Tehran: Ministry of Health and Medical Education IR Iran, 2003; 21
- 3 **Ganji A,** Safavi M, Nouraie SM, Nasser-Moghadam S, Merat Sh, Vahedi H, Malekzadeh R. Digestive and Liver Diseases Statistics in Several Referral Centers in Tehran, 2000-2004. *Govaresh* 2006; **11**: 33-38
- 4 **Amini S,** Mahmoodi MF, Andalibi S, Solati AA. Seroepidemiology of hepatitis B, delta and human immunodeficiency virus infections in Hamadan province, Iran: a population based study. *J Trop Med Hyg* 1993; **96**: 277-287
- 5 **Alizadeh AH,** Ranjbar M, Ansari S, MirArab A, Alavian SM, Mohammad K, Adibi P, Sadri GH, Keramat F, Ardalan A, Arabi M, Gharekhani S, Ataei A, Amraei GR, Hosseinzadeh M, Hatami S, Zali M. Seroprevalence of hepatitis B in Nahavand, Islamic Republic of Iran. *East Mediterr Health J* 2006; **12**: 528-537
- 6 **Gholamreza R,** Shahryar S, Abbasali K, Hamidreza J, Abdolvahab M, Khodabardi K, Danyal R, Nafiseh A.

- Seroprevalence of hepatitis B virus and its co-infection with hepatitis D virus and hepatitis C virus in Iranian adult population. *Indian J Med Sci* 2007; **61**: 263-268
- 7 **Pourshams A**, Malekzadeh R, Monavvari A, Akbari MR, Mohamadkhani A, Yarahmadi S, Seddighi N, Mohamadnejad M, Sotoudeh M, Madjlessi A. Prevalence and etiology of persistently elevated alanine aminotransferase levels in healthy Iranian blood donors. *J Gastroenterol Hepatol* 2005; **20**: 229-233
  - 8 **Ghavanini AA**, Sabri MR. Hepatitis B surface antigen and anti-hepatitis C antibodies among blood donors in the Islamic Republic of Iran. *East Mediterr Health J* 2000; **6**: 1114-1116
  - 9 **Ansar MM**, Kooloobandi A. Prevalence of hepatitis C virus infection in thalassemia and haemodialysis patients in north Iran-Rasht. *J Viral Hepat* 2002; **9**: 390-392
  - 10 **Azimi K**, Sarafi M, Alavian SM, Alawi M, Mikaeli J, Malekzadeh R. Causes of cirrhosis in cirrhotic patients in Shariati hospital. *Govaresh* 2002: 19-26
  - 11 **Ziad Alizadeh B**, Taheri H, Malekzadeh R. Defining the etiologies of chronic Hepatitis in patients visited in several referral centers in Tehran. *Govaresh* 1998: 13-23
  - 12 **Merat S**, Malekzadeh R, Rezvan H, Khatibian M. Hepatitis B in Iran. *Arch Iranian Med* 2000; **3**: 192-201
  - 13 **Azizi F**, Salehi P, Etemadi A, Zahedi-Asl S. Prevalence of metabolic syndrome in an urban population: Tehran Lipid and Glucose Study. *Diabetes Res Clin Pract* 2003; **61**: 29-37
  - 14 **Karmen A**, Wroblewski F, Ladue JS. Transaminase activity in human blood. *J Clin Invest* 1955; **34**: 126-131
  - 15 **US Department of Health and Human Services**. Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults. Washington, DC: US Department of Health and Human Services, 1998: 15
  - 16 **Mc Gahan JP**, Goldenberg BB. Liver. In: McGahan JP, Goldberg BB, eds. *Diagnostic Ultrasound: a Logical Approach*. Philadelphia: Lippincott-Raven, 1998: 599-691
  - 17 **Mohamadnejad M**, Pourshams A, Malekzadeh R, Mohamadkhani A, Rajabiani A, Asgari AA, Alimohamadi SM, Razjooyan H, Mamar-Abadi M. Healthy ranges of serum alanine aminotransferase levels in Iranian blood donors. *World J Gastroenterol* 2003; **9**: 2322-2324
  - 18 **Ruhl CE**, Everhart JE. Determinants of the association of overweight with elevated serum alanine aminotransferase activity in the United States. *Gastroenterology* 2003; **124**: 71-79
  - 19 **Clark JM**, Brancati FL, Diehl AM. The prevalence and etiology of elevated aminotransferase levels in the United States. *Am J Gastroenterol* 2003; **98**: 960-967
  - 20 **Anderson NA**, Raafat A, Shwe KH, Barbara J, Contreras M, Fraser ID, Gunson HH, Martlew V, Mijovic V, Goldie DJ. U.K. multicentre study on blood donors for surrogate markers of non-A non-B hepatitis. Part I: Alanine transferase and anti-HBc testing. *Transfus Med* 1992; **2**: 301-310
  - 21 **Saadeh S**, Younossi ZM, Remer EM, Gramlich T, Ong JP, Hurley M, Mullen KD, Cooper JN, Sheridan MJ. The utility of radiological imaging in nonalcoholic fatty liver disease. *Gastroenterology* 2002; **123**: 745-750
  - 22 **Feldman M**, Friedman L S, Brandt L J. Nonalcoholic fatty liver disease. *Gastrointestinal and Liver Disease*. 8th ed. Canada: Saunders, 2006: 1793-1805
  - 23 **Neuschwander-Tetri BA**, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. *Hepatology* 2003; **37**: 1202-1219
  - 24 **Bellentani S**, Saccoccio G, Masutti F, Croce LS, Brandi G, Sasso F, Cristanini G, Tiribelli C. Prevalence of and risk factors for hepatic steatosis in Northern Italy. *Ann Intern Med* 2000; **132**: 112-117
  - 25 **Sotoudehmanesh R**, Sotoudeh M, Ali-Asgari A, Abedi-Ardakani B, Tavangar SM, Khakinejad A, Sadeghi Z, Malekzadeh R. Silent liver diseases in autopsies from forensic medicine of Tehran. *Arch Iran Med* 2006; **9**: 324-328
  - 26 **Chitturi S**, Farrell GC, George J. Non-alcoholic steatohepatitis in the Asia-Pacific region: future shock? *J Gastroenterol Hepatol* 2004; **19**: 368-374
  - 27 **Pourshams A**, Saadatian-Elahi M, Nouraie M, Malekshah AF, Rakhshani N, Salahi R, Yoonessi A, Semnani S, Islami F, Sotoudeh M, Fahimi S, Sadjadi AR, Nasrollahzadeh D, Aghcheli K, Kamangar F, Abnet CC, Saidi F, Sewram V, Strickland PT, Dawsey SM, Brennan P, Boffetta P, Malekzadeh R. Golestan cohort study of oesophageal cancer: feasibility and first results. *Br J Cancer* 2005; **92**: 176-181
  - 28 **Bahrami H**, Sadatsafavi M, Pourshams A, Kamangar F, Nouraei M, Semnani S, Brennan P, Boffetta P, Malekzadeh R. Obesity and hypertension in an Iranian cohort study; Iranian women experience higher rates of obesity and hypertension than American women. *BMC Public Health* 2006; **6**: 158
  - 29 **Hsiao TJ**, Chen JC, Wang JD. Insulin resistance and ferritin as major determinants of nonalcoholic fatty liver disease in apparently healthy obese patients. *Int J Obes Relat Metab Disord* 2004; **28**: 167-172
  - 30 **Dixon JB**, Bhathal PS, O'Brien PE. Nonalcoholic fatty liver disease: predictors of nonalcoholic steatohepatitis and liver fibrosis in the severely obese. *Gastroenterology* 2001; **121**: 91-100
  - 31 **Marchesini G**, Bugianesi E, Forlani G, Cerrelli F, Lenzi M, Manini R, Natale S, Vanni E, Villanova N, Melchionda N, Rizzetto M. Nonalcoholic fatty liver, steatohepatitis, and the metabolic syndrome. *Hepatology* 2003; **37**: 917-923
  - 32 **Ford ES**, Giles WH, Dietz WH. Prevalence of the metabolic syndrome among US adults: findings from the third National Health and Nutrition Examination Survey. *JAMA* 2002; **287**: 356-359
  - 33 **Abnet CC**, Saadatian-Elahi M, Pourshams A, Boffetta P, Feizzadeh A, Brennan P, Taylor PR, Kamangar F, Dawsey SM, Malekzadeh R. Reliability and validity of opiate use self-report in a population at high risk for esophageal cancer in Golestan, Iran. *Cancer Epidemiol Biomarkers Prev* 2004; **13**: 1068-1070

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RAPID COMMUNICATION

## A simple and rapid method for extracting bacterial DNA from intestinal microflora for ERIC-PCR detection

Jin-Long Yang, Ming-Shu Wang, An-Chun Cheng, Kang-Cheng Pan, Chuan-Feng Li, Shu-Xuan Deng

Jin-Long Yang, Chuan-Feng Li, Shu-Xuan Deng, Avian Diseases Research Center, College of Veterinary Medicine of Sichuan Agricultural University, Yaan 625014, Sichuan Province, China

An-Chun Cheng, Ming-Shu Wang, Kang-Cheng Pan, Avian Diseases Research Center, College of Veterinary Medicine of Sichuan Agricultural University; Key Laboratory of Animal Diseases and Human Health of Sichuan Province, Yaan 625014, Sichuan Province, China

Ming-Shu Wang, College of Life Science and Technology of Southwest University for Nationalities, Chengdu 610041, Sichuan Province, China

**Author contributions:** Yang JL, Wang MS, Cheng AC and Pan KC contributed equally to this work; Cheng AC, Wang MS designed the research; Yang JL, Pan KC, Li CF and Deng SX performed the research; Li CF and Deng SX analyzed the data; Yang JL, Cheng AC and Wang MS wrote the paper.

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**Correspondence to:** Professor An-Chun Cheng, Avian Diseases Research Center, College of Veterinary Medicine of Sichuan Agricultural University, Yaan 625014, Sichuan Province, China. [chenganchun@vip.163.com](mailto:chenganchun@vip.163.com)

Telephone: +86-835-2885774 Fax: +86-835-2885774

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### Abstract

**AIM:** To develop a simple and convenient method for extracting genomic DNA from intestinal microflora for enterobacterial repetitive intergenic consensus (ERIC)-PCR detection.

**METHODS:** Five methods of extracting bacterial DNA, including Tris-EDTA buffer, chelex-100, ultrapure water, 2% sodium dodecyl sulfate and 10% Triton-100 with and without sonication, were compared with the commercial fecal DNA extraction kit method, which is considered as the gold standard for DNA extraction. The comparison was based on the yield and purity of DNA and the indexes of the structure and property of micro-organisms that were reflected by ERIC-PCR.

**RESULTS:** The yield and purity of DNA obtained by the chelex method was similar to that obtained with the fecal DNA kit. The ERIC-PCR results obtained for the DNA extracted by the chelex method and those obtained for DNA extracted with the fecal DNA kit were basically the same.

**CONCLUSION:** The chelex method is recommended for ERIC-PCR experiments in view of its simplicity and cost-effectiveness; and it is suitable for extracting total DNA from intestinal micro-organisms, particularly for handling a large number of samples.

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**Key words:** DNA extraction; Intestinal microflora

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### INTRODUCTION

The intestinal tract of animals harbors a large, active, and complex community of microbes<sup>[1]</sup>. There are at least 400-500 different microbial species, constituting a complex ecosystem<sup>[2]</sup>. They play significant roles in colonization resistance, i.e., the prevention of colonization of pathogens in the gastrointestinal tract and the growth of autochthonous opportunistic micro-organisms<sup>[3]</sup>. Intestinal bacteria that synthesize or metabolize potential carcinogens and produce anti-tumorigenic products may be related to colorectal cancer, which is the second most common cause of cancer death in the USA<sup>[4]</sup>.

Probiotics are defined as living organisms which, on ingestion in certain numbers, exert health benefits beyond the inherent basic nutrition in the host<sup>[5,6]</sup>. In humans, probiotics are effective in preventing the onset and relapse of pouchitis<sup>[7,8]</sup>, and in mice, they effectively prevent experimental colitis and reduce gut bacterial translocation<sup>[9,10]</sup>.

Commensal intestinal microflora have been monitored by cultivation-based techniques and molecular detections<sup>[11,12]</sup>. Enterobacterial repetitive intergenic consensus (ERIC)-PCR uses oligonucleotides targeting short repetitive sequences that are dispersed throughout various bacterial genomes<sup>[11]</sup>. Commensal intestinal microflora can be identified at the genus, species and strain levels based on the electrophoretic pattern of amplified products<sup>[13-15]</sup>.



The capacity of these assays to monitor bacteria in animal intestines depends on the efficiency of PCR. One of the key factors in obtaining the PCR fingerprinting patterns from the intestinal microflora is the efficiency of the DNA extraction procedure.

Usually, two factors have to be particularly considered during the extraction procedure. The first is to maximize the DNA yield. The second is to ensure that the extracted DNA is amenable to several enzymatic treatments like PCR amplification<sup>[16]</sup>. In other words, the greatest challenge is the extraction of high-quality PCR-compatible DNA from the intestinal microflora. Several methods have been evaluated for bacterial cell wall lysis and DNA extraction using detergents, proteolytic enzymes, lysozyme, mechanical disruption, temperature changes alone or in various combinations, DNA stool mini- kit, *etc.* Although the DNA stool mini-kit method is convenient, rapid and highly efficient, it is not widely applied on account of its high cost<sup>[17-20]</sup>.

In this study, 5 methods of extracting bacterial DNA were compared with that using the fecal DNA kit (FDK). The purpose of this study was to establish an economical, simple, and convenient method for extracting genomic DNA from the intestinal microflora for ERIC-PCR detection.

## MATERIALS AND METHODS

### Collection and preparation of fecal samples

Fresh fecal samples were collected from 20 10-d-old healthy goslings. No subject had received antibiotics, probiotics, or prebiotics. Gosling care and experimental procedures were performed in compliance with the Institutional Animal Care and Use Guidelines provided by the Centers for Disease Control and Prevention. Two goslings were randomly selected and their fecal samples from rectum were collected separately. The samples were collected in sterile bags, refrigerated, and immediately taken to the laboratory.

### Total DNA extraction

Each of the six extraction methods was repeated 3 times. Each sample (20 mg) was first thawed and suspended in 5.0 mL phosphate-buffered saline (PBS; pH 7.2). Centrifugation was carried out at 100 *g* for 15 min at 4°C in order to remove the fecal pellets; and the obtained supernatant was centrifuged at 13000 *g* at 4 °C for 10 min. The pellet was then washed 3 times by suspending it in 1.5 mL acetone. Each preparation was centrifuged at 13000 *g* for 10 min at 4°C in order to remove potential PCR inhibitors in stool<sup>[21]</sup>. The supernatant was discarded, and the pellet was processed for each procedure as follows. (1) The TE boil extraction method (T method): It is a modification of the bacterial DNA extraction protocol described by Li *et al.*<sup>[22]</sup>. The pellet was suspended in 200 µL TE buffer [10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA]<sup>[23]</sup>, and the mixture was briefly mixed on a vortex mixer. The suspension was placed in a boiling water bath for 1 min, subjected to 3 freeze-thaw cycles alternating between -70°C for 3 min and 100°C for 2 min, and then centrifuged at 10000 *g* for 5 min. A 100 µL aliquot of the

supernatant was transferred to a sterile tube and stored at -20°C until PCR testing. (2) The ultrapure water method (UW method): A 200 µL aliquot of ultrapure water was added to the pellet, and the suspension was treated as described above for the TE buffer. (3) The chelex method (C method): This method is a modification of bacterial DNA extraction protocol described by Emi Suenaga<sup>[24]</sup>. A 200 µL aliquot of Chelex-100 (5%) and 0.2 mg protease K was added to the pellet and the sample was incubated at 56°C for 30 min in a water bath. The mixture was then briefly mixed on a vortex mixer and centrifuged at 10000 *g* for 5 min. A 100 µL aliquot of the supernatant was transferred to a sterile tube and stored at -20°C until PCR testing. (4) SDS method: The pellets were treated as described above for the TE buffer, except that 200 µL of the nonionic detergent mix (2% SDS containing 10% Triton X-100) was substituted for the TE buffer<sup>[18]</sup>. (5) The SDSS method: A 200 µL aliquot of 2% SDS containing 10% Triton X-100 was added to the pellet, and the mixture was briefly agitated on a vortex mixer. The suspension was sonicated for 15 min and then treated as described above for the TE buffer<sup>[18]</sup>. (6) The FDK method: The pellets were processed using the Fecal DNA Kit™ (Tianli, China) and the DNA was purified with a spin column according to the manufacturer's instructions.

### Measurement of DNA concentration and purity

The concentration and purity of DNA were determined spectrophotometrically (BIO-RAD Smart Spec 3000; USA); for this purpose, DNA absorbance was measured at 260 nm (µg DNA/g sample; 1  $A_{260}$  = 50 µg/mL DNA) and protein impurities were checked at 280 nm<sup>[25]</sup>. The concentration and purity of each DNA extraction method was statistically analyzed by Excel 2003 for Windows.

### Genomic DNA detection by different extraction methods

For each method tested, the presence and quality of the extracted genomic DNA from one of the triplicate samples was analyzed using a 0.5% agarose gel containing ethidium bromide at 4°C. Ten microliters of the DNA extracted by each method was added into the gel and electrophoresed for 30 min at 150 V. Gel images were acquired as tagged image file format (TIFF) files with a Gel Imaging System (Bio-Rad). Unless mentioned otherwise, the following molecular procedures were carried out with the extracted genomic DNA obtained from 1 or 2 of the triplicate samples.

### ERIC-PCR and statistical analysis

For fingerprinting the bacterial population in fecal samples, the total fecal DNA was used as a template for ERIC-PCR, and the sequence of the ERIC primers were E1 (ERIC1R): 5'-ATGTAAGCTCCTGGGGATTACAC-3' and E2 (ERIC2): 5'-AAGTAAGTGACTGGGGTGAGCG-3' which was described by Versalovic *et al.*<sup>[11]</sup>. The ERIC-PCRs were performed under the conditions described by Di Giovanni *et al.*<sup>[26]</sup>.

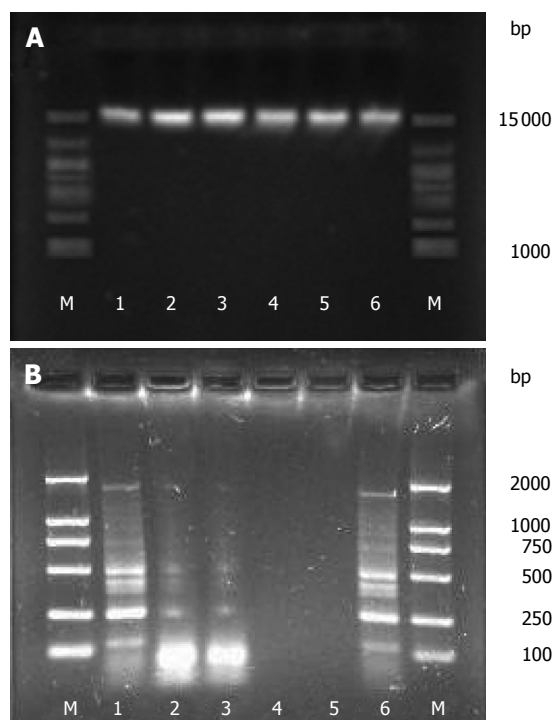
We analyzed the PCR products by agarose gel electrophoresis as described by Melanie W Syrmis<sup>[27]</sup>. We acquired gel images as TIFF files using a Gel Imaging System (Bio-Rad).

**Table 1** DNA yields and purity obtained by six DNA extraction methods ( $\mu\text{g}$  DNA/g sample)

Samples	T-method	C-method	UW-method	SDS-method	SDSS-method	FDK-method
Yield	601.52	1301.52	586.03	1710.95	1820.12	1326.07
Purity ( $A_{260/280}$ )	1.58	1.84	1.6	1.54	1.49	1.82

**Table 2** Cs matrix values of ERIC-PCR fingerprint of the samples extracted by different methods (%)

Samples	T-method	C-method	UW-method	SDS-method	SDSS-method	FDK-method
C-method	25	-	25	0	0	92.1
UW-method	100	25	-	0	0	25
SDS-method	0	0	0	-	100	0
SDSS-method	0	0	0	100	-	0
FDK-method	25	92.1	25	25	25	-

**Figure 1** Electrophoresis results with the samples extracted by different methods. **A:** The genomic DNA; **B:** The ERIC-PCR products. M: marker (TIANYI, China, D-15000); 1: C-method; 2: T-method; 3: UW-method; 4: SDS-method; 5: SDSS-method; 6: FDK-method.

To interpret the ERIC-PCR fingerprint profiles, the Quantity One software package (Applied Bio-Rad) was used with pairwise similarity coefficient ( $C_s$ )<sup>[28]</sup>. Fingerprints were assigned to a different type when any band differences were observed. Variations in band intensity were not considered as band differences. The fingerprints were considered similar when the  $C_s$  was more than 90%, different when the  $C_s$  was 0, and identical when the  $C_s$  was 100%<sup>[28]</sup>.

## RESULTS

### Yield, purity and quality of DNA obtained by different methods

The yield, purity, and quality of genomic DNA obtained from the six extraction methods are shown in Table 1. The quality of the extracted DNA was evaluated by the  $A_{260/280}$  ratio, and values close to 1.8 indicate a good DNA extract with little protein contamination<sup>[25]</sup>. Although high-molecular-weight DNA (approximately 15 kb) was acquired by all the six methods (Figure 1A), discrepancies in the yield and purity of DNA were observed among different methods. For instance, the SDSS and SDS

methods yielded the maximum amount of DNA (mean, 1820.12  $\mu\text{g/g}$  and 1710.95  $\mu\text{g/g}$ , respectively), but the lowest  $A_{260/280}$  ratio (mean, 1.49 and 1.54, respectively), thus implying the lowest purity of DNA. The T and UW methods yielded the least amount of DNA (mean, 601.52  $\mu\text{g/g}$  and 586.03  $\mu\text{g/g}$ , respectively) and lower purity of DNA (mean  $A_{260/280}$  ratio, 1.58 and 1.60, respectively). The C and FDK methods yielded similar amounts of DNA (mean, 1301.52  $\mu\text{g/g}$  and 1326.07  $\mu\text{g/g}$ , respectively) and similar  $A_{260/280}$  ratios (mean, 1.84 and 1.82, respectively). The purity of the DNA obtained by the two methods was the highest.

### Analysis of ERIC-PCR fingerprints

The amplifications of ERIC-PCR were confirmed by agarose gel electrophoresis. Except for the SDSS and SDS methods, the DNA extracted by the remaining methods was effectively amplified (Figure 1B).

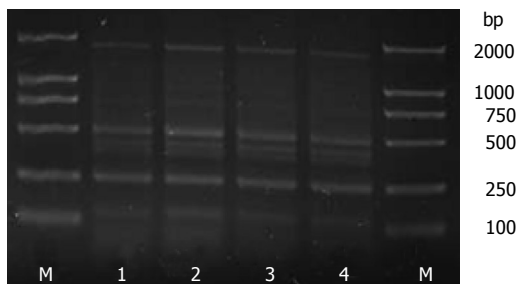
The ERIC-PCR fingerprint profiles of the genomic DNA of intestinal bacterium extracted from the same sample by the six methods showed that the results of the C and FDK methods were consistent, and the  $C_s$  value for these methods was 92.1%. However, the  $C_s$  values by other methods were lower than 30%, and the  $C_s$  by SDSS and SDS methods was 0 (Table 2).

The ERIC-PCR fingerprint profiles of the intestinal bacterial genomic DNA extracted from the same sample by the C and FDK methods showed that the C method (1) had a 100%  $C_s$  value when compared with the C method (2), and the FDK method (1) had a 100%  $C_s$  value when compared with FDK method (2) (Figure 2 and Table 3). These results indicated that the stability and reproducibility of the C and FDK methods were equally good.

## DISCUSSION

Although extensive researches with ERIC-PCR have been conducted on the bacterial microflora in the intestine, the effect of the method selected for DNA extraction on the analysis of a bacterial community has barely been considered. This study is important as DNA extraction is one of the most commonly used procedures in genetics, molecular biology, and biochemistry.

Some important factors that should be considered when choosing a DNA extraction method are the time required to complete the extraction, the cost of extraction, and the safety of the chemical reagents employed. Moreover, DNA fragmentation should be avoided during the extraction.



**Figure 2** Electrophoresis for ERIC-PCR fingerprint of the samples extracted by C-method and FDK-method. Lanes: M: marker (TIANYI, China, DL-2000); 1 and 2: C-method; 3 and 4: FDK-method.

From the extraction methods already published for various bacteria, we chose and compared six methods for extracting DNA from the intestinal microflora: the T, C, UW, SDSS, SDS, and commercial FDK methods.

The aim of an extraction procedure is to obtain a high quality and high yield of DNA from the samples. The extracted DNA should contain the least amount of proteins, RNA, or any other PCR inhibitors such as bile salt and cholerythrin, which are present in the feces<sup>[28,29]</sup>. Removing those inhibitors is one of the key factors for a successful PCR. In this study, we used acetone to remove the inhibitors from the stool samples, as reported by Zhong Hua *et al.*<sup>[21]</sup>. Our ERIC-PCR results was found successful in removal of these inhibitors.

DNA absorbance was measured at 260 nm ( $A_{260}$ ) to evaluate the quantity of the extracted DNA, and the ratio of the absorbance at 260 nm to that at 280 nm ( $A_{260/280}$ ) was used to evaluate the DNA quality. This method was employed previously by other researchers to compare different DNA extraction methods<sup>[30,31]</sup>.

In this study, the results of the concentration and the purity for each method were correlated. The SDSS and SDS methods seemed to yield a greater quantity of DNA, but their  $A_{260/280}$  ratio indicated a high protein contamination. On the other hand, the concentrations of the DNA obtained using the C and FDK methods were lower, but the  $A_{260/280}$  ratio showed a high purity of the DNA obtained. When applied to engorged individuals, the C method resulted in 100% successful DNA amplification that was similar to the FDK method. In our study, the C method provided the best results with a Cs close to 92.1% when compared with the FDK method.

Bacterial lysis is the key to obtain bacterial DNA. Although the SDS and SDSS methods can provide the highest DNA yield, the SDS residue inhibits the PCR process. This result is consistent with that reported by Khan<sup>[18]</sup> and Wade<sup>[17]</sup>. The excessive SDS above 0.01% has been shown to inhibit PCR by denaturing the Taq polymerase.

Many reports have described DNA extraction from tissues of other organisms using chelex-100. The results vary by different methods<sup>[32,33]</sup>. The use of chelex-100 has been recommended for DNA extraction in some papers, but other reports have not regarded it as being optimum because of its lowest efficiency for DNA amplification<sup>[32]</sup>. However, we found that the modified chelex-100 protocol was the best method to extract DNA from stool. Although

**Table 3** Cs matrix values of ERIC-PCR fingerprint of the samples extracted by C-method and FDK-methods (%)

Samples	C-method (1)	C-method (2)	FDK-method (1)	FDK-method (2)
C-method (2)	100.0		92.1	92.1
FDK-method (1)	92.1	92.1		100.0
FDK-method (2)	92.1	92.1	100.0	

there are methodological differences between our study and others<sup>[32,33]</sup>, the conclusion is the same that this method is simple and efficient for PCR.

Theoretically, column-purified DNA should be the cleanest, containing the least PCR-inhibitory substances. One purpose of our study was to identify a method for rapid DNA extraction that did not compromise PCR sensitivity. We used the FDK extraction method as the gold standard for PCR purification. Our results showed that for PCR, column purification was unnecessary for DNA extracted from the intestinal microflora. Compared to FDK that requires 2 h, DNA extraction with chelex-100 can be completed within less than 1 h, and it does not involve 3-4 transfers of samples to new tubes.

In conclusion, of the 5 extraction methods evaluated, the chelex method is technically simpler, less expensive, and more rapid than the FDK method; it is the best method for extracting genomic DNA from the intestinal microflora. Although the extraction with TE buffer and ultrapure water is extremely easy and inexpensive, the DNA yield is the lowest. The SDS and SDSS methods consistently inhibit PCR, therefore, they cannot be recommended for DNA extraction from the intestinal microflora.

## COMMENTS

### Background

The term probiotic is a relatively new term that implies "for life". Currently, it is used to refer to intestinal bacteria that have beneficial effects on humans and animals. Probiotics can play an important role in immunological, digestive, and respiratory functions and could have a significant effect in alleviating infectious diseases in children and other high-risk populations. Thus, knowledge about probiotics could contribute to improving human health. It is necessary to understand the bacterial flora in the gastrointestinal tract, particularly at the species level of bacterium. The DNA-based techniques often represent the most appropriate approach in this regard.

### Research frontiers

To date, the chelex method has mainly been used for medicolegal investigations. Since it is a rapid, simple, and inexpensive technique for extracting genomic DNA from intestinal microflora, it will be an ideal method to study the microflora in the gastrointestinal tract.

### Innovations and breakthroughs

In previous studies on DNA extraction from the intestinal microflora, researchers used the traditional DNA extraction process that involved proteinase K digestion by phenol chloroform extraction or the FDK method. However, the chemical products employed in these experiments were either toxic or expensive. Through the present study, we provide a significantly improved method for the rapid, safe, simple and economical extraction of DNA from the intestinal microflora.

### Applications

This study details a new, simple protocol for extracting DNA from the intestinal



bacterial microflora and may ultimately provide new insights in the field of probiotics.

### Peer review

In this study, through the development of new methods for extracting bacterial DNA from the intestinal microflora, the authors found that the chelex method is a simpler, more rapid, and a less expensive method than the FDK method. Moreover, the results presented herein may lead to the development of a novel convenient approach for monitoring the intestinal microflora by molecular detection methods.

## REFERENCES

- Steinhoff U. Who controls the crowd? New findings and old questions about the intestinal microflora. *Immunol Lett* 2005; **99**: 12-16
- Moore WE, Cato EP, Holdeman LV. Some current concepts in intestinal bacteriology. *Am J Clin Nutr* 1978; **31**: S33-S42
- Falk PG, Hooper LV, Midtvedt T, Gordon JI. Creating and maintaining the gastrointestinal ecosystem: what we know and need to know from gnotobiology. *Microbiol Mol Biol Rev* 1998; **62**: 1157-1170
- Nechvatal JM, Ram JL, Basson MD, Namprachan P, Niec SR, Badsha KZ, Matherly LH, Majumdar AP, Kato I. Fecal collection, ambient preservation, and DNA extraction for PCR amplification of bacterial and human markers from human feces. *J Microbiol Methods* 2008; **72**: 124-132
- Guarner F, Schaafsma GJ. Probiotics. *Int J Food Microbiol* 1998; **39**: 237-238
- Food and Agriculture Organization of the United Nations/World Health Organization. Health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. World Health Organization online, 2001-10-01, cited 2007-11-08. Available from: URL: [http://www.who.int/foodsafety/publications/fs\\_management/en/probiotics.pdf](http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf)
- Mimura T, Rizzello F, Helwig U, Poggioli G, Schreiber S, Talbot IC, Nicholls RJ, Gionchetti P, Campieri M, Kamm MA. Once daily high dose probiotic therapy (VSL#3) for maintaining remission in recurrent or refractory pouchitis. *Gut* 2004; **53**: 108-114
- Gionchetti P, Rizzello F, Helwig U, Venturi A, Lammers KM, Brigidi P, Vitali B, Poggioli G, Miglioli M, Campieri M. Prophylaxis of pouchitis onset with probiotic therapy: a double-blind, placebo-controlled trial. *Gastroenterology* 2003; **124**: 1202-1209
- Shen TY, Qin HL, Gao ZG, Fan XB, Hang XM, Jiang YQ. Influences of enteral nutrition combined with probiotics on gut microflora and barrier function of rats with abdominal infection. *World J Gastroenterol* 2006; **12**: 4352-4358
- Foligne B, Nutton S, Granette C, Dennin V, Goudercourt D, Poiret S, Dewulf J, Brassart D, Mercenier A, Pot B. Correlation between in vitro and in vivo immunomodulatory properties of lactic acid bacteria. *World J Gastroenterol* 2007; **13**: 236-243
- Versalovic J, Koeuth T, Lupski JR. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacterial genomes. *Nucleic Acids Res* 1991; **19**: 6823-6831
- Finegold SM, Attebery HR, Sutter VL. Effect of diet on human fecal flora: comparison of Japanese and American diets. *Am J Clin Nutr* 1974; **27**: 1456-1469
- Rodriguez-Barradas MC, Hamill RJ, Houston ED, Georgiour PR, Clarridge JE, Regnery RL, Koehler JE. Genomic fingerprinting of Bartonella species by repetitive element PCR for distinguishing species and isolates. *J Clin Microbiol* 1995; **33**: 1089-1093
- Sampaio JL, Viana-Niero C, de Freitas D, Hofling-Lima AL, Leao SC. Enterobacterial repetitive intergenic consensus PCR is a useful tool for typing Mycobacterium chelonae and Mycobacterium abscessus isolates. *Diagn Microbiol Infect Dis* 2006; **55**: 107-118
- de Bruijn FJ. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of Rhizobium meliloti isolates and other soil bacteria. *Appl Environ Microbiol* 1992; **58**: 2180-2187
- Spaniolas S, Tsachaki M, Bennett MJ and Tucker GA. Evaluation of DNA extraction methods from green and roasted coffee beans. *Food Control* 2008; **3**: 257-262
- Aldous WK, Pounder JL, Cloud JL, Woods GL. Comparison of six methods of extracting Mycobacterium tuberculosis DNA from processed sputum for testing by quantitative real-time PCR. *J Clin Microbiol* 2005; **43**: 2471-2473
- Khan IU, Yadav JS. Development of a single-tube, cell lysis-based, genus-specific PCR method for rapid identification of mycobacteria: optimization of cell lysis, PCR primers and conditions, and restriction pattern analysis. *J Clin Microbiol* 2004; **42**: 453-457
- Rantakokko-Jalava K, Jalava J. Optimal DNA isolation method for detection of bacteria in clinical specimens by broad-range PCR. *J Clin Microbiol* 2002; **40**: 4211-4217
- Buck GE, O'Hara LC, Summersgill JT. Rapid, simple method for treating clinical specimens containing Mycobacterium tuberculosis to remove DNA for polymerase chain reaction. *J Clin Microbiol* 1992; **30**: 1331-1334
- Zhong H, Lai XL, Wei RP, Liu ZL. An improved protocol for DNA extraction from the faeces of giant panda. *Acta Zool Sin* 2003; **49**: 670-674
- Li M, Gong J, Cottrill M, Yu H, de Lange C, Burton J, Topp E. Evaluation of QIAamp DNA Stool Mini Kit for ecological studies of gut microbiota. *J Microbiol Methods* 2003; **54**: 13-20
- Wang RF, Cao WW, Cerniglia CE. PCR detection and quantitation of predominant anaerobic bacteria in human and animal fecal samples. *Appl Environ Microbiol* 1996; **62**: 1242-1247
- Suenaga E, Nakamura H. Evaluation of three methods for effective extraction of DNA from human hair. *J Chromatogr B Analyt Technol Biomed Life Sci* 2005; **820**: 137-141
- Samuel M, Lu M, Pachuk CJ, Satishchandran C. A spectrophotometric method to quantify linear DNA. *Anal Biochem* 2003; **313**: 301-306
- Di Giovanni GD, Watrud LS, Seidler RJ, Widmer F. Comparison of Parental and Transgenic Alfalfa Rhizosphere Bacterial Communities Using Biolog GN Metabolic Fingerprinting and Enterobacterial Repetitive Intergenic Consensus Sequence-PCR (ERIC-PCR). *Microb Ecol* 1999; **37**: 129-139
- Syrmis MW, O'Carroll MR, Sloots TP, Coulter C, Wainwright CE, Bell SC, Nissen MD. Rapid genotyping of Pseudomonas aeruginosa isolates harboured by adult and paediatric patients with cystic fibrosis using repetitive-element-based PCR assays. *J Med Microbiol* 2004; **53**: 1089-1096
- Sidransky D, Tokino T, Hamilton SR, Kinzler KW, Levin B, Frost P, Vogelstein B. Identification of ras oncogene mutations in the stool of patients with curable colorectal tumors. *Science* 1992; **256**: 102-105
- Deuter R, Pietsch S, Hertel S, Muller O. A method for preparation of fecal DNA suitable for PCR. *Nucleic Acids Res* 1995; **23**: 3800-3801
- Tan H, Wang J, Zhao ZK. Purification and refolding optimization of recombinant bovine enterokinase light chain overexpressed in Escherichia coli. *Protein Expr Purif* 2007; **56**: 40-47
- Ki JS, Chang KB, Roh HJ, Lee BY, Yoon JY, Jang GY. Direct DNA isolation from solid biological sources without pretreatments with proteinase-K and/or homogenization through automated DNA extraction. *J Biosci Bioeng* 2007; **103**: 242-246
- Desloire S, Valiente Moro C, Chauve C, Zenner L. Comparison of four methods of extracting DNA from D. gallinae (Acari: Dermatyssidae). *Vet Res* 2006; **37**: 725-732
- Aranishi F, Okimoto T. A simple and reliable method for DNA extraction from bivalve mantle. *J Appl Genet* 2006; **47**: 251-254

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## Hepatitis C virus core proteins derived from different quasispecies of genotype 1b inhibit the growth of Chang liver cells

Xue-Bing Yan, Lei Mei, Xia Feng, Mei-Rong Wan, Zhi Chen, Nicole Pavio, Christian Brechot

Xue-Bing Yan, Lei Mei, Department of Infectious Diseases, the First Affiliated Hospital of Xuzhou Medical College, Xuzhou 221002, Jiangsu Province, China

Lei Mei, Changshu No. 2 People's Hospital, 68 Haiyu South Road, Changshu 215500, Jiangsu Province, China

Xia Feng, Mei-Rong Wan, Department of Central Laboratory, the First Affiliated Hospital of Xuzhou Medical College, Xuzhou 221002, Jiangsu Province, China

Zhi Chen, Institute of Infectious Diseases, the First Affiliated Hospital, College of Medical Science, Zhejiang University; Key Lab of Infectious Diseases, Ministry of Public Health, Hangzhou, 310003, Zhejiang Province, China

Nicole Pavio, Christian Brechot, Inserm, U785, Villejuif, F-94804, France; Université Paris sud, Centre Hepato-Biliaire, Hôpital Paul Brousse, Villejuif F-94804, France

Author contributions: Yan XB and Mei L contributed equally to this work; Yan XB, Mei L and Chen Z designed the research; Mei L, Feng X and Wan MR performed the experiments and Pavio N and Brechot C contributed the plasmids.

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Correspondence to: Xue-Bing Yan, Department of Infectious Diseases, The First Affiliated Hospital of Xuzhou Medical College, Xuzhou 221002, Jiangsu Province, China. [yxbxuzhou@126.com](mailto:yxbxuzhou@126.com)

Telephone: +86-516-85802180 Fax: +86-516-85802215

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### Abstract

**AIM:** To investigate the influence of different quasispecies of hepatitis C virus (HCV) genotype 1b core protein on growth of Chang liver cells.

**METHODS:** Three eukaryotic expression plasmids (pEGFP-N1/core) that contained different quasispecies truncated core proteins of HCV genotype 1b were constructed. These were derived from tumor (T) and non-tumor (NT) tissues of a patient infected with HCV and C191 (HCV-J6). The core protein expression plasmids were transiently transfected into Chang liver cells. At different times, the cell cycle and apoptosis was assayed by flow cytometry, and cell proliferation was assayed by methyl thiazolyl tetrazolium (MTT) assay.

**RESULTS:** The proportion of S-phase Chang liver cells transfected with pEGFP-N1/core was significantly lower than that of cells transfected with blank plasmid at three different times after transfection (all  $P < 0.05$ ). The proliferation ratio of cells transfected with pEGFP-N1/core

was significantly lower than that of cells transfected with blank plasmid. Among three different quasispecies, T, NT and C191 core expression cells, there was no significant difference in the proportion of S- and G<sub>0</sub>/G<sub>1</sub>-phase cells. The percentage of apoptotic cells was highest for T (T > NT > C191), and apoptosis was increased in cells transfected with pEGFP-N1/core as the transfection time increased (72 h > 48 h > 24 h).

**CONCLUSION:** These results suggest that HCV genotype 1b core protein induces apoptosis, and inhibits cell-cycle progression and proliferation of Chang liver cells. Different quasispecies core proteins of HCV genotype 1b might have some differences in the pathogenesis of HCV persistent infection and hepatocellular carcinoma.

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**Key words:** Hepatitis C virus; Core protein; Chang liver cells; Cell cycle; Apoptosis

**Peer reviewer:** Baumert F Thomas, Professor, Inserm U748, Louis Pasteur University, 3 Rue Koeberle, 67000 Strasbourg, France

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### INTRODUCTION

Hepatitis C virus (HCV) infection is prevalent worldwide. HCV infection may lead to the development of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC)<sup>[1]</sup>. HCV is a positive-strand RNA virus that belongs to the family Flaviviridae. The HCV RNA genome is 9.6 kb in length and encodes a precursor polyprotein of 3000 amino acids. The precursor polyprotein is cleaved by host and viral proteases and produces a series of structural and non-structural proteins. In addition, HCV genome has the property of variation, and it usually exists as different quasispecies of the same genotype in HCV-infected patients<sup>[2,3]</sup>. The function of different quasispecies of HCV is not fully identical<sup>[4,5]</sup>.

HCV core protein is important as a viral structural component in nucleocapsid formation, and it is highly conserved among different HCV genotypes. Several studies have established that HCV core protein can regulate cell signal transduction, such as MAPK-, JAK-STAT-, NF- $\kappa$ B-, AP-1- and SRE-associated pathways<sup>[6-8]</sup>. Hence, it plays important roles in the pathogenesis of HCV persistent infection, as well as HCC. However, the mechanism of persistent infection and HCC due to HCV core protein infection remains unclear. Although several studies have investigated the influence of the HCV core protein on cell growth and apoptosis, the results of these investigations have been inconsistent and mutually conflicting.

In most studies, HCV core protein has been expressed in single clones of permanently transfected cell lines, while more recently, transiently transfected cells have also been used. These studies have found that core protein affects normal cellular functions, such as proliferation and death, which are involved, directly or indirectly, in HCV hepatocarcinogenesis. Because we have found that different quasispecies core proteins of HCV genotype 1b have some differences in the pathogenesis of HCV persistent infection and HCC. HCV is a hepatotropic virus, it is therefore more appropriate to use a hepatocyte cell line to study the function of HCV core protein.

To further clarify the *in vivo* role of truncated HCV core protein from different genotype 1b quasispecies, we expressed three different truncated forms of HCV core protein derived from tumor tissue (T), non-tumor tissue (NT) and C191 (HCV-J6), which all belong to HCV genotype 1b, in transiently transfected Chang liver cells, an immortalized non-tumor hepatic cell line. Cell cycle and apoptosis were assayed by flow cytometry, and cell proliferation was assayed by methyl thiazolyl tetrazolium (MTT) assay.

## MATERIALS AND METHODS

### Plasmid constructs

Three different truncated HCV core protein eukaryotic expression plasmids, pEGFP-N1/core, were constructed. Truncated core protein nucleotide sequences were amplified from pGEX 4T-1/HCV-core, which contained core sequences from T, NT and C191, respectively. Sequence analysis revealed that T and NT were all HCV genotype 1b. The primers were designed according to the core protein gene sequence of T, NT and C191 (Table 1). PCR reaction system: 50  $\mu$ L: water 40.75  $\mu$ L; PCR reaction buffer (10  $\times$ ), 5  $\mu$ L; template (45 ng), 1  $\mu$ L; primer up 20 pmol/L, 1  $\mu$ L; primer down 20 pmol/L, 1  $\mu$ L; dNTP 20 mmol/L, 1  $\mu$ L, Expand high enzyme 0.25  $\mu$ L, 94°C for 2 min, 94°C for 30 s, 50°C for 30 s, 72°C for 30 s, 10 cycles; 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, 20 cycles; and 72°C for 7 min. PCR products (Figure 1) were purified and cleaved with restriction enzymes *Nhe* I and *Eco*R I, and cloned into pEGFP-N1 to yield GFP-core fusion protein plasmids.

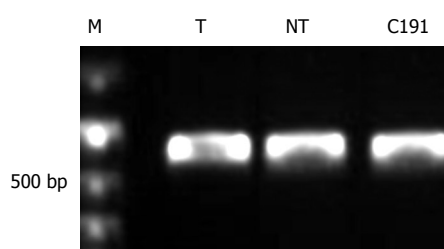
### Cell culture and transfection

Chang liver cells were grown in Dulbecco's modified Eagle's medium (Gibco, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) at 37°C in 5% CO<sub>2</sub>. Cells were incubated for 18 h and then transfected with Lipofectamine

**Table 1** Primers of different quasispecies core proteins of HCV genotype 1b

	Primer up (location)	Primer down (location)
T: 1-172	CGCGCTAGCATGAGG CACGAATCC (1-14)	CCGGAATTCGCAACCGG GCAG (505-516)
NT: 1-172	CGCGCTAGCATGAGG ACGAATCC (1-14)	CCGGAATTCGGCAACCG GGCAGATTC (505-516)
C191: 1-172	CGCGCTAGCATGAGG ACAAATCC (1-14)	CCGGAATTCGGCAACCG GGCAAATTC (505-516)

GCTAGC, *Nhe* I site; GAATTC, *Eco*R I site. Primer designed according to the sequence of different core protein genes.



**Figure 1** PCR product of core protein genes.

2000 (Invitrogen, USA) following the manufacturer's instructions. Two micrograms of plasmids were used to achieve transfection. After 6 h, the medium was changed and cells were collected at different times.

### Flow cytometry analysis

The cell cycle and apoptosis was assayed by flow cytometry at 24, 48 and 72 h after transfection. Ten  $\times 10^5$  cells were trypsinized, pelleted by centrifugation, and resuspended in PBS, and then cells were fixed with 70% ethanol and stored at 4°C overnight. After washing twice, cells were incubated in propidium iodine for 20 min at room temperature. The cell suspension was analyzed by flow cytometry using Cell Quest software (Becton Dickinson, USA).

### Cell growth analysis

Cell proliferation was assayed by MTT assay and a growth curve was created.  $10^4$  cells were seeded per well on a 96-well plate. Zero, 24, 48, 72 and 96 h after transfection, 10  $\mu$ L water-soluble tetrazolium reagent (MTT; Sigma, USA) was added to 100  $\mu$ L culture medium. Cells were then incubated at 37°C in 5% CO<sub>2</sub> for 4 h.  $A_{570}$  was measured. The assay was done in quadruplicate.

### Statistical analysis

The values were expressed as mean  $\pm$  SD. Experimental data were analyzed by SPSS 13.0 software (SPSS Inc, Chicago, IL, USA). Comparison of different groups was analyzed by one-way analysis of variance (ANOVA).  $P < 0.05$  was considered statistically significant.

## RESULTS

### Different HCV genotype 1b quasispecies core proteins inhibited Chang liver cell cycle

As shown in Tables 2-4, three different quasispecies

**Table 2** Core proteins of different quasiespecies of HCV genotype 1b inhibited Chang liver cell cycle by impairing G1 to S transition at 24 h after transfection

	G0/G1 phase	S phase	G2/M phase
T	67.53 ± 5.47 <sup>a</sup>	17.23 ± 4.21 <sup>a</sup>	15.25 ± 1.96
NT	65.49 ± 5.98 <sup>a</sup>	18.56 ± 2.49 <sup>a</sup>	15.95 ± 2.59
C191	66.72 ± 6.82 <sup>a</sup>	17.89 ± 2.54 <sup>a</sup>	15.38 ± 2.16
pEGFP-N1	59.76 ± 5.33	24.33 ± 3.16	15.93 ± 1.42

<sup>a</sup>*P* < 0.05 vs pEGFP-N1.**Table 3** Core proteins of different quasiespecies of HCV genotype 1b inhibited Chang liver cell cycle by impairing G1 to S transition at 48 h after transfection

	G0/G1 phase	S phase	G2/M phase
T	68.23 ± 6.54 <sup>a</sup>	15.30 ± 3.12 <sup>a</sup>	16.46 ± 2.57
NT	66.14 ± 5.33 <sup>a</sup>	16.38 ± 2.21 <sup>a</sup>	17.47 ± 3.74
C191	66.41 ± 3.02 <sup>a</sup>	15.92 ± 2.93 <sup>a</sup>	16.99 ± 2.43
pEGFP-N1	58.72 ± 2.23	25.69 ± 2.41	15.62 ± 1.28

<sup>a</sup>*P* < 0.05 vs pEGFP-N1.**Table 4** Core proteins of different quasiespecies of HCV genotype 1b inhibited Chang liver cell cycle by impairing G1 to S transition at 72 h after transfection

	G0/G1 phase	S phase	G2/M phase
T	68.45 ± 4.98 <sup>a</sup>	14.79 ± 3.76 <sup>a</sup>	16.75 ± 3.21
NT	67.21 ± 5.47 <sup>a</sup>	16.17 ± 2.55 <sup>a</sup>	16.63 ± 2.95
C191	66.77 ± 4.32 <sup>a</sup>	16.38 ± 2.11 <sup>a</sup>	16.86 ± 2.19
pEGFP-N1	56.97 ± 3.29	27.48 ± 3.37	15.54 ± 1.93

<sup>a</sup>*P* < 0.05 vs pEGFP-N1.

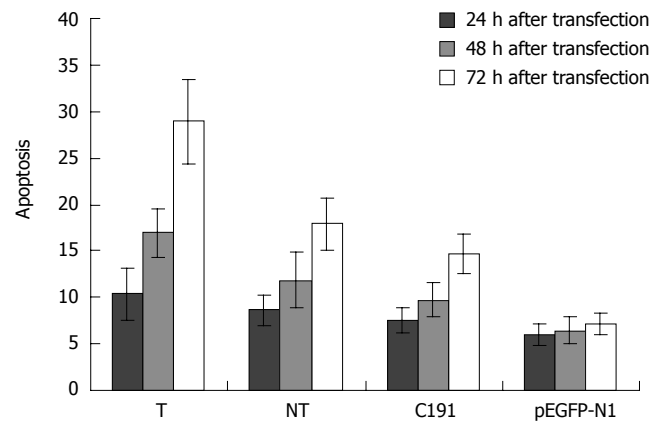
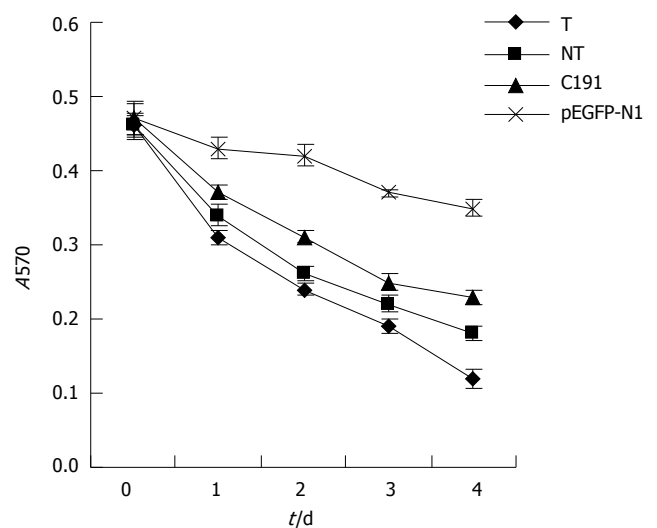
truncated core proteins inhibited Chang liver cell cycle progression by impairing G1- to S-phase transition. The proportion of S- and G0/G1-phase Chang liver cells transfected with pEGFP-N1/core was significantly lower than that of cells transfected with blank plasmid at 24, 48 and 72 h after transfection (*P* = 0.002, *P* = 0.001, *P* = 0.001, respectively), but there were no significant differences among cells expressing the three different quasiespecies HCV truncated core proteins.

#### Different HCV genotype 1b quasiespecies core proteins induced Chang liver cell apoptosis

As shown in Figure 2, three different quasiespecies truncated core proteins induced apoptosis at different levels. The apoptotic ratio of Chang liver cells transfected with pEGFP-N1/core was significantly higher than that of cells transfected with blank plasmid. The apoptotic percentage of T was the highest, and C191 was the lowest (T > NT > C191). The apoptosis ratio increased in cells transfected with pEGFP-N1/core as transfection time increased (72 h > 48 h > 24 h).

#### Different HCV genotype 1b quasiespecies core proteins inhibited Chang liver cell proliferation

We found that different HCV genotype 1b quasiespecies

**Figure 2** Core proteins of different quasiespecies of HCV genotype 1b induced Chang liver cell apoptosis at 24, 48 and 72 h after transfection.**Figure 3** Core proteins from different HCV genotype 1b quasiespecies inhibited proliferation of Chang liver cells.

core proteins inhibited the Chang liver cell cycle by impairing G1- to S-phase transition and induced apoptosis at different times after transfection. Chang liver cell proliferation was further analyzed using MTT assay. As shown in Figure 3, different HCV genotype 1b quasiespecies core proteins inhibited Chang liver cell proliferation. Among the three different HCV genotype 1b quasiespecies core proteins, that of T inhibited Chang liver cell proliferation more obviously than NT and C191 (T > NT > C191) at 24, 48, 72 and 96 h.

## DISCUSSION

HCV core protein is a multifunctional protein that can modulate a number of cellular processes, including transcription, inhibition or stimulation of cell cycle and apoptosis, and suppression of host immunity. In this study, using flow cytometry and MTT assay, we investigated the influence of different quasiespecies of truncated HCV genotype 1b core proteins on growth of Chang liver cells, an immortalized non-tumor hepatic cell line. We found that HCV genotype 1b core protein HCV induced apoptosis, and inhibited the cell cycle and cell proliferation. Among

the three different quasispecies core proteins, the rates of inducing apoptosis and inhibiting cell proliferation were different, which suggests that different quasispecies of HCV genotype 1b core proteins might have some differences in their pathogenesis of HCV persistent infection and HCC.

Regulation of cell cycle and apoptosis is essential to maintain normal cell growth. Several studies have reported that HCV core protein can influence cell cycle progression. Two important checkpoints of the cell cycle, G1/S and G2/M, are responsible for accumulation of detrimental mutations that may result in cell transformation. The mechanism of cell cycle regulation is quite complex, and involves a number of proteins, such as p53, p21, PRb, E2F and c-myc<sup>[9,10]</sup>. Ruggieri *et al* have reported that stable expression of core protein in unsynchronized HepG2 cells induces perturbation of the cell cycle by increasing the S-phase fraction. c-myc protein stability is increased, which is one of the regulatory molecules of the cell cycle<sup>[11]</sup>. Honda *et al* have found that core protein promotes cell cycle progression in stable CHO transformation by upregulation of c-myc<sup>[12]</sup>. Ohkawa *et al* have reported that core protein impairs G1 to S transition by using core expression stable CL2 cell, a murine normal liver-derived cell line. E2F-mediated transcription, and PRb, CDK4 and CDK2 activities were suppressed by HCV core protein<sup>[13]</sup>. Different studies have come to different conclusions. We found that transient expression of HCV genotype 1b truncated core proteins could impair G1/S in the Chang liver cell cycle. Different quasispecies core proteins from T, NT and C191 have no significant difference when it comes to impairing G1/S. The above studies suggest that core protein might be a double-function protein, promoting cell cycle progression and inhibiting cell cycle progression. However, the exact mechanisms of how different quasispecies core proteins affect the G1/S checkpoint remain unclear.

Apoptosis is defined as programmed cell death and it is an orderly process. Apoptosis can be induced by a variety of stimuli, including oxidative stress, heat shock, ionizing radiation, cytokines and virus infection. The apoptotic process appears to be a host defense mechanism against viral infections. HCV might lead to viral persistence by inhibiting hepatocyte apoptosis and inducing monocyte apoptosis<sup>[14]</sup>. Although diverse effects of the core protein on apoptosis have been reported, the underlying mechanisms are not fully understood<sup>[15]</sup>. Core protein exhibits both pro- and anti-apoptotic actions. Transient expression of the core protein can inhibit tumor necrosis factor (TNF)- $\alpha$ -mediated apoptosis in MCF7 cells, a cell line derived from breast carcinoma tissue<sup>[16]</sup>. In contrast, several studies have indicated that core protein can promote apoptosis induced by Fas or TNF- $\alpha$  in stable expression systems in HepG2, HeLa and Jurkat T cell lines<sup>[17-19]</sup>. In our study, we found that different quasispecies of HCV genotype 1b truncated core proteins could induce Chang liver cell apoptosis, and the ability to induce apoptosis was different (T > NT > C191). Apoptosis was increased depending on prolongation of transfection time (72 h > 48 h > 24 h). Moreover, the cell proliferation induced by HCV core protein was consistent with our finding that HCV core protein induced cell apoptosis. Nevertheless, the present results allow us to

propose an alternative, and in fact contrasting mechanism for HCV core protein. Our observations imply that the liver cell proliferation observed during liver carcinogenesis is associated with the selection of viral genomes whose core products can resist apoptosis.

This apparently paradoxical finding should be discussed in the light of two important observations. Consistent with previous reports<sup>[20-23]</sup>, in HCV-related HCC, strong arguments exist in favor of the importance of the immune response to HCV proteins in liver cell destruction during acute or chronic HCV infection. Our findings therefore suggest that cell apoptosis may reduce HCV protein expression and the elimination of infected cells, thus favoring viral persistence. A sustained reduction in HCV replication during the later stages of liver cancer development probably depends on other mechanisms, such as a lack in dedifferentiated cancer cells of the tissue-specific cellular factors necessary for HCV polyprotein expression and maturation. This favors the selection of a cell subset that acquires resistance to HCV apoptosis and whose differentiation status maintains down-regulated HCV multiplication.

Cell-cycle progression and apoptosis are balanced processes regulated by a number of cellular factors. Several cell-cycle-regulatory proteins are involved in signal transduction pathways of apoptosis<sup>[24-26]</sup>. Core protein impaired G1/S transition, which might be advantageous to cells that are entering the apoptotic process. The influence of core protein on cell cycle and apoptosis is different, depending on different cell systems and expression systems used<sup>[27-30]</sup>. However, in most studies, human hepatoma-derived cell lines Huh-7 or HepG2 were used, but in our study, Chang liver cell and transient transfection were used. As an immortalized non-tumor cell line, the biological function of Chang liver cells is similar to that of normal human hepatocytes, so it is suitable for studying virus-host interactions *in vitro*.

In summary, we found that HCV genotype 1b core protein could induce apoptosis, and inhibit cell-cycle progression and cell proliferation. Among three different quasispecies core proteins, the capability of inducing apoptosis and inhibiting cell proliferation was different.

## COMMENTS

### Background

It is known that hepatitis C virus (HCV) core protein plays an important role in the pathogenesis of HCV persistent infection and hepatocellular carcinoma (HCC). HCV core protein derived from different quasispecies of the same HCV genotype 1b might have different functions in their pathogenesis.

### Research frontiers

HCV core protein is involved in regulation of host cell growth, including the cell cycle and apoptosis, but the results are conflicting. Recent studies have shown that the influence of HCV core protein on cell growth might be associated with viral persistence and generation of liver carcinogenesis.

### Innovations and breakthroughs

We used Chang liver cells, an immortalized non-tumor hepatic cell line, different from the human hepatoma-derived cell lines in most studies. Moreover, different quasispecies of HCV genotype 1b core proteins are found to have different biological functions.



## Applications

Our findings further show the importance of HCV core proteins on the pathogenesis of HCV persistent infection, as well as HCC. It might provide new ideas for the cure and prevention of HCV infection.

## Peer review

This work investigated the role of the core protein of HCV from different quasispecies on the pathogenesis of Chang liver cells. The findings are potentially interesting and point towards the ability of HCV core protein to induce apoptosis and inhibit cell-cycle progression in Chang liver cells. The intensity of these observed effects seems to be dependent on the quasispecies of the HCV core protein isolated.

## REFERENCES

- 1 Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, Furuta S, Akahane Y, Nishioka K, Purcell RH. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. *Hepatology* 1990; **12**: 671-675
- 2 Lerat H, Rumin S, Habersetzer F, Berby F, Traub MA, Trepo C, Inchauspe G. In vivo tropism of hepatitis C virus genomic sequences in hematopoietic cells: influence of viral load, viral genotype, and cell phenotype. *Blood* 1998; **91**: 3841-3849
- 3 Ruster B, Zeuzem S, Krump-Konvalinkova V, Berg T, Jonas S, Severin K, Roth WK. Comparative sequence analysis of the core- and NS5-region of hepatitis C virus from tumor and adjacent non-tumor tissue. *J Med Virol* 2001; **63**: 128-134
- 4 Delhem N, Sabile A, Gajardo R, Podevin P, Abadie A, Bleton MA, Kremsdorf D, Beretta L, Brechot C. Activation of the interferon-inducible protein kinase PKR by hepatocellular carcinoma derived-hepatitis C virus core protein. *Oncogene* 2001; **20**: 5836-5845
- 5 Pavo N, Battaglia S, Boucreux D, Arnulf B, Sobesky R, Hermine O, Brechot C. Hepatitis C virus core variants isolated from liver tumor but not from adjacent non-tumor tissue interact with Smad3 and inhibit the TGF-beta pathway. *Oncogene* 2005; **24**: 6119-6132
- 6 Kato N, Yoshida H, Ono-Nita SK, Kato J, Goto T, Otsuka M, Lan K, Matsushima K, Shiratori Y, Omata M. Activation of intracellular signaling by hepatitis B and C viruses: C-viral core is the most potent signal inducer. *Hepatology* 2000; **32**: 405-412
- 7 Hayashi J, Aoki H, Kajino K, Moriyama M, Arakawa Y, Hino O. Hepatitis C virus core protein activates the MAPK/ERK cascade synergistically with tumor promoter TPA, but not with epidermal growth factor or transforming growth factor alpha. *Hepatology* 2000; **32**: 958-961
- 8 Heim MH, Moradpour D, Blum HE. Expression of hepatitis C virus proteins inhibits signal transduction through the Jak-STAT pathway. *J Virol* 1999; **73**: 8469-8475
- 9 Kwun HJ, Jung EY, Ahn JY, Lee MN, Jang KL. p53-dependent transcriptional repression of p21(waf1) by hepatitis C virus NS3. *J Gen Virol* 2001; **82**: 2235-2241
- 10 Cho J, Baek W, Yang S, Chang J, Sung YC, Suh M. HCV core protein modulates Rb pathway through pRb down-regulation and E2F-1 up-regulation. *Biochim Biophys Acta* 2001; **1538**: 59-66
- 11 Ruggieri A, Murdolo M, Harada T, Miyamura T, Rapicetta M. Cell cycle perturbation in a human hepatoblastoma cell line constitutively expressing Hepatitis C virus core protein. *Arch Virol* 2004; **149**: 61-74
- 12 Honda M, Kaneko S, Shimazaki T, Matsushita E, Kobayashi K, Ping LH, Zhang HC, Lemon SM. Hepatitis C virus core protein induces apoptosis and impairs cell-cycle regulation in stably transformed Chinese hamster ovary cells. *Hepatology* 2000; **31**: 1351-1359
- 13 Ohkawa K, Ishida H, Nakanishi F, Hosui A, Ueda K, Takehara T, Hori M, Hayashi N. Hepatitis C virus core functions as a suppressor of cyclin-dependent kinase-activating kinase and impairs cell cycle progression. *J Biol Chem* 2004; **279**: 11719-11726
- 14 Kountouras J, Zavos C, Chatzopoulos D. Apoptosis in hepatitis C. *J Viral Hepat* 2003; **10**: 335-342
- 15 Marusawa H, Hijikata M, Chiba T, Shimotohno K. Hepatitis C virus core protein inhibits Fas- and tumor necrosis factor alpha-mediated apoptosis via NF-kappaB activation. *J Virol* 1999; **73**: 4713-4720
- 16 Ray RB, Meyer K, Steele R, Shrivastava A, Aggarwal BB, Ray R. Inhibition of tumor necrosis factor (TNF-alpha)-mediated apoptosis by hepatitis C virus core protein. *J Biol Chem* 1998; **273**: 2256-2259
- 17 Yamanaka T, Kodama T, Doi T. Subcellular localization of HCV core protein regulates its ability for p53 activation and p21 suppression. *Biochem Biophys Res Commun* 2002; **294**: 528-534
- 18 Hahn CS, Cho YG, Kang BS, Lester IM, Hahn YS. The HCV core protein acts as a positive regulator of fas-mediated apoptosis in a human lymphoblastoid T cell line. *Virology* 2000; **276**: 127-137
- 19 Zhu N, Khoshnan A, Schneider R, Matsumoto M, Dennert G, Ware C, Lai MM. Hepatitis C virus core protein binds to the cytoplasmic domain of tumor necrosis factor (TNF) receptor 1 and enhances TNF-induced apoptosis. *J Virol* 1998; **72**: 3691-3697
- 20 Soguero C, Joo M, Chianese-Bullock KA, Nguyen DT, Tung K, Hahn YS. Hepatitis C virus core protein leads to immune suppression and liver damage in a transgenic murine model. *J Virol* 2002; **76**: 9345-9354
- 21 Large MK, Kittlesen DJ, Hahn YS. Suppression of host immune response by the core protein of hepatitis C virus: possible implications for hepatitis C virus persistence. *J Immunol* 1999; **162**: 931-938
- 22 Kimball P, Verbeke S, Shiffman M. HCV core protein augments cyclosporine immunosuppression. *Transplant Proc* 2005; **37**: 652-653
- 23 Yao ZQ, Nguyen DT, Hiotellis AI, Hahn YS. Hepatitis C virus core protein inhibits human T lymphocyte responses by a complement-dependent regulatory pathway. *J Immunol* 2001; **167**: 5264-5272
- 24 Shrivastava A, Manna SK, Ray R, Aggarwal BB. Ectopic expression of hepatitis C virus core protein differentially regulates nuclear transcription factors. *J Virol* 1998; **72**: 9722-9728
- 25 Fukuda K, Tsuchihara K, Hijikata M, Nishiguchi S, Kuroki T, Shimotohno K. Hepatitis C virus core protein enhances the activation of the transcription factor, Elk1, in response to mitogenic stimuli. *Hepatology* 2001; **33**: 159-165
- 26 Tai DI, Tsai SL, Chen YM, Chuang YL, Peng CY, Sheen IS, Yeh CT, Chang KS, Huang SN, Kuo GC, Liaw YF. Activation of nuclear factor kappaB in hepatitis C virus infection: implications for pathogenesis and hepatocarcinogenesis. *Hepatology* 2000; **31**: 656-664
- 27 Cho JW, Baek WK, Suh SI, Yang SH, Chang J, Sung YC, Suh MH. Hepatitis C virus core protein promotes cell proliferation through the upregulation of cyclin E expression levels. *Liver* 2001; **21**: 137-142
- 28 Giannini C, Caini P, Giannelli F, Fontana F, Kremsdorf D, Brechot C, Zignego AL. Hepatitis C virus core protein expression in human B-cell lines does not significantly modify main proliferative and apoptosis pathways. *J Gen Virol* 2002; **83**: 1665-1671
- 29 Sacco R, Tsutsumi T, Suzuki R, Otsuka M, Aizaki H, Sakamoto S, Matsuda M, Seki N, Matsuura Y, Miyamura T, Suzuki T. Antiapoptotic regulation by hepatitis C virus core protein through up-regulation of inhibitor of caspase-activated DNase. *Virology* 2003; **317**: 24-35
- 30 Nguyen H, Mudryj M, Guadalupe M, Dandekar S. Hepatitis C virus core protein expression leads to biphasic regulation of the p21 cdk inhibitor and modulation of hepatocyte cell cycle. *Virology* 2003; **312**: 245-253

S- Editor Zhong XY L- Editor Kerr C E- Editor Ma WH



RAPID COMMUNICATION

# Control of gallbladder contractions by cholecystokinin through cholecystokinin-A receptors on gallbladder interstitial cells of cajal

Dan Xu, Bao-Ping Yu, He-Sheng Luo, Ling-Dan Chen

Dan Xu, Bao-Ping Yu, He-Sheng Luo, Ling-Dan Chen, Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China

**Author contributions:** Xu D designed and performed the research; Luo HS contributed new reagents/analytic tools; Xu D and Chen LD analyzed the data; Xu D and Yu BP wrote the paper.

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**Correspondence to:** Bao-Ping Yu, Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China. [yubaoping62@yahoo.com.cn](mailto:yubaoping62@yahoo.com.cn)

Telephone: +86-27-68759391 Fax: +86-27-68758766

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## Abstract

**AIM:** To identify the cholecystokinin (CCK)-A receptors (CCK-AR) on the guinea pig gallbladder interstitial cells of cajal (ICC) and to study CCK-8 induced gallbladder muscle strip contractions through the CCK-AR.

**METHODS:** The existence of CCK-AR was examined by immunohistofluorescence on sectioned tissue and cultured cells. *In vitro* contractile response of guinea pig gallbladder muscle strips and the strips with ICC removed were also studied with CCK-8 receptors added.

**RESULTS:** In tissue sections, intensely CCKAR-immunoreactive interstitial cells were found mainly in the muscular layers. In cultured cell sections, distinctive double staining of C-kit and CCK-AR ICCs were found. When we removed the ICC of the gallbladder, CCK-8 induced muscle strip contraction dose response curve significantly shifted to the right.

**CONCLUSION:** We proved that both the existence of CCK-AR on the guinea pig gallbladder ICC and CCK evoked contraction are mediated through direct action on CCK-AR on the gallbladder ICC.

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**Key words:** Gallbladder; Interstitial cells of cajal; Cholecystokinin-A receptor; C-kit; Immunohistofluorescence

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Xu D, Yu BP, Luo HS, Chen LD. Control of gallbladder contractions by cholecystokinin through cholecystokinin-A receptors on gallbladder interstitial cells of cajal. *World J Gastroenterol* 2008; 14(18): 2882-2887 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2882.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2882>

## INTRODUCTION

Cholecystokinin (CCK) is released from mucosal endocrine cells in the proximal small intestine in response to a meal<sup>[1]</sup>, and it is classically known to stimulate the gallbladder contraction. The CCK-induced gallbladder contractions were once believed to be brought about by direct stimulation of gallbladder smooth muscle. The CCK receptors have been identified by using isolated gallbladder muscle membranes and autoradiographical analysis<sup>[2-4]</sup>. Two CCK receptors, CCK-A (CCK-AR) and CCK-B receptors (CCK-BR) have been identified<sup>[5]</sup>. It is well established that CCK-AR and CCK-BR regulate a number of physiological functions, e.g. gallbladder contraction<sup>[6]</sup>, pancreatic enzyme release<sup>[7]</sup>, gastric acid secretion<sup>[8]</sup> and pyloric sphincter closure<sup>[9,10]</sup>. In 1892, a special kind of cell which is now called "interstitial cells of Cajal" (ICC) in the gastrointestinal tract was first described by the famous Spanish neuro-histologist Ramon y Cajal<sup>[11]</sup>. It has been approved that ICC play an important role in gastrointestinal tissues by generating and propagating electrical slow waves to gastrointestinal muscles, and it mediates signals from the enteric nervous system<sup>[12-15]</sup>. ICCs were found in the murine<sup>[16]</sup>, *Homo sapiens*<sup>[17]</sup> and guinea pig gallbladders<sup>[18]</sup>. Both the CCK-AR and CCK-BR mediate the contraction of the guinea pig ileum<sup>[19-21]</sup>, whereas the guinea pig gallbladder contraction is mediated solely by CCK-AR<sup>[22]</sup>. It is considered that CCK-induced gallbladder contractions are controlled through CCK-ARs both on the vagal nerve in stimulating endogenous release of acetylcholine and on the gallbladder to directly stimulate muscle contraction in dogs<sup>[23]</sup>. The recent availability of antibodies directed against the CCK-AR has made it possible to identify the receptor by immunocytochemistry<sup>[24]</sup> and immunohistochemistry<sup>[25]</sup>. It has been reported that some ICCs in the rat pylorus express CCK-ARs<sup>[26]</sup>. Therefore, we used an immunohistochemical approach to localize the CCK-AR in the gallbladder. Surprisingly, we found that the interstitial cells of Cajal express CCKARs to a high degree. We also measured the effect of atropine,

tetrodotoxin and CCK on the gallbladder muscle strips, and the contractions of the muscle strips with ICC removed in the guinea pig. This is the first study in which the CCK-induced gallbladder contraction through CCK-AR on the gallbladder ICC was investigated using the guinea pig as the experimental animal.

## MATERIALS AND METHODS

### Animals

Adult guinea-pigs (250-300 g) were obtained by mail from Animal Experiment Center of Wuhan University. The animal-testing protocol used in the present investigation was approved by the Institutional Animal Care and the Use Committee of the Wuhan University.

### Chemicals

All the chemicals and bovine serum albumin were obtained from Sigma. The rat monoclonal antibodies against C-kit were purchased from R&D. The Rabbit anti-CCK-AR polyclonal antibody was purchased from Chemicon. M199 were purchased from Sigma. All the secondary antibodies were bought from Beijing Zhongshan Biotechnology CO., LTD.

### Experimental procedures

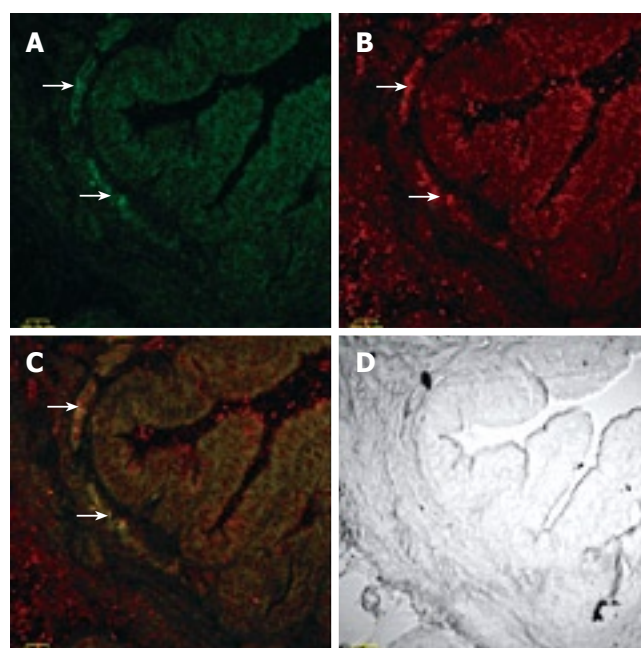
**Demonstration of CCK-AR on the gallbladder tissue by immunohistofluorescence:** The gallbladder was fixed with 4% paraformaldehyde at 4°C for 16 h. Following fixation, the tissues were embedded and cut for immunohistochemistry. Next, they were washed and incubated with normal bovine serum albumin for 10 min. They were subsequently incubated with rat anti-kit monoclonal antibody, with rabbit anti-CCK-AR polyclonal antibody added to the anti-kit solution at 4°C for 24 h. The sections were incubated with the secondary antibody after the first antibody. CCK-AR polyclonal antibody was visualized using the goat anti-rabbit IgG conjugated to Cy3 and the C-kit was visualized using the rabbit anti-rat antibody conjugated to FITC for 2 h at room temperature. A negative control without primary antibody was performed.

**Demonstration of CCK-AR on the gallbladder ICCs by immunohistofluorescence:** The surrounding tissue of guinea pig's gallbladder was cleaned off and cut longitudinally. The tissue was pinned to the base of a dish filled with Sylgard elastomer with the mucosal side facing up. The mucosa was removed by sharp dissection, and the muscular layer tissues were cut out and incubated at 37°C for 23 min in a  $\text{Ca}^{2+}$ -free physiological saline solution (PSS mmol/L: NaCl 135.0, KCl 5.0, glucose 10.0, Hepes 10.0, and  $\text{MgCl}_2$  1.2; pH set to 7.4 with NaOH) containing type II collagenase (1.2 mg/mL), soybean trypsin inhibitor (2.0 mg/mL) and bovine serum albumin (BSA; 2.0 mg/mL). The pieces of tissue were then rinsed five times with  $\text{Ca}^{2+}$ -free PSS and gently triturated with a wide-bore glass pipette to yield the isolated cells. Aliquots of the suspension of the cells were then placed in glass coverslips, and they were left at 4°C for 45-60 min to attach onto the bottom of coverslips for culturing. Cells were washed

four times with Krebs-Ringer buffer (KRB mmol/L: NaCl 118.5, KCl 4.5,  $\text{MgCl}_2$  1.2,  $\text{NaHCO}_3$  23.8,  $\text{KH}_2\text{PO}_4$  1.2, dextrose 11.0 and  $\text{CaCl}_2$  2.4; pH at 7.4) containing penicillin (200 U/mL), streptomycin (200 mg/mL) and amphotericin B (0.5 mg/mL), and they were placed in M199 medium (Sigma) containing similar concentrations of antibiotics and antimycotic. The cells were incubated at 37°C (90% humidity and 95%  $\text{O}_2$ -5%  $\text{CO}_2$ ) for 1 d. Cells were fixed with 4% paraformaldehyde at 4°C for 16 h, and then it was washed and incubated with normal bovine serum albumin for 10 min. Tissues were processed with protocol identical to the cells. The cells and tissues were examined under the TCS SP2 MP laser scanning confocal microscope (Leica, Germany). With an excitation beam (488 nm) produced by argon, the emitted fluorescence was captured using Leica Confocal software running on Windows XP work station.

**Contractile response of the gallbladder muscle strip to CCK-8:** (1) CCK-Induced Contractions on Common Muscle Strips. The adult guinea-pigs sent in were killed by cervical dislocation. The gallbladder was removed and opened, and it was washed several times in Krebs solution to remove bile. Four strips of each gallbladder measuring approximately 0.2 cm  $\times$  0.8 cm were mounted in organ baths containing Krebs solution (30 mL) with the following composition (mmol/L): NaCl 120.35, KCl 5.9,  $\text{MgCl}_2$  2.5,  $\text{NaH}_2\text{PO}_4$  1.2,  $\text{NaHCO}_3$  15.5,  $\text{CaCl}_2$  2.5, and glucose 11.5; pH 7.4. The organ bath was maintained at 37°C and gassed with 95%  $\text{O}_2$  and 5%  $\text{CO}_2$ . A resting 1 g pre-load was applied to each muscle strip and was allowed to equilibrate for 1 h, and during which time the Krebs solution was changed every 20 min. During the equilibration period, the tension was adjusted to 1 g when required. Mechanical activity was recorded using isometric transducers (World Precision Instruments, Stevenage, Herts, U.K.). Tension was continuously monitored and recorded using a MacLab data acquisition system (AD Instruments Ltd., Hastings, U.K.). A dose-response curve for CCK-8 was constructed by adding CCK-8 to the medium in a cumulative way, and the effect of pretreatment with  $10^{-6}$  mol/L atropine,  $3 \times 10^{-7}$  mol/L tetrodotoxin (ITX) on CCK-8 induced gallbladder contraction was also examined. (2) CCK-Induced Contractions on Muscle Strips with ICCs Removed. Methylene blue staining is a nonspecific but effective and time-dependent staining method for ICCs. After the removal of the mucosa, muscle strips were washed with fresh oxygenated Krebs solution (in mmol/L: NaCl 120.35, KCl 5.9,  $\text{MgCl}_2$  2.5,  $\text{NaH}_2\text{PO}_4$  1.2,  $\text{NaHCO}_3$  15.5,  $\text{CaCl}_2$  2.5, and glucose 11.5; pH at 7.4) and then incubated in Krebs solution containing 50  $\mu\text{mol/L}$  methylene blue at 37°C bubbled with 95%  $\text{O}_2$ -5%  $\text{CO}_2$  (v/v) for 40 min in the dark with intermittent visual checking of the staining intensity. After staining, the muscle strips were immediately put under the light (50 mW/cm<sup>2</sup>) for 5 min. Next, CCK-induced contractions on muscle strips with ICCs removed were done as mentioned above for the common muscle strips. Results were expressed as mean  $\pm$  SE. (3) Transmission Electron Microscopy. We used transmission electron microscopy to identify the effect of methylene blue





**Figure 1** Overview of CCKAR-immunoreactive ICC seen on cross section of guinea pig gallbladder. **A:** Low-magnification montage showing C-kit positive cells (arrow) distributed over a band of gallbladder muscle adjacent to the mucosa; **B:** Low-power montage showing CCKAR-immunoreactive cells distribute mainly in the muscular layers of guinea pig gallbladder; **C:** Compound micrographs from (A) and (B). Some, but not all C-kit immunoreactive (green) ICC colocalize CCKAR (red), meanwhile, not all CCKAR-immunoreactive cells are ICC; **D:** Phase-contrast micrographs of the same gallbladders wall at the same magnification.

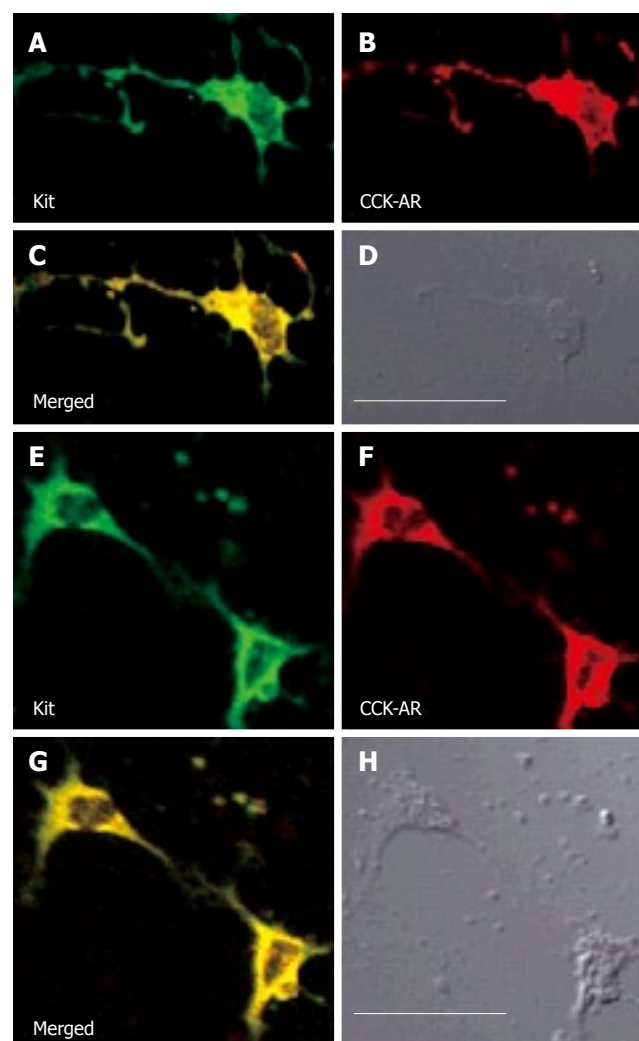
with light on gallbladder ICC. The muscle strips removed ICC were fixed in 2.5% GA in 0.1 mol/L (PB) for 2 h or overnight. They were then washed in 0.1 mol/L PB ( $2 \times 15$  min) and post-fixed with 2% osmium tetroxide in 0.1 mol/L PB (2 h). After another wash with 0.1 mol/L PB ( $2 \times 15$  min), they were dehydrated through ascending grades of ethanol (50, 75, 95 and  $2 \times 100\%$ , 15-20 min each) and embedded in an epoxy resin, Quetol-812. Ultra-thin sections were cut using a Reichert OMU4 ultramicrotome with a diamond-cutting knife. Sections were picked up on 200 mesh copper grids and stained with 2% uranyl acetate in 30% ethanol, and this was followed by Sato's lead staining. Grids with sections were viewed under the OPTON EM10C transmission electron microscope.

## RESULTS

### *Demonstration of CCK-AR on the gallbladder*

**Tissue sections immunohistochemistry:** In cross sections of the gallbladder, intensely CCK-AR immunoreactive interstitial cells were found mainly in the muscular layers. Labeling in the most intensely stained cells was already optimal at an antibody concentration of 1:8000. At low antibody concentrations, there were very little staining found on smooth muscle cells of the gallbladder muscle, yet, at higher concentrations, the gallbladder muscle showed considerable immunoreactivity, particularly in its inner portions (Figure 1).

**Cultured cells immunohistochemistry:** CCK-AR



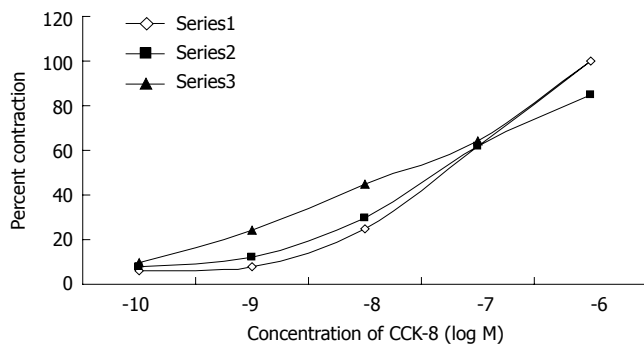
**Figure 2** Colocalization of CCKAR immunoreactivity with markers for ICC, kit in guinea pig gallbladder cultured ICC. CCK-AR immunoreactive ICC in the muscle of the gallbladder is distinctive of their labeled processes. ICC possessed several processes (A-D); C-kit immunoreactive (green) ICC colocalize CCKAR (red); **C:** Compound micrographs from (A) and (B); **D:** Phase-contrast micrographs of the same gallbladder ICC at the same magnification; Often two or more labeled ICC were aggregated into a network (E-H), making it difficult to discern the individual cells; C-kit immunoreactive (green) ICCs colocalize CCKAR (red); **G:** Compound micrographs from (E) and (F); **H:** Phase-contrast micrographs of the same gallbladder ICCs at the same magnification; All these ICCs were big and had a large and angular nucleus; Bar (in D) 30  $\mu$ m for (A-D) and (in H) 30  $\mu$ m for (E-H).

immunoreactive ICC in the muscle of the gallbladder differed in the complexity and shape of their labeling processes (Figure 2). In one extreme end, the processes showed multiplicity and the results were frequently subdivided into fine filaments and peaks (Figure 2A-D). In another end, ICCs possessed only three smooth processes and two labeled ICC were aggregated into the network (Figure 2E-H).

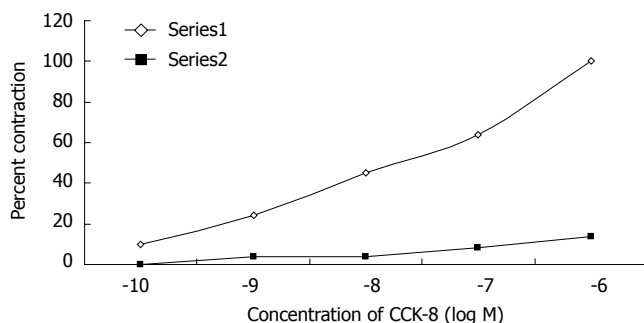
### *CCK-8-induced contractions on gallbladder muscle strips*

The contractile responses of muscle strip to CCK-8 was observed at  $10^{-10}$  mol/L, and they reached the maximum at  $10^{-6}$  mol/L as shown in Figures 3 and 4. CCK-8 stimulated tonic contraction in a concentration-dependent manner. The dose response curve for CCK-8 was not affected





**Figure 3** Effect of atropine and TTX on CCK-8 induced contraction of gallbladder muscle strips. The addition of atropine ( $10^{-6}$  mol/L) or TTX ( $3 \times 10^{-7}$  mol/L) to the medium had no effect on the control dose-response curve for CCK-8. Each value is the mean  $\pm$  SE obtained in 5 observations and observed at  $10^{-10}$ ,  $10^{-9}$ ,  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  mol/L. Series 1: CCK-8+TTX; Series 2: CCK-8+atropine; Series 3: CCK-8 alone.



**Figure 4** Effects of CCK-8 on contraction of gallbladder muscle strips *in vitro*. Removing the ICC of the gallbladder significantly shifted the control dose-response curve to the right. Each value is the mean  $\pm$  SE obtained in 5 observations and observed at  $10^{-10}$ ,  $10^{-9}$ ,  $10^{-8}$ ,  $10^{-7}$ ,  $10^{-6}$  mol/L. Series 1: Common gallbladder muscle strip; Series 2: Gallbladder muscle strip removed ICC.

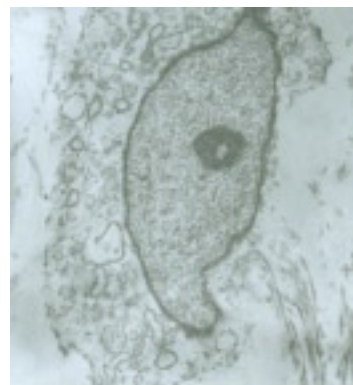
by pretreatment of the muscle strips with atropine ( $10^{-6}$  mol/L) or TTX ( $3 \times 10^{-7}$  mol/L) (Figure 3). The purpose of using atropine and TTX is to eliminate the effect of nervous system on the contractions of the guinea pig gallbladder. When we removed the ICCs from the gallbladder muscle strip, it significantly shifted the dose response curve to the right (Figure 4).

### Transmission electron microscopy

Methylene blue with light destroyed the structure of the gallbladder ICCs. The ICCs were swollen, and they showed low contrast for the cytoplasm. Cells became smaller and processes diminished. All these changes result in the reduction of contractile function of the gallbladder (Figure 5).

## DISCUSSION

In this study, we demonstrated that: (1) CCK-AR are present on the gallbladder interstitial cells of Cajal; (2) The gallbladder contractions evoked by CCK-8 through the CCK-AR on the gallbladder ICC. This is the first report on on ICC of guinea pig *in vitro* studies on the mechanism of gallbladder contraction evoked by CCK-8 through CCK-AR.



**Figure 5** The effect of methylene blue with light on gallbladder ICC. After incubation with methylene and light, ICC on gallbladder was swollen and had low cytoplasmic, and the nucleolus became smaller and processes diminished.

The study has clearly shown that CCK-AR are present on gallbladder ICCs by *in vitro* study with immunohistochemistry, immunocytochemistry and muscle strip trials. The CCK receptors on the gallbladder have been demonstrated previously by autoradiography in the guinea pig. In this study, we have clearly shown that CCK-AR exists in the gallbladder smooth muscle layer in the guinea pig, and to the best of our knowledge, the CCK-AR has not yet been demonstrated by any other studies. When we initially used high primary antibody concentrations, the entire gallbladder exhibited immunoreactivity. With lower antibody concentration of 1:8000, there were little immunoreactivity associated with the smooth muscle, but intense staining of ICC in the gallbladder sections. CCK may bind to CCK-ARs on both smooth muscle and ICC.

We demonstrate here that a population of guinea pig gallbladder ICC strongly expresses CCK-AR immunoreactivity. Identification of ICC was based on their typical morphological traits such as clusters of mitochondria, well-developed smooth endoplasmic reticulum, dense body, lysosomes, distinctive caveolae and abundant fibers around the lysosomes. In addition, co-localization of C-kit double labeling with CCK-AR showed that only a subpopulation was localized mainly to a contiguous band of circular muscle of the guinea pig.

In the gallbladder, ICCs intimately contact with smooth muscle cells with gap junction, and they are responsible for the generation of smooth muscle rhythmic activity<sup>[18]</sup>. The finding that gallbladder ICCs strongly express CCK-AR suggests a CCK-induced gallbladder activity. ICCs may mediate some of the effect of CCK on gallbladder pressure. It was previously shown that the gallbladder muscle strip contractions evoked by CCK are mediated by a direct myogenic action<sup>[27,28]</sup> and inhibited by a specific CCK-AR antagonist in some species<sup>[29]</sup>. The next question is how CCK action is mediated to the gallbladder through the CCK-AR on the ICC. When we removed the ICCs in the gallbladder, the CCK-8 induced contraction dose response curve shifted significantly to the right. Liu<sup>[30]</sup> showed that intense illumination after incubation with methylene blue resulted in specific lesions of dogs' ICCs, and it was verified by electron microscopy. The ICCs were swollen and had low cytoplasmic contrast. They had barely discernible mitochondria with few caveolae, and the plasma membranes was punctuated so that they were presumably ruptured. All changes in ICCs result in the abolishing of the slow wave. The effects of methylene

blue with light in ICC were specific only to ICCs, but not the associated smooth muscle cells. In our study, the contractile ability of the guinea pig gallbladder muscle strips without the evocation of CCK-8 was sharply weakened. This phenomenon implied that CCK-8 evoked the contraction of the gallbladder through the gallbladder ICC.

Immunohistofluorescence and muscle strip trials proved that CCK acted not only on the CCK-AR on the smooth muscle, but also on the CCK-AR on the gallbladder ICC. This may be the first indication that ICC do not only generate slow-wave pacemaker activity and mediate effect of enteric neurotransmitters, but also mediate the effects of circulating hormones on smooth muscle activity.

In conclusion, we have shown that the CCK-evoked contractions are mediated through direct action on CCK-AR on the gallbladder ICC.

## COMMENTS

### Background

It has been convinced that the interstitial cells of Cajal (ICC) in rat gastric pylorus expresses CCK-ARs to a high degree. The fact that some ICC in the rat pylorus expresses CCK-ARs suggests the possibility that circulating CCK can influence gastrointestinal motility via ICC. It is well known that CCK regulates the guinea pig gallbladder contraction by CCK-AR. The ICC were also found in the murine, human and guinea pig gallbladder. It deserved us to study that if there are CCK-ARs on the guinea pig gallbladder ICC and how CCK regulates gallbladder contraction through CCK-AR on ICC.

### Research frontiers

ICC does not only generate slow-wave pacemaker activity and mediate effects of enteric neurotransmitters, but also mediate effects of circulating hormones such as CCK on smooth muscle activity.

### Innovations and breakthroughs

In the present study, it was demonstrated that: (1) CCK-AR is present on the gallbladder interstitial cells of Cajal; (2) The gallbladder contractions evoked by CCK-8 through the CCK-AR on the gallbladder ICC. This is the first report on guinea pig *in vitro* studies on the mechanism of gallbladder contraction evoked by CCK-8 through CCK-AR on ICC.

### Applications

It has been shown that CCK evoked contraction is mediated through direct action on CCK-AR on the gallbladder ICC. This maybe some use of study in gallbladder motility disorders, such as cholelithiasis.

### Peer review

This manuscript demonstrates CCK-AR receptor immunoreactivity in the gallbladder of the guinea pig. Furthermore, the authors demonstrate that administration of CCK8 to gallbladder muscle strips caused muscle contraction. This study is of important clinical significance and should be of interest to the readers.

## REFERENCES

- Lewis LD, Williams JA. Regulation of cholecystokinin secretion by food, hormones, and neural pathways in the rat. *Am J Physiol* 1990; **258**: G512-G518
- Schjoldager B, Shaw MJ, Powers SP, Schmalz PF, Szurszewski J, Miller LJ. Bovine gallbladder muscularis: source of a myogenic receptor for cholecystokinin. *Am J Physiol* 1988; **254**: G294-G299
- von Schrenck T, Moran TH, Heinz-Erian P, Gardner JD, Jensen RT. Cholecystokinin receptors on gallbladder muscle and pancreatic acinar cells: a comparative study. *Am J Physiol* 1988; **255**: G512-G521
- Tokunaga Y, Cox KL, Coleman R, Concepcion W, Nakazato P, Esquivel CO. Characterization of cholecystokinin receptors on the human gallbladder. *Surgery* 1993; **113**: 155-162
- West SD, Mercer DW. Cholecystokinin-induced gastroprotection: a review of current protective mechanisms. *Dig Dis Sci* 2004; **49**: 361-369
- Srivastava A, Pandey SN, Dixit M, Choudhuri G, Mittal B. Cholecystokinin receptor A gene polymorphism in gallstone disease and gallbladder cancer. *J Gastroenterol Hepatol* 2008; **23**: 970-975
- Yamamoto M, Otani M, Jia DM, Fukumitsu K, Yoshikawa H, Akiyama T, Otsuki M. Differential mechanism and site of action of CCK on the pancreatic secretion and growth in rats. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**: G681-G687
- Chen D, Zhao CM, Hakanson R, Samuelson LC, Rehfeld JF, Friis-Hansen L. Altered control of gastric acid secretion in gastrin-cholecystokinin double mutant mice. *Gastroenterology* 2004; **126**: 476-487
- Scheurer U, Varga L, Drack E, Burki HR, Halter F. Measurement of cholecystokinin octapeptide-induced motility of rat antrum, pylorus, and duodenum *in vitro*. *Am J Physiol* 1983; **244**: G261-G265
- Scheurer U, Varga L, Drack E, Burki HR, Halter F. Mechanism of action of cholecystokinin octapeptide on rat antrum, pylorus, and duodenum. *Am J Physiol* 1983; **244**: G266-G272
- Junquera C, Martinez-Ciriano C, Castiella T, Serrano P, Azanza MJ, Junquera SR. Immunohistochemical and ultrastructural characteristics of interstitial cells of Cajal in the rabbit duodenum. Presence of a single cilium. *J Cell Mol Med* 2007; **11**: 776-787
- Thuneberg L. Interstitial cells of Cajal: intestinal pacemaker cells? *Adv Anat Embryol Cell Biol* 1982; **71**: 1-130
- Huizinga JD, Thuneberg L, Kluppel M, Malysz J, Mikkelsen HB, Bernstein A. W/kit gene required for interstitial cells of Cajal and for intestinal pacemaker activity. *Nature* 1995; **373**: 347-349
- Suzuki N, Prosser CL, Dahms V. Boundary cells between longitudinal and circular layers: essential for electrical slow waves in cat intestine. *Am J Physiol* 1986; **250**: G287-G294
- Wang XY, Sanders KM, Ward SM. Intimate relationship between interstitial cells of cajal and enteric nerves in the guinea-pig small intestine. *Cell Tissue Res* 1999; **295**: 247-256
- Sun X, Yu B, Xu L, Dong W, Luo H. Interstitial cells of Cajal in the murine gallbladder. *Scand J Gastroenterol* 2006; **41**: 1218-1226
- Hinescu ME, Ardeleanu C, Gherghiceanu M, Popescu LM. Interstitial Cajal-like cells in human gallbladder. *J Mol Histol* 2007; **38**: 275-284
- Lavoie B, Balemba OB, Nelson MT, Ward SM, Mawe GM. Morphological and physiological evidence for interstitial cell of Cajal-like cells in the guinea pig gallbladder. *J Physiol* 2007; **579**: 487-501
- Lucaites VL, Mendelsohn LG, Mason NR, Cohen ML. CCK-8, CCK-4 and gastrin-induced contractions in guinea pig ileum: evidence for differential release of acetylcholine and substance P by CCK-A and CCK-B receptors. *J Pharmacol Exp Ther* 1991; **256**: 695-703
- Dal Forno G, Pietra C, Urciuoli M, van Amsterdam FT, Toson G, Gaviraghi G, Trist D. Evidence for two cholecystokinin receptors mediating the contraction of the guinea pig isolated ileum longitudinal muscle myenteric plexus. *J Pharmacol Exp Ther* 1992; **261**: 1056-1063
- Patel M, Spraggs CF. Functional comparisons of gastrin/cholecystokinin receptors in isolated preparations of gastric mucosa and ileum. *Br J Pharmacol* 1992; **106**: 275-282
- Bishop LA, Gerskowitch VP, Hull RA, Shankley NP, Black JW. Combined dose-ratio analysis of cholecystokinin receptor antagonists, devazepide, lorglumide and loxiglumide in the guinea-pig gall bladder. *Br J Pharmacol* 1992; **106**: 61-66
- Sonobe K, Sakai T, Satoh M, Haga N, Itoh Z. Control of gallbladder contractions by cholecystokinin through cholecystokinin-A receptors in the vagal pathway and gallbladder in the dog. *Regul Pept* 1995; **60**: 33-46
- Fischer de Toledo C, Roettger BF, Morys-Wortmann C,

- Schmidt WE, Miller LJ. Cellular handling of unoccupied and agonist-stimulated cholecystokinin receptor determined by immunolocalization. *Am J Physiol* 1997; **272**: G488-G497
- 25 **Sternini C**, Wong H, Pham T, De Giorgio R, Miller LJ, Kuntz SM, Reeve JR, Walsh JH, Raybould HE. Expression of cholecystokinin A receptors in neurons innervating the rat stomach and intestine. *Gastroenterology* 1999; **117**: 1136-1146
- 26 **Patterson LM**, Zheng H, Ward SM, Berthoud HR. Immunohistochemical identification of cholecystokinin A receptors on interstitial cells of Cajal, smooth muscle, and enteric neurons in rat pylorus. *Cell Tissue Res* 2001; **305**: 11-23
- 27 **Amer MS**. Studies with cholecystokinin *in vitro*. 3. Mechanism of the effect on the isolated rabbit gall bladder strips. *J Pharmacol Exp Ther* 1972; **183**: 527-534
- 28 **Yau WM**, Makhlouf GM, Edwards LE, Farrar JT. Mode of action of cholecystokinin and related peptides on gallbladder muscle. *Gastroenterology* 1973; **65**: 451-456
- 29 **Gully D**, Frehel D, Marcy C, Spinazze A, Lespy L, Neliat G, Maffrand JP, Le Fur G. Peripheral biological activity of SR 27897: a new potent non-peptide antagonist of CCKA receptors. *Eur J Pharmacol* 1993; **232**: 13-19
- 30 **Liu LW**, Thuneberg L, Huizinga JD. Selective lesioning of interstitial cells of Cajal by methylene blue and light leads to loss of slow waves. *Am J Physiol* 1994; **266**: G485-G496

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RAPID COMMUNICATION

## Serum leptin and soluble leptin receptor in non-alcoholic fatty liver disease

Xiao-Dong Huang, Yan Fan, Hen Zhang, Ping Wang, Jing Ping Yuan, Ming-Jie Li, Xi-Yan Zhan

Xiao-Dong Huang, Yan Fan, Hen-Zhang, Ping-Wang, Department of Gastroenterology, The Central Hospital of Wuhan, Wuhan 430014, Hubei Province, China

Jing-Ping Yuan, Department of Pathology, The Central Hospital of Wuhan, Wuhan 430014, Hubei Province, China

Ming-Jie Li, Department of Hepatobiliary Surgery, The Central Hospital of Wuhan, Wuhan 430014, Hubei Province, China

Xi-Yan Zhan, Department of Clinical Laboratory, The Central Hospital of Wuhan, Wuhan 430014, Hubei Province, China

Author contributions: Huang XD and Fan Y contributed equally to this work; Fan Y, Zhang H, Wang P, Yuan JP, Li MJ and Zhan XY performed the research; Fan Y analyzed the data; and Huang XD wrote the paper.

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Correspondence to: Xiao-Dong Huang, Department of Gastroenterology, The Central Hospital of Wuhan, Wuhan 430014, Hubei Province, China. [fanyanyanyan@163.com](mailto:fanyanyanyan@163.com)

Telephone: +86-27-82811080 Fax: +86-27-82811446

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feature of steatosis, and serum leptin seems to increase as hepatocyte steatosis develops. An enhanced release of leptin is accompanied by an decrease in sOB-R concentration, which suggests higher resistance of peripheral tissues towards the action of leptin.

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**Key words:** Leptin; Soluble leptin receptor; Non-alcoholic fatty liver disease

**Peer reviewer:** Philip Abraham, Professor, Consultant Gastroenterologist & Hepatologist, PD Hinduja National Hospital & Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai 400 016, India

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### Abstract

**AIM:** To determine the role of leptin system in non-alcoholic fatty liver disease (NAFLD) development by delineating the changes in serum levels of leptin and soluble leptin receptor (sOB-R).

**METHODS:** Blood samples were collected from 30 consecutive patients with liver-biopsy-proven NAFLD and 30 patients with cholecystolithiasis (stationary phase) as controls. Serum leptin levels were determined by radio-immunoassay and concentration of sOB-R was measured by ELISA. Body mass index (BMI) was calculated for all subjects, and serum insulin, C-peptide, and lipoprotein levels were also detected.

**RESULTS:** Mean serum leptin level and BMI in the NAFLD group were significantly higher than in the controls (both  $P < 0.001$ ), but mean sOB-R level was lower in the NAFLD group when compared to the controls. Both men and women in the NAFLD group had higher mean serum leptin levels and lower sOB-R levels than did the men and women in the control group (all  $P < 0.001$ ). There was a significant negative correlation between serum leptin and sOB-R levels ( $r = -0.725$ ,  $P < 0.001$ ). Multivariate analysis showed that the percentage of hepatocyte steatosis, sex, BMI, and homeostasis model assessment of insulin resistance (HOMA IR) were independently related to serum leptin levels.

**CONCLUSION:** Elevated serum leptin seems to be a

### INTRODUCTION

Leptin - the ob gene product - is a circulating 16-kDa peptide hormone secreted mainly by adipocytes of white fat tissue. It regulates food intake, body fat, insulin action, thermogenesis, induction of angiogenesis, and modulation of the immune system. Leptin synthesis in adipocytes is regulated by several hormones<sup>[1]</sup>. Leptin action in peripheral tissues involves interaction with specific transmembrane receptors. The leptin receptor (Ob-R), which is a member of the class-1 cytokine receptor family, may be an important determinant of leptin sensitivity. Ob-R was originally demonstrated in hypothalamic neurons, through which leptin regulates food intake and body weight<sup>[2]</sup>. Alongside several membrane-bound isoforms of Ob-R, with varying cytoplasmic length and with the same extracellular domain, a soluble form of the leptin receptor (sOb-R) can be demonstrated. sOb-R represents the main leptin-binding compound in plasma, which results in fractions of bound and free leptin in plasma<sup>[3]</sup>. The balance between the free form, the rapidly bioavailable compartment, and bound leptin regulates leptin bioavailability<sup>[4]</sup>. However, the precise pathophysiological role of sOb-R has not yet been defined.

Non-alcoholic fatty liver disease (NAFLD) is increasingly recognized as a common and potentially severe condition often associated with obesity, type 2 diabetes and



hyperlipidemia<sup>[5]</sup>. However, the pathogenesis of NAFLD remains unclear. Increased synthesis of fatty acids in the liver, increased delivery of free fatty acids to the liver, and decreased  $\beta$ -oxidation of free fatty acids may cause the accumulation of fat in the liver<sup>[6]</sup>. Therefore, fat in hepatocytes causes cellular dysfunction and may render the liver more vulnerable to any factor that leads to inflammation. Furthermore, hepatic steatosis, the hallmark of NAFLD<sup>[7]</sup>, may damage liver parenchyma.

The close relationship of leptin with adipose tissue and fat stores of the body suggests its involvement in the etiology and pathogenesis of NAFLD. Chitturi *et al.*<sup>[8]</sup> have shown that serum leptin levels are significantly higher in subjects with non-alcoholic steatohepatitis (NASH), when compared to controls. Furthermore, they have shown that serum leptin is independently associated with the degree of hepatic steatosis but not hepatic inflammation or fibrosis. In addition to this study, there have been three other published studies that have yielded conflicting results regarding the role of leptin in the pathogenesis of NASH<sup>[9-11]</sup>. Thus, the role of leptin in NAFLD is not yet clear. Also, the relationship between sOB-R and NAFLD is an unresolved issue. Since concentrations of sOB-R are decreased in obese subjects when compared with lean controls<sup>[12]</sup>, an interaction between sOB-R and NAFLD is suspected. However, currently no data on the association of sOB-R with NAFLD disease are available. Therefore, the objective of this study was to evaluate the association between serum leptin and sOB-R levels and NAFLD.

## MATERIALS AND METHODS

### Patients

A total of 30 consecutive patients with liver-biopsy-proven NAFLD were enrolled in the study from May 2007 to September 2007. NAFLD patients were admitted or referred to our department with incidentally found liver enzyme elevations. For the diagnosis of NAFLD and to rule out other possible liver diseases, all patients underwent liver biopsy. Thirty patients with cholecystolithiasis (stationary phase) were recruited as controls. Liver biopsies were obtained during an examination that preceded laparoscopic cholecystectomy, using a 16-gauge bard core biopsy needle. Informed consent was obtained from all patients.

All subjects underwent a detailed clinical and laboratory evaluation, including liver function tests, hepatitis markers, and autoantibodies, in addition to upper abdominal ultrasonography. Alcohol consumption was absent in all subjects. Subjects with previous or current history of acute or chronic viral hepatitis; malignant disease; acute infections; pituitary, adrenal, thyroid and pancreatic disease, or evidence for any other endocrine disorder; or prolonged use of corticosteroids or sex hormones were excluded. The controls had normal liver enzymes and no clinical, laboratory or imaging evidence of liver disease.

### Clinic and laboratory evaluation

Overnight (12 h) fasting samples of serum obtained after centrifugation were stored in aliquots at -70°C until assayed. Clinical and laboratory data were collected on the

date the liver biopsy was performed. A complete medical history and physical examination was accomplished in all patients and controls. Body mass index (BMI) was calculated. Laboratory evaluation included liver enzymes, glucose, complete blood count, total cholesterol, triglycerides, viral serology for hepatitis B and C, and autoantibodies. C-peptide was measured by a direct, double antibody sequential radioimmunoassay (RIA) (North Bio, Beijing, China). Serum insulin was measured by enzyme immunoassay kits (North Bio). The insulin resistance (IR) index was calculated on the basis of fasting values of plasma glucose and insulin according to the homeostasis model assessment for insulin sensitivity (HOMA) model formula:  $\text{HOMA IR} = \text{fasting insulin (mUI/L)} \times \text{fasting glucose (mmol/L)} / 22.5$ .

Leptin levels were measured by RIA kits (North Bio). The test utilizes <sup>125</sup>I-labelled human leptin and human leptin antiserum to determine the level of total (free of and bound to serum proteins) leptin in serum. The test was 100% specific for human leptin and the sensitivity was 0.45 ng/mL. The coefficient of variation (CV) was < 8.3% and < 6.2%, respectively, for intra- and inter-assay variations ( $n = 5$ ). The total concentration of sOB-R was determined using photometric ELISA kits (BioVendor Laboratory Medicine Inc, Brno, Czech Republic), which utilizes double monoclonal antihuman leptin receptor antibodies labeled with horseradish peroxidase. This test was 100% specific for human leptin receptors and the sensitivity was 0.4 U/mL. The intra-assay (CV < 5.6%) and inter-assay precision was good (CV < 5.5%). Samples were diluted three times according to the manufacturer's recommendation, and results higher than the higher standard were run again at another dilution.

### Liver histology

Liver biopsy specimens were at least 15 mm in length, and had an appropriate number of portal tracts to make a confident evaluation of histological features and diagnosis<sup>[13]</sup>. Slides were routinely stained with hematoxylin-eosin, Masson's trichrome and special stains for iron and copper. Liver biopsies were read by a single liver pathologist who was unaware of the patients' clinical and laboratory data. Degree of steatosis was assessed on a scale of 1-3: 1, mild (5%-33% of hepatocytes affected); 2, moderate (33%-66% of hepatocytes affected); and 3, severe (> 66% of hepatocytes affected). The grades of inflammation and stages of fibrosis were modified to two categories as follows: mild inflammation (Kleiner grades 0 and 1) and moderate-to-severe inflammation (Kleiner grades 2 and 3), mild fibrosis (stages 0-2), and advanced fibrosis (stages 3 and 4). NASH was defined as steatosis plus lobular inflammation, plus either ballooning of hepatocytes or abnormal fibrosis (stages 1-4).

### Statistical analysis

Hepatic steatosis was assessed as categorical (absent or present) or a continuous variable from 0%-100%. Comparisons between the NAFLD group and controls were made using Student's *t* test for continuous variables and the  $\chi^2$  or Fisher's exact probability test for categorical data. All values are presented as mean  $\pm$  SD.  $P < 0.05$  was

**Table 1** Clinical and laboratory data of patients with NAFLD and controls

	NAFLD	Control	P
n	30	30	
Gender (F/M)	15/15	16/14	0.8
Age	41.97 ± 8.36	43.90 ± 8.35	0.374
BMI (kg/m <sup>2</sup> )	26.47 ± 3.11	23.97 ± 1.88	< 0.001
Total cholesterol (mmol/L)	4.11 ± 0.85	3.97 ± 0.63	0.446
Triglyceride (mmol/L)	1.54 ± 0.62	1.30 ± 0.38	0.075
C-peptide (nmol/L)	1.73 ± 0.29	1.78 ± 0.34	0.505
Glucose (mmol/L)	5.42 ± 0.94	5.03 ± 0.68	0.072
Insulin (mU/L)	14.22 ± 2.66	8.64 ± 1.88	< 0.001
HOMA IR	3.46 ± 1.04	1.92 ± 0.45	< 0.001
Leptin (ng/mL)	10.90 ± 3.39	5.55 ± 1.96	< 0.001
sOB-R (ng/mL)	18.00 ± 3.74	23.51 ± 2.62	< 0.001

considered to be statistically significant. Multiple linear regression has been used in multivariate analysis of factors associated with leptin levels, including age, sex, steatosis, BMI, sOB-R, C-peptide and IR.

## RESULTS

### Comparison of patients and controls

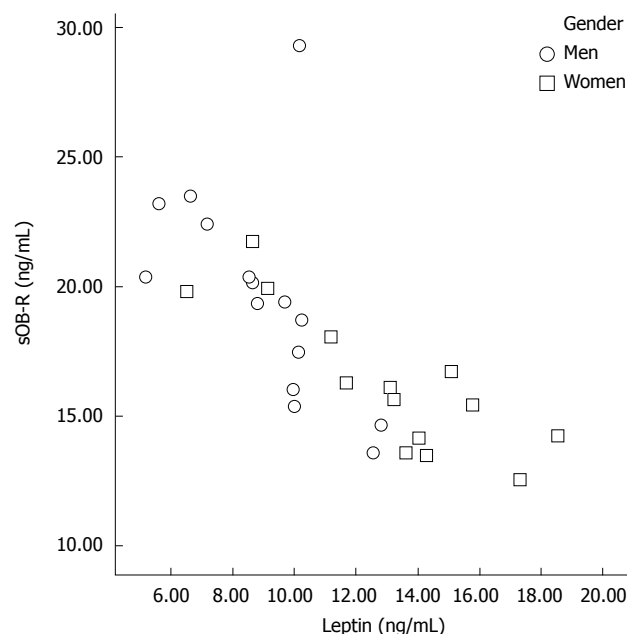
Clinical and biochemical characteristics of the patients with NAFLD and controls are compared in Table 1. Serum leptin levels were found to be significantly higher in patients with NAFLD as compared with the controls ( $P < 0.001$ ), and subjects with NAFLD had significantly lower levels of sOB-R, and higher levels of insulin and HOMA than the controls. There was no significant difference in serum total cholesterol, triglyceride, C-peptide or glucose levels in patients with NAFLD when compared with the controls.

### Gender difference in serum leptin and sOB-R levels

As expected, serum leptin levels were significantly higher in women than in men in the NAFLD group ( $P = 0.002$ ). In contrast, sOB-R levels were significantly lower in women compared with men in the NAFLD group ( $P = 0.018$ ). For this reason, comparisons were made between men and women separately. Both men and women in the NAFLD group had higher mean serum leptin levels and lower sOB-R levels than did the men and women in the control group (all  $P < 0.001$ , Table 2).

### Histological Evaluation

Upon histological staging, steatosis was mild in 17 patients, moderate to severe in 13; inflammation was mild in 16, and moderate to severe in 14; and fibrosis was absent in nine, mild in 21, and advanced in one patient. As summarized in Table 3, patients with moderate to severe steatosis had significantly higher levels of leptin than those with mild steatosis. Similarly, patients with moderate to severe steatosis were more insulin resistant as indicated by higher values of HOMA-IR. sOB-R levels were higher in the mild steatosis group than in the moderate to severe steatosis group, but the difference was not significant ( $P = 0.098$ ).

**Figure 1** Correlation between serum leptin and sOB-R.

### Correlation between serum leptin and sOB-R

There was a significant negative correlation between serum leptin and sOB-R levels ( $r = -0.725$ ,  $P < 0.001$ ; Figure 1).

### Relationship of serum leptin and sOB-R levels with BMI

The proportion of obese patients was rather low in the study population. Therefore, to determine the relationship between obesity and leptin more clearly, the NAFLD patients were divided into two groups according to mean BMI (26.5) as a cut-off point. Thus, patients with a BMI of 26.5 were defined as overweight. There were 12 patients with a BMI  $\geq 26.5$ , and there were 18 patients with a BMI  $< 26.5$ . The serum leptin levels and HOMA IR values were significantly higher in the overweight group than in the normal weight group ( $P < 0.001$  and  $P = 0.01$ , respectively). In addition, the sOB-R levels were significantly lower in the overweight group than in the normal weight group ( $P < 0.001$ , Table 4). Age, sex, BMI, HOMA IR, C-peptide and steatosis were tested in an overall multiple linear regression model, and the percentage of hepatocyte steatosis, sex, and BMI were independently related to serum leptin levels (Table 5).

## DISCUSSION

The findings of the present study show that the adipocyte-derived hormone leptin is involved in metabolic abnormalities that lead to steatosis in NAFLD patients. Significantly high levels of serum leptin in NAFLD patients were found. In contrast to increased leptin level, the concentration of sOB-R significantly decreased in patients with NAFLD as compared to controls. As expected, the serum leptin level was significantly higher in women. Such a gender difference in serum leptin levels has been reported previously<sup>[14]</sup>. Leptin is exclusively expressed in adipose tissue and secreted from white adipose cells<sup>[15]</sup>. The difference is,

Table 2 Leptin and sOB-R results according to gender

	Male			Female		
	NAFLD	Control	P	NAFLD	Control	P
n	15	14		15	16	
Leptin (ng/mL)	9.07 ± 2.22	4.89 ± 1.73	< 0.001	12.73 ± 3.43	6.13 ± 2.01	< 0.001
sOB-R (ng/mL)	19.58 ± 4.04	23.92 ± 2.36	< 0.001	16.41 ± 2.70	23.15 ± 2.86	< 0.001

Table 3 Leptin and sOB-R results in relation to degree of steatosis

	Steatosis (n = 30)		
	Mild	Moderate to severe	P
n	17	13	
Gender (F/M)	9/8	6/7	0.7
Leptin (ng/mL)	9.42 ± 2.76	12.84 ± 3.23	0.004
sOB-R (ng/mL)	18.99 ± 2.95	16.70 ± 4.36	0.098
HOMA IR	2.98 ± 0.86	4.20 ± 0.83	0.001

Table 4 The results of BMI, leptin and sOB-R tests in NASH patients with normal weight and overweight

	Overweight (BMI ≥ 26.5)	Normal weight (BMI < 26.5)	P
n	12	18	
Female/Male	8/4	9/9	0.5
Leptin (ng/mL)	13.73 ± 2.96	9.02 ± 2.14	< 0.001
sOB-R (ng/mL)	15.16 ± 2.10	19.89 ± 3.40	< 0.001
HOMA IR	4.06 ± 1.06	3.14 ± 0.86	0.01

therefore, in part, due to the higher percentage body fat in women. In addition, our study showed that serum leptin levels were strongly correlated with BMI or fat mass, consistent with a number of previous studies<sup>[16-18]</sup>. Also, the above results suggest a significant difference between normal and overweight NAFLD patients with regard to serum leptin levels.

The antisteatotic effects of leptin, which can curtail intrahepatic lipid accumulation (lipotoxicity), have been demonstrated in rodents. Thus, we considered whether leptin could play a similar role in NAFLD. Steatosis is a feature of clinical states characterized by defective leptin signaling. Steatosis is therefore often present in obese individuals<sup>[19]</sup> who are generally leptin resistant, and also in patients with congenital generalized lipodystrophy, a syndrome of leptin deficiency<sup>[20]</sup>. One of the important results of the study was that higher serum leptin levels were detected in patients with moderate to severe steatosis, compared with mild steatosis. The finding was inconsistent with a simple antisteatotic effect of leptin, which assumes that serum leptin values correlate with leptin biological activity in hepatocytes. There are two most likely explanations for the above finding. Firstly, leptin is inextricably related to IR. It has been suggested that leptin may contribute to hepatic steatosis by promoting IR and by altering insulin signaling in hepatocytes, so as to promote increased intracellular

Table 5 Multiple linear regression analysis ( $R^2 = 0.895$ ) with serum leptin levels as a dependent variable in patients with NAFLD

Variable	Coefficient	SE	t	P
Age	-0.040	-0.099	-1.371	0.183
Sex	2.128	0.319	4.295	< 0.001
BMI (kg/m <sup>2</sup> )	0.529	0.485	5.838	< 0.001
C-peptide (nmol/L)	-0.866	-0.075	-1.072	0.295
HOMA IR	0.744	0.227	2.399	0.025
Steatosis (%)	1.329	0.247	2.675	0.014

fatty acids<sup>[21]</sup>. Moreover, at a later stage, leptin may cause hepatic steatosis to turn into steatohepatitis by amplifying selected proinflammatory responses<sup>[21]</sup>. This explanation is mainly based on the effect of insulin on hepatic fat metabolism. In this study, an elevation of serum insulin level and HOMA IR in NAFLD patients, in comparison to the controls, was detected, which supports the latter suggestion. Secondly, those patients who develop NAFLD differ from obese individuals who do not develop NASH by having a state of peripheral (hepatic) leptin resistance (inadequate leptin signaling or/and decreased levels of sOB-R).

The biological relationship between leptin and its soluble receptor has previously been well established in animal models, as well as in various other clinical conditions, such as obesity, puberty<sup>[22]</sup>, pregnancy<sup>[23]</sup> and inflammation<sup>[24]</sup>. However, to the best of our knowledge, the serum level of sOB-R has not previously been described in NAFLD patients. In this study, a strong negative correlation was found between serum levels of leptin and sOB-R in patients with NAFLD.

The low sOB-R levels in NAFLD patients may be part of a feedback mechanism aimed at reducing the increase in leptin. High free leptin levels were observed in morbidly obese subjects, probably caused by high leptin release by adipocytes due to abundant food intake. Interestingly, during food restriction, circulating leptin levels in lean and obese subjects drop dramatically within 1 d after onset of starvation<sup>[25]</sup>, which indicates that leptin release is strongly reduced. On the other hand, plasma sOB-R might function like the soluble IL-6 receptor for the cytokine IL-6 (also a member of the gp-130 receptor family) and enhance leptin signaling. An increase in the bioavailability of leptin in ob/ob mice by overexpression of sOB-R leads to an improved weight-reducing effect of exogenous leptin<sup>[26]</sup>. It has been suggested that decreased serum levels of sOB-R result in adequate leptin signaling in NAFLD. Furthermore, the origin of sOB-R in plasma is also unclear at present. It may originate from alternative splicing of the OB-R or from

full-length functional OB-Rs released by enzymatic cleavage. In line with this, sOB-R levels in plasma may reflect the amount of OB-R expressed by tissues. The finding of decreased serum levels of sOB-R in NAFLD suggests an increased resistance of peripheral tissues to the action of leptin, due to decreased expression and secretion of the OB-R. This might be in agreement with the proposed leptin resistance in morbidly obese subjects<sup>[27-29]</sup>.

In conclusion, this study shows that increased serum concentrations of leptin are involved in NAFLD. Enhanced release of leptin is accompanied by a decrease in sOB-R concentration, which suggests higher resistance of peripheral tissues towards the action of leptin. However, the precise pathophysiological role of leptin and sOB-R has not yet been defined. We suggest that further studies should focus on local expression of leptin and its receptor in tissues of non-adipose origin, since the expression of leptin has been shown to be non-specific for adipocytes.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

The exact pathogenesis in non-alcoholic fatty liver disease (NAFLD) is poorly understood, but some studies have analyzed the role of a variety of soluble peptides and other mediators. The close relationship of leptin with adipose tissue and fat stores of the body suggests its possible involvement in the etiology and pathogenesis of NAFLD.

### Research frontiers

There have been several studies on the association between leptin and NAFLD, which have yielded conflicting results regarding the role of leptin in the pathogenesis of NASH. Since concentrations of the sOB-R are decreased in obese subjects when compared with lean controls, interaction between sOB-R and NAFLD is suspected. However, currently no data on the association of sOB-R with NAFLD disease are available.

### Innovations and breakthroughs

The study showed elevated levels of leptin with a corresponding decrease in the concentration of sOB-R in patients with NAFLD. Although the results of laboratory experiments are not readily applicable to the clinical situation, these findings offer further insight into the role of leptin in the pathogenesis of NAFLD.

### Applications

The results from this study confirm higher resistance of liver towards the action of leptin. This encourages us to study promising new drugs for treating NAFLD to improve leptin resistance in future clinical trials.

### Peer review

This study aimed to investigate the role of leptin system in NAFLD development by delineating the changes in serum levels of leptin and sOB-R. The authors found a higher resistance of peripheral tissues towards the action of leptin. This is an interesting subject.

## REFERENCES

- 1 Stenvinkel P, Lonnqvist F, Schalling M. Molecular studies of leptin: implications for renal disease. *Nephrol Dial Transplant* 1999; **14**: 1103-1112
- 2 Tartaglia LA, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Wool EA, Monroe CA, Tepper RI. Identification and expression cloning of a leptin receptor, OB-R. *Cell* 1995; **83**: 1263-1271
- 3 Lammert A, Kiess W, Bottner A, Glasow A, Kratzsch J. Soluble leptin receptor represents the main leptin binding activity in human blood. *Biochem Biophys Res Commun* 2001; **283**: 982-988
- 4 Lahlou N, Clement K, Carel JC, Vaisse C, Lotton C, Le Bihan Y, Basdevant A, Lehouc Y, Froguel P, Roger M, Guy-Grand B. Soluble leptin receptor in serum of subjects with complete resistance to leptin: relation to fat mass. *Diabetes* 2000; **49**: 1347-1352
- 5 Angulo P. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231
- 6 Sheth SG, Gordon FD, Chopra S. Nonalcoholic steatohepatitis. *Ann Intern Med* 1997; **126**: 137-145
- 7 Day CP, James OF. Hepatic steatosis: innocent bystander or guilty party? *Hepatology* 1998; **27**: 1463-1466
- 8 Chitturi S, Farrell G, Frost L, Kriketos A, Lin R, Fung C, Liddle C, Samarasinghe D, George J. Serum leptin in NASH correlates with hepatic steatosis but not fibrosis: a manifestation of lipotoxicity? *Hepatology* 2002; **36**: 403-409
- 9 Uygun A, Kadayifci A, Yesilova Z, Erdil A, Yaman H, Saka M, Devenci MS, Bagci S, Gulsen M, Karaeren N, Dagalp K. Serum leptin levels in patients with nonalcoholic steatohepatitis. *Am J Gastroenterol* 2000; **95**: 3584-3589
- 10 Giannini E, Botta F, Cataldi A, Tenconi GL, Ceppa P, Barreca T, Testa R. Leptin levels in nonalcoholic steatohepatitis and chronic hepatitis C. *Hepatogastroenterology* 1999; **46**: 2422-2425
- 11 Nakao K, Nakata K, Ohtsubo N, Maeda M, Moriuchi T, Ichikawa T, Hamasaki K, Kato Y, Eguchi K, Yukawa K, Ishii N. Association between nonalcoholic fatty liver, markers of obesity, and serum leptin level in young adults. *Am J Gastroenterol* 2002; **97**: 1796-1801
- 12 van Dielen FM, van't Veer C, Buurman WA, Greve JW. Leptin and soluble leptin receptor levels in obese and weight-losing individuals. *J Clin Endocrinol Metab* 2002; **87**: 1708-1716
- 13 Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Ferrell LD, Liu YC, Torbenson MS, Unalp-Arida A, Yeh M, McCullough AJ, Sanyal AJ. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**: 1313-1321
- 14 Couillard C, Mauriege P, Prud'homme D, Nadeau A, Tremblay A, Bouchard C, Despres JP. Plasma leptin concentrations: gender differences and associations with metabolic risk factors for cardiovascular disease. *Diabetologia* 1997; **40**: 1178-1184
- 15 Friedman JM, Halaas JL. Leptin and the regulation of body weight in mammals. *Nature* 1998; **395**: 763-770
- 16 Ankarberg-Lindgren C, Dahlgren J, Carlsson B, Rosberg S, Carlsson L, Wikland KA, Norjavaara E. Leptin levels show diurnal variation throughout puberty in healthy children, and follow a gender-specific pattern. *Eur J Endocrinol* 2001; **145**: 43-51
- 17 Ahmed ML, Ong KK, Watts AP, Morrell DJ, Preece MA, Dunger DB. Elevated leptin levels are associated with excess gains in fat mass in girls, but not boys, with type 1 diabetes: longitudinal study during adolescence. *J Clin Endocrinol Metab* 2001; **86**: 1188-1193
- 18 Horlick MB, Rosenbaum M, Nicolson M, Levine LS, Fedun B, Wang J, Pierson RN Jr, Leibel RL. Effect of puberty on the relationship between circulating leptin and body composition. *J Clin Endocrinol Metab* 2000; **85**: 2509-2518
- 19 James OF, Day CP. Non-alcoholic steatohepatitis (NASH): a disease of emerging identity and importance. *J Hepatol* 1998; **29**: 495-501
- 20 Chandalia M, Garg A, Vaitch F, Nizzi F. Postmortem findings in congenital generalized lipodystrophy. *J Clin Endocrinol Metab* 1995; **80**: 3077-3081
- 21 Kaplan LM. Leptin, obesity, and liver disease. *Gastroenterology* 1998; **115**: 997-1001
- 22 Quinton ND, Smith RF, Clayton PE, Gill MS, Shalet S, Justice SK, Simon SA, Walters S, Postel-Vinay MC, Blakemore AI,



- Ross RJ. Leptin binding activity changes with age: the link between leptin and puberty. *J Clin Endocrinol Metab* 1999; **84**: 2336-2341
- 23 **Lewandowski K**, Horn R, O'Callaghan CJ, Dunlop D, Medley GF, O'Hare P, Brabant G. Free leptin, bound leptin, and soluble leptin receptor in normal and diabetic pregnancies. *J Clin Endocrinol Metab* 1999; **84**: 300-306
- 24 **Voegelings S**, Fantuzzi G. Regulation of free and bound leptin and soluble leptin receptors during inflammation in mice. *Cytokine* 2001; **14**: 97-103
- 25 **Kolaczynski JW**, Considine RV, Ohannesian J, Marco C, Opentanova I, Nyce MR, Myint M, Caro JF. Responses of leptin to short-term fasting and refeeding in humans: a link with ketogenesis but not ketones themselves. *Diabetes* 1996; **45**: 1511-1515
- 26 **Pelleymounter MA**, Cullen MJ, Healy D, Hecht R, Winters D, McCaleb M. Efficacy of exogenous recombinant murine leptin in lean and obese 10- to 12-mo-old female CD-1 mice. *Am J Physiol* 1998; **275**: R950-R959
- 27 **Arch JR**, Stock MJ, Trayhurn P. Leptin resistance in obese humans: does it exist and what does it mean? *Int J Obes Relat Metab Disord* 1998; **22**: 1159-1163
- 28 **Zimmet P**, Boyko EJ, Collier GR, de Courten M. Etiology of the metabolic syndrome: potential role of insulin resistance, leptin resistance, and other players. *Ann N Y Acad Sci* 1999; **892**: 25-44
- 29 **Martin RL**, Perez E, He YJ, Dawson R Jr, Millard WJ. Leptin resistance is associated with hypothalamic leptin receptor mRNA and protein downregulation. *Metabolism* 2000; **49**: 1479-1484

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RAPID COMMUNICATION

## Down-regulation of transforming growth factor $\beta$ 1/activin receptor-like kinase 1 pathway gene expression by herbal compound 861 is related to deactivation of LX-2 cells

Li Li, Xin-Yan Zhao, Bao-En Wang

Li Li, Xin-Yan Zhao, Bao-En Wang, Liver Research Center, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China

Author contributions: Wang BE, Li L and Zhao XY designed the research; Li L and Zhao XY performed the research; Li L, Zhao XY and Wang BE analyzed the data; and Li L wrote the paper.

Correspondence to: Bao-En Wang, MD, Liver Research Center, Beijing Friendship Hospital, Capital Medical University, Beijing 100050, China. [bjfhlc@126.com](mailto:bjfhlc@126.com)

Telephone: +86-10-63164411 Fax: +86-10-63162233

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### Abstract

**AIM:** To investigate the effect of herbal compound 861 (Cpd861) on the transforming growth factor- $\beta$ 1 (TGF $\beta$ 1)/activin receptor-like kinase 1 (ALK1, type I receptor) signaling-pathway-related gene expression in the LX-2 cell line, and the inhibitory mechanism of Cpd861 on the activation of LX-2 cells.

**METHODS:** LX-2 cells were treated with TGF $\beta$ 1 (5 ng/mL) Cpd861 (0.1 mg/mL), TGF $\beta$ 1 (5 ng/mL) plus Cpd861 (5 ng/mL) for 24 h to investigate the effect of Cpd861 on the TGF $\beta$ 1/ALK1 pathway. Real-time PCR was performed to examine the expression of  $\alpha$ -SMA ( $\alpha$ -smooth muscle actin), *ALK1*, *Id1* (inhibitor of differentiation 1). Western blotting was carried out to measure the levels of  $\alpha$ -SMA and phosphorylated Smad1, and immunocytochemical analysis for the expression of  $\alpha$ -SMA.

**RESULTS:** In LX-2 cells, TGF $\beta$ 1/ALK1-pathway-related gene expression could be stimulated by TGF $\beta$ 1, which led to excessive activation of the cells. Cpd861 decreased the activation of LX-2 cells by reducing the expression of  $\alpha$ -SMA mRNA and protein expression. This effect was related to inhibition of the above TGF $\beta$ 1/ALK1-pathway-related expression of genes such as *Id1* and *ALK1*, and phosphorylation of Smad1 in LX-2 cells, even with TGF $\beta$ 1 co-treatment for 24 h.

**CONCLUSION:** Cpd861 can restrain the activation of LX-2 cells by inhibiting the TGF $\beta$ 1/ALK1/Smad1 pathway.

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**Key words:** Herbal compound 861; LX-2 cell; Activin

receptor-like kinase 1; Inhibitor of differentiation 1; Smad1

**Peer reviewers:** Shannon S Glaser, Dr, Department of Internal Medicine, Scott & White Hospital, 702 SW HK Dodgen Loop, Medical Research Building, Temple 76504, United States; Scott A Waldman, MD, PhD, FCP, Division of Clinical Pharmacology, Departments of Medicine and Biochemistry and Molecular Pharmacology, Jefferson Medical College, Thomas Jefferson University, 1100 Walnut Street, MOB Suite 813, Pennsylvania 19107, Philadelphia, United States

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### INTRODUCTION

Hepatic fibrosis is a reversible scarring process that is characterized by increased and altered deposition of extracellular matrix in the liver. During the past 20 years, much research has proved that the activation of hepatic stellate cells (HSCs) triggers fibrogenesis<sup>[1-3]</sup> and TGF $\beta$ 1 has a pivotal regulating role in this process<sup>[1,4,5]</sup>. Currently, TGF $\beta$ 1/ALK1/Smad1 signaling is found in HSCs and is involved in their activation<sup>[6-8]</sup>.

Herbal compound 861 (Cpd861) is a traditional Chinese medicines that is used to treat liver diseases and has been demonstrated to have anti-fibrotic effects and to reverse cirrhosis, especially in the early stage<sup>[9-11]</sup>. Cpd861 is an extract of 10 herbs. A randomized, double-blinded, placebo-controlled clinical trial has demonstrated that Cpd861 can significantly delay and reverse the process of clinical hepatic fibrosis in patients with liver fibrosis and early cirrhosis due to HBV infection, as diagnosed with liver biopsy performed before and after treatment<sup>[11,12]</sup>.

Our previous studies have shown that Cpd861 can inhibit the activation of HSCs to exert its anti-fibrotic effect<sup>[13]</sup>. In this study, we selected the TGF $\beta$ 1/ALK1/Smad1 signaling pathway in an attempt to elucidate the molecular mechanism of Cpd861 in the deactivation of LX-2 cells. The effect of Cpd861 on the expression of

genes in this pathway, such as *ALK1*, *Id1* and the protein level of phosphorylated Smad1 was investigated.

## MATERIALS AND METHODS

### Materials

LX-2 cells were a gift from Dr. Friedman of Mount Sinai School of Medicine, New York, USA. They are activated human HSCs that are generated by spontaneous immortalization in low-serum conditions<sup>[14]</sup>. Cpd861 powder (*Radix Salviae Miltiorrhiae*, *Radix Astragali*, *Suberect Spatholobus*, *Flos Carthami*, *Rhizoma Chuanxiong*, *Radix Paeoniae Rubra*, *Rhizoma Cyperi*, *Pericarpium Citri Reticulatae*, *Radix Angelicae Sinensis*, *Radix bupleuri*, patent No. 99103265.9) was from Jiangyin Pharmaceutical Company, Jiangsu Province, China. Powder (2500 mg) was dissolved in 100 mL PBS, and the final concentration was 0.1 mg/mL in LX-2 cell-culture medium. After centrifugation at 3000 r/min for 10 min, the solution was sterilized at 105°C for 20 min. Dulbecco's modified Eagle's medium (DMEM), L-glutamine, streptomycin and fetal bovine serum (FBS) were purchased from Gibco, NY, USA. Penicillin was from Sigma, St. Louis, USA. Oligo (dT) primers, M-MLV (Moloney murine leukemia virus) reverse transcriptase, recombinant RNasin ribonuclease inhibitor and dNTP were acquired from Promega (Madison, WI, USA). TRIzol reagent was from Invitrogen, Carlsbad, CA, USA. Power SYBR Green PCR Master Mix was purchased from Applied Biosystems, Warrington, UK. The CO<sub>2</sub> incubator was from SANYO, Sakata, Japan. Cell culture wells (6 cm<sup>2</sup>) were from Corning Incorporation, NY, USA. Phospho-Smad1 antibody (60 kDa) was from Cell Signaling Company, Danvers, MA, USA. Mouse anti-human α-SMA (42 kDa) was from Zhongshan Goldenbridge Biotechnology, Beijing, China.

### Cell culture and group design

LX-2 cells were cultivated in DMEM supplemented with 50 mL/L FBS, 200 mmol/L L-glutamine, penicillin G (100 U/mL) and streptomycin (100 U/mL) in a humidified incubator at (37°C, 5% CO<sub>2</sub>). Cells were cultivated in 25 cm<sup>2</sup> culture flasks to 70% confluence or on glass coverslips in six-well culture dishes to 50% confluence. After serum starvation for 16 h, LX-2 cells were treated with Cpd861 0.1 mg/mL<sup>[13]</sup>, TGFβ1 (5 ng/mL)<sup>[13]</sup> and TGFβ1 (5 ng/mL) plus Cpd861 0.1 mg/mL. Untreated cells served as a control group. All four groups were harvested after 24 h treatment to investigate the related gene expression and phosphorylation of Smad1 protein. Cells on glass coverslips in six-well culture dishes were fixed in 4% paraformaldehyde in PBS after 24 h for immunocytochemical analysis. Every group included six samples.

### Immunocytochemistry

Cells were fixed in 4% paraformaldehyde and permeabilized with PBS containing 0.1% Triton X-100 for 15 min. They were incubated with blocking solution (5% Bovine Serum Albumin in PBS) for 30 min. Primary antibodies (mouse anti-human α-SMA, diluted 1:100 in blocking solution) were incubated with the cells overnight.

Table 1 Primers sequence for real time-PCR

Gene	Oligonucleotide primer	Fragment sequence size (bp)
α-SMA (target)		
Sense	CGCATCCTCATCTCCCT	268
Anti-sense	GGCCGTGATCTCCTTCTG	
ALK1 (target)		
Sense	AGACCCCAACCATCCCTA	67
Anti-sense	CGCATCATCTGAGCTAGG C	
Id1 (target)		
Sense	CCAGAACCGCAAGGTGAG	62
Anti-sense	GGTCCCTGATGTAGTCGATGA	
Gapdh (household)		
Sense	GGCTCTCCAGAACATCATCC	187
Anti-sense	GCTTACCACCTTCTTGATG	

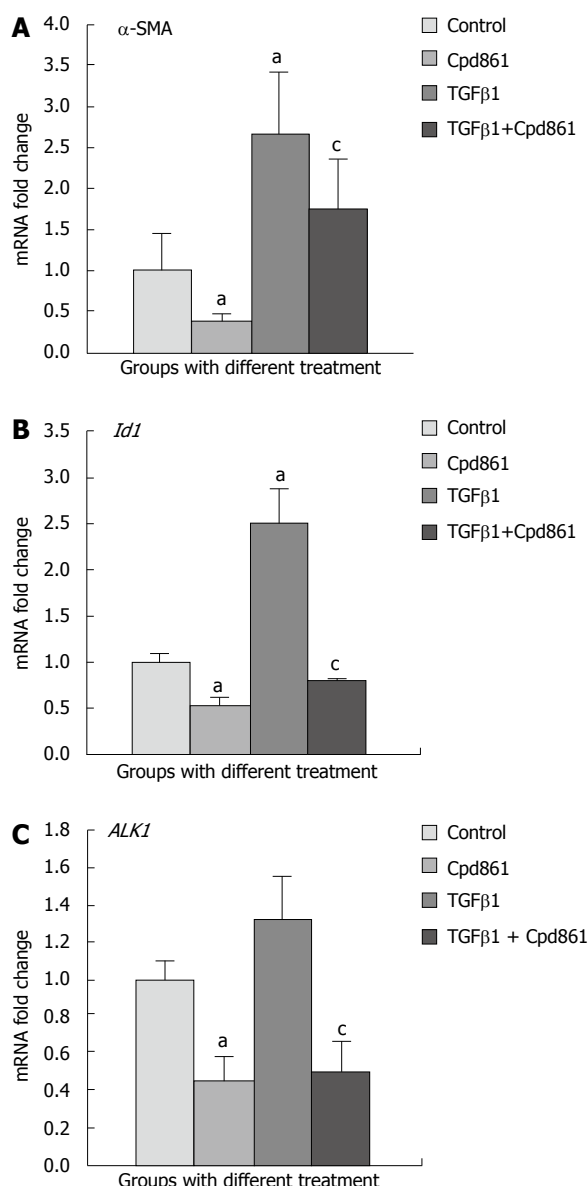
After three washes with PBST (0.2% Tween 20 in PBS) for 15 min, cells were incubated with the secondary antibody (goat anti-mouse IgG labeled with biotin, with streptavidin labeled with horseradish peroxidase), and then with DAB (Diaminobenzidine), Beijing Zhongshan Goldenbridge Co.). PBS (0.01 mol/L) was substituted for primary antibody as a negative control. The α-SMA positive cells presented as brownish yellow. Cells were counterstained with hematoxylin to identify nuclei. Cells were viewed with an Olympus DP71 microscope (Tokyo, Japan) at 20 × magnification.

### RNA isolation and reverse transcription

Total RNA was extracted from LX-2 cells using TRIzol reagent as the lysis buffer. cDNA was synthesized using the Reverse Transcription System (Promega). RNA (1 μg) in 7.7 μL nuclease-free water was added to 2.5 μL 10× transcriptase buffer, 2.5 μL 10 mmol/L dNTP, 0.5 μL RNase inhibitor, and 1 μL M-MLV reverse transcriptase. The reaction was performed for 60 min at 42°C (cDNA synthesis), and 5 min at 95°C (enzyme denaturation).

### Real-time PCR of mRNA

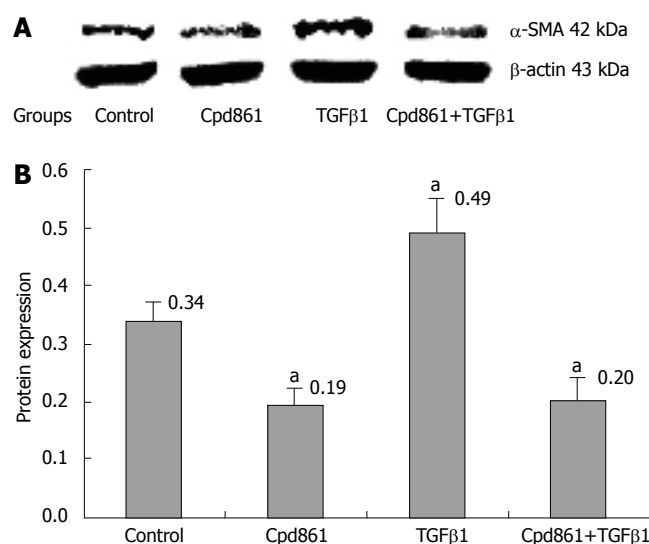
The cDNA samples were analyzed with the Applied Biosystems 7300/7500 real-time PCR system (Applied Biosystems, Foster city, CA, USA) for detecting the gene expression of α-SMA, *ALK1* and *Id1*. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. The primer sequences were obtained from SaiBaiSheng Biocompany, Beijing, China (Table 1). Each experiment was performed in 20 μL reaction volume. The PCR program consisted of an initial activation step at 50°C for 2 min, followed by 95°C for 10 min, then 40 cycling steps of denaturing for 15 s at 95°C, annealing and extension at 60°C for 1 min (data collected at this stage). The PCR data were analyzed using SDS 2.1 software (Applied Biosystems). mRNA levels were normalized relative to GAPDH values. Fold expression changes and standard errors were calculated using the equation  $2^{-\Delta\Delta C_t}$  (Ct, threshold cycle). Each group had six wells of cells for examining the relative fold change of gene expression. Three replicate reactions per sample and endogenous control were used to ensure statistical significance.



**Figure 1** Effect of different treatment on  $\alpha$ -SMA, *Id1*, *ALK1* expression after 24 h. Cpd861 could decrease the level of all these genes compared with untreated LX-2 cells ( $^aP < 0.05$  vs control LX-2 cell group). Even with TGF $\beta$ 1, Cpd861 could also exert its inhibitory roles in these genes expression ( $^cP < 0.05$  vs TGF $\beta$ 1 treated group). Samples were obtained from 6 wells of cells for examining the relative fold change of  $\alpha$ -SMA (A), *Id1* (B), *ALK1* (C) expression. Three replicate reactions for per sample. Error bars, SD.

### Western blot analysis

Cells were lysed on ice by 80  $\mu$ L lysis buffer for 30 min. The cell lysate was centrifuged at 10000 r/min for 10 min and the supernatant was collected for Western blot analysis. Protein concentration was measured using BCA Protein Assay kit (Pierce Company, Rockford, IL, USA) following the manufacturer's instructions. Protein samples (40  $\mu$ g/lane) were subjected to 10% SDS-PAGE and then transferred onto a PVDF nitrocellulose membrane by electro-blotting. The membrane was incubated in 25 mL blocking buffer for 1 h at room temperature. After blocking, the membrane was incubated in 10 mL blocking buffer containing 1:1000 dilution of rabbit anti-phospho-Smad1 or anti-SMA at 4°C overnight. After gentle washing with blocking buffer, the membrane was incubated in



**Figure 2** The level of  $\alpha$ -SMA protein expression with different treatment after 24 h. Western blot was used as described in Materials and Methods. A: Representative Western blot results of  $\alpha$ -SMA. The positions of protein size markers were given; B: Densitometry of Western blot analyzed by Gel-pro software. The levels of  $\alpha$ -SMA were normalized to the level of  $\beta$ -actin protein. Six independent experiments were performed.  $^aP < 0.05$  vs control LX-2 cell group. Error bars, SD.

rabbit anti- $\beta$ -actin polyclonal antibody (1:200 dilution). After vigorous washing, the membrane was incubated with a 1:5000 dilution of horseradish peroxidase-conjugated anti-rabbit IgG antibody. The intensity of the bands was determined by scanning video densitometry. The levels of phospho-Smad1 and SMA were normalized to the level of  $\beta$ -actin protein. Six independent experiments were performed. The intensity of the bands was determined using Gel-Pro Analyzer Version 3.0 (Media Cybernetics, Silver Spring, MD, USA).

### Statistical analysis

Data were expressed as mean  $\pm$  SD and the statistical significance was assessed by one-way analysis of variance followed by Student-Newman-Keul tests.  $P < 0.05$  was considered to be significant.

## RESULTS

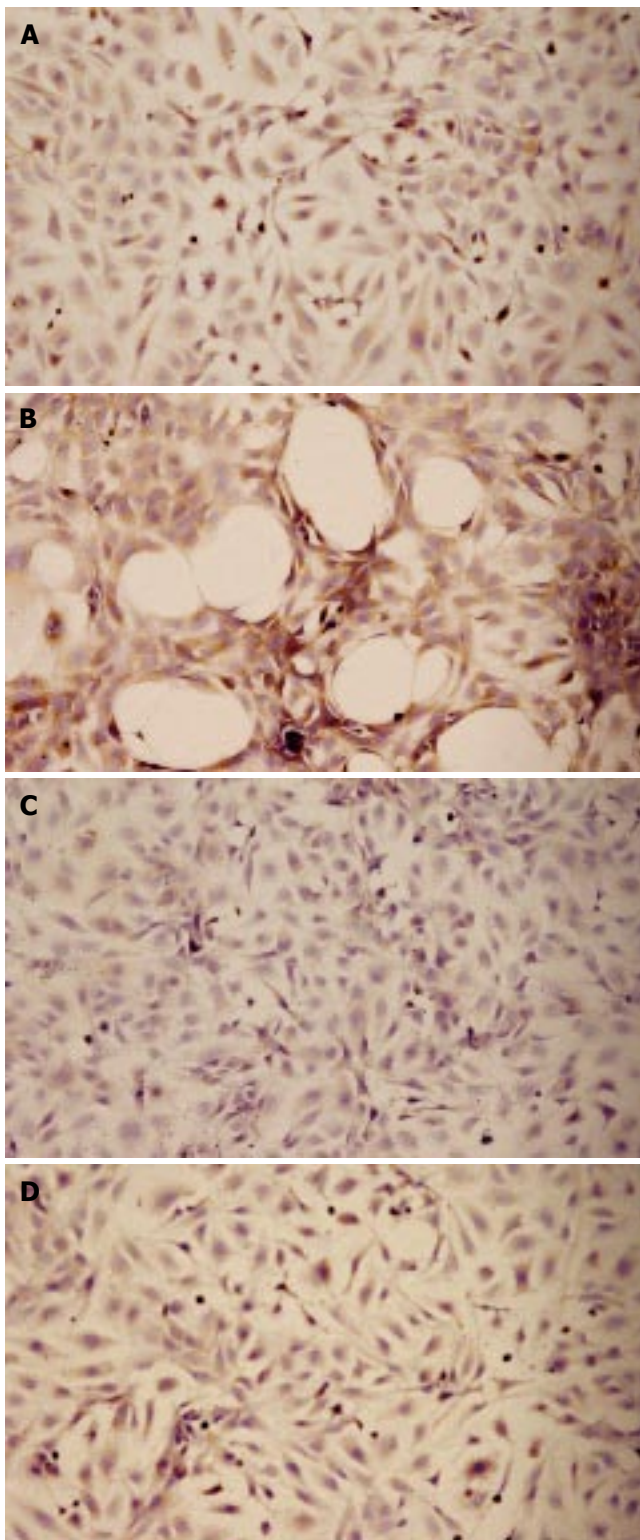
### Effect of TGFβ1 and Cpd861 on $\alpha$ -SMA expression

Changes in gene or protein expression of  $\alpha$ -SMA are often used to study the extent of HSC activation<sup>[16,17]</sup>. In this study, the effect of TGF $\beta$ 1 and Cpd861 on the activation of the LX-2 cell line was investigated by examining changes in gene and protein expression of  $\alpha$ -SMA.

Real-time PCR analysis showed that after exogenous TGF $\beta$ 1 (5 ng/mL) stimulation for 24 h,  $\alpha$ -SMA mRNA expression reached 2.65 fold ( $P < 0.05$ ) compared with that in control LX-2 cells, whereas Cpd861 inhibited the expression to 0.38-fold (24 h,  $P < 0.05$ ) (Figure 1A).  $\alpha$ -SMA mRNA expression in Cpd861 and TGF $\beta$ 1 co-treated cells was 1.73-fold compared to that in the controls.

Western blot analysis confirmed the trend of  $\alpha$ -SMA gene expression changes (Figure 2A and B). In the TGF $\beta$ 1 treatment group,  $\alpha$ -SMA protein expression





**Figure 3** Expression of  $\alpha$ -SMA in different treatment groups. Qualitative expression of  $\alpha$ -SMA in control LX-2 cells (A), treated with TGF $\beta$ 1 (B), Cpd861 (C) and Cpd861 together with TGF $\beta$ 1 (D) for 24 h using immunohistochemical staining.  $\alpha$ -SMA presented brown color in cytoplasm ( $\times 200$ ).

increased significantly compared with that in the controls (photodensity: 0.49 in TGF $\beta$ 1 group and 0.34 in control respectively), whereas its expression was inhibited by Cpd861 (0.19 *vs* 0.49), even in the presence of TGF $\beta$ 1 (0.20 *vs* 0.34) (Figure 2).

Immunocytochemical analysis showed that

$\alpha$ -SMA protein was expressed in the cytoplasm, which was stained brown in low-power fields (Figure 3A). An interesting phenomenon was that after TGF $\beta$ 1 treatment for 24 h, many various-sized, round spaces were present. Cells around the spaces were fusiform in shape, with dark brown cytoplasm. We presumed that it was a result of contraction of adjacent cells, which acquired the contractive property from excessive activation (Figure 3B). Cells treated with Cpd861, however, had a lighter brown cytoplasm (Figure 3C). In the Cpd861 and TGF $\beta$ 1 co-treatment group, the cytoplasm presented lighter brown than in the TGF $\beta$ 1-treated group and no spaces were seen (Figure 3D).

These results confirmed that gene and protein expression and function of  $\alpha$ -SMA in LX-2 cells, markers of activation of LX-2 cells, were enhanced by TGF $\beta$ 1 and inhibited by Cpd861. This is consistent with a previous study, which showed that Cpd861 can inhibit activation of LX-2 cells<sup>[13]</sup>.

#### **Effect of TGF $\beta$ 1 and Cpd861 on TGF $\beta$ 1/ALK1 signaling pathway in LX-2 cell line**

TGF $\beta$ 1/ALK1/Smad1 signaling has been found in HSCs<sup>[18,19]</sup>. In this pathway, ALK1 phosphorylated Smad1 leads to gene expression of *Id1*<sup>[20]</sup>. *Id1* proteins have a helix-loop-helix (HLH) domain but lack ability to bind DNA that acts as an important dominant negative antagonist of the bHLH family of transcription factors<sup>[21,22]</sup>. Recently, the *Id1* gene was identified as a novel target gene that promotes the expression and polymerization of  $\alpha$ -SMA, therefore involving the transdifferentiation of HSCs.

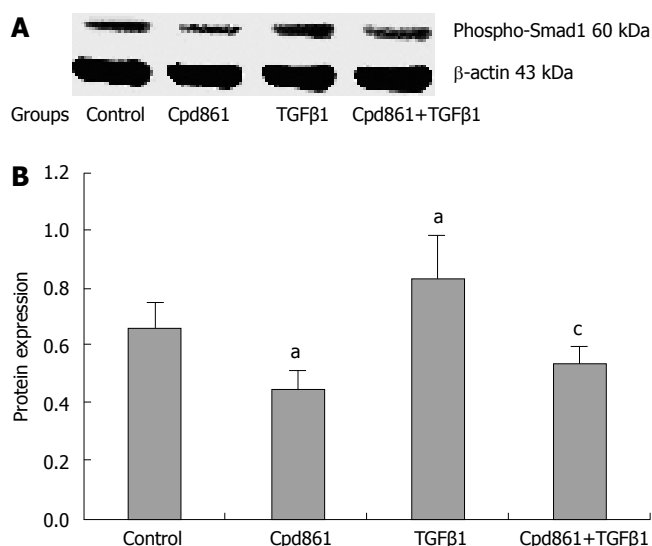
Here, we examined the level of phosphorylated Smad1 (the marker of activation of the pathway) using Western blot analysis. The level of phosphorylated Smad1 increased after TGF $\beta$ 1 treatment and was reduced by Cpd861 or TGF $\beta$ 1 and Cpd861 co-treatment (photodensity was 0.66 in control, 0.45 in Cpd861-treated cells, 0.84 in TGF $\beta$ 1-treated cells, and 0.55 in TGF $\beta$ 1 and Cpd861 co-treated cells, Figure 4).

Stimulation of LX-2 cells with exogenous TGF $\beta$ 1 strongly induced *Id1* gene expression by 2.5-fold, whereas Cpd861 reduced *Id1* expression by 0.53-fold ( $P < 0.05$ ). The fold change of *Id1* expression in Cpd861 and TGF $\beta$ 1 co-treatment was 0.79 compared with that in the controls, but it was significantly lower than that with TGF $\beta$ 1 treatment alone (Figure 1B).

The *ALK1* gene was constitutively expressed in LX-2 cells and TGF $\beta$ 1 did not further increase its expression, but Cpd861 reduced it significantly (Figure 2C). The change in gene expression of the receptor might be an important inhibitory mechanism of Cpd861 in the pathway.

## **DISCUSSION**

Our data indicated that the TGF $\beta$ 1/ALK1/Smad1 pathway could be activated by TGF $\beta$ 1 and abrogated by Cpd861 in LX-2 cells. The TGF $\beta$ 1/ALK1/Smad1 pathway is one of the TGF $\beta$  superfamily signaling branches<sup>[23]</sup>. Several studies have indicated that activation of the



**Figure 4** The level of Phosphorylated Smad1 with different treatment after 24 h. Western blot was used as described. **A:** Representative Western blot results of Phosphorylated Smad1. The positions of protein size markers were given; **B:** Densitometry of Western-blot analyzed by Gel-pro software. The levels of Phospho-Smad1 were normalized to the level of  $\beta$ -actin protein. Six independent experiments were performed.  $^aP < 0.05$  vs untreated LX-2 cell group,  $^cP < 0.05$  vs TGF $\beta$ 1 treated group. Error bars, SD.

pathway might play an important role in development of organ fibrosis<sup>[24-27]</sup>. Recent research has demonstrated that the existence of ALK1 and the TGF $\beta$ 1/ALK1/Smad1 pathway is critical during the transdifferentiation process of primary rat HSCs to myofibroblast (MFB)<sup>[20]</sup>. The present study demonstrated that activation of the TGF $\beta$ 1/ALK1/Smad1 pathway may be enhanced by TGF $\beta$ 1 in LX-2 cells. It was shown that Cpd861 inhibited activation of the TGF $\beta$ 1/ALK1/Smad1 pathway, and this might have been mediated by reducing *ALK1* expression. However, effects of Cpd861 on ALK1 protein expression and on the affinity of TGF $\beta$ 1 for ALK1 need further investigation.

Cpd861 has been demonstrated to be effective for treatment of patients with hepatic fibrosis<sup>[28]</sup>. Previous studies have shown that Cpd861 has multiple anti-fibrotic mechanisms, such as stimulating apoptosis of activated HSCs<sup>[29]</sup>, down-regulating tissue inhibitor of metalloproteinase 1 gene expression<sup>[30]</sup>, reducing expression of collagen and fibrosis-related cytokines. This study presented another possible anti-fibrotic mechanism of Cpd861, and also showed that the TGF $\beta$ 1/ALK1/Smad1 pathway may represent a potential target for antifibrotic therapy. However, which components in the compound play the inhibitory roles needs to be further investigated.

## ACKNOWLEDGMENTS

We thank Dr. Friedman and Mount Sinai, School of Medicine, for their help in providing the LX-2 cell line.

## COMMENTS

### Background

Activation of hepatic stellate cells (HSCs) triggers fibrogenesis and TGF $\beta$ 1/ALK1/

Smad1 signaling has been shown to be involved in the transdifferentiation of HSCs. Herbal compound 861 (Cpd861) can inhibit activation of HSCs to exert its anti-fibrotic effect, but the mechanism is not very clear.

### Research frontiers

The effect of TGF $\beta$ 1/ALK1/Smad1 signaling on the activation of HSCs has recently been reported. This study chose the pathway as a means to establishing the molecular mechanism of Cpd861 in the deactivation of LX-2 cells.

### Innovations and breakthroughs

This is believed to be the first attempt to describe the molecular mechanism of the traditional herbal medicine Cpd861, which has been demonstrated to have anti-fibrotic effects and to reverse liver disease caused by HBV infection. This is believed to be the first time that TGF $\beta$ 1/ALK1/Smad1 has been selected as a pathway to study the effects of deactivation of LX-2 HSCs.

### Applications

This study presents another possible anti-fibrotic mechanism of Cpd861 and shows that the TGF $\beta$ 1/ALK1/Smad1 pathway may represent a potential target for the anti-fibrotic therapy.

### Terminology

ALK1 is activin receptor-like kinase 1, a type I receptor, which is typically activated by the bone morphogenetic proteins, and then phosphorylates Smad1, Smad5 and Smad8 for signal transduction. Id1 is inhibitor of differentiation 1, which has an helix-loop-helix (HLH) domain but lacks a DNA-binding domain. It was first shown to act as a dominant negative antagonist of the basic HLH family of transcription factors, which positively regulate differentiation in many cell lineages. It is an important part of signaling pathways involved in development, cell cycle and tumorigenesis.

### Peer review

This study demonstrates that Cpd861 can down-regulate the TGF $\beta$ 1/ALK1 pathway, which results in the deactivation of HSCs. The study presents novel findings on the anti-fibrotic mechanism of Cpd861 and its possible application in anti-fibrotic therapy.

## REFERENCES

- Gressner AM, Weiskirchen R. Modern pathogenetic concepts of liver fibrosis suggest stellate cells and TGF-beta as major players and therapeutic targets. *J Cell Mol Med* 2006; **10**: 76-99
- Friedman SL. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem* 2000; **275**: 2247-2250
- Iredale JP. Hepatic stellate cell behavior during resolution of liver injury. *Semin Liver Dis* 2001; **21**: 427-436
- Gressner AM, Weiskirchen R, Breitkopf K, Dooley S. Roles of TGF-beta in hepatic fibrosis. *Front Biosci* 2002; **7**: d793-d807
- Parsons CJ, Takashima M, Rippe RA. Molecular mechanisms of hepatic fibrogenesis. *J Gastroenterol Hepatol* 2007; **22** Suppl 1: S79-S84
- Ouyang XS, Wang X, Ling MT, Wong HL, Tsao SW, Wong YC. Id-1 stimulates serum independent prostate cancer cell proliferation through inactivation of p16(INK4a)/pRB pathway. *Carcinogenesis* 2002; **23**: 721-725
- Lindert S, Wickert L, Sawitz A, Wiercinska E, Gressner AM, Dooley S, Breitkopf K. Transdifferentiation-dependent expression of alpha-SMA in hepatic stellate cells does not involve TGF-beta pathways leading to coinduction of collagen type I and thrombospondin-2. *Matrix Biol* 2005; **24**: 198-207
- Shen H, Fan J, Burczynski F, Minuk GY, Cattini P, Gong Y. Increased Smad1 expression and transcriptional activity enhances trans-differentiation of hepatic stellate cells. *J Cell Physiol* 2007; **212**: 764-770
- Jia JD, Bauer M, Cho JJ, Ruehl M, Milani S, Boigk G, Riecken EO, Schuppan D. Antifibrotic effect of silymarin in rat secondary biliary fibrosis is mediated by downregulation of procollagen alpha1(I) and TIMP-1. *J Hepatol* 2001; **35**: 392-398
- Kayano K, Sakaida I, Uchida K, Okita K. Inhibitory effects of

- the herbal medicine Sho-saiko-to (TJ-9) on cell proliferation and procollagen gene expressions in cultured rat hepatic stellate cells. *J Hepatol* 1998; **29**: 642-649
- 11 **Yin SS**, Wang BE, Wang TL, Jia JD, Qian LX. [The effect of Cpd 861 on chronic hepatitis B related fibrosis and early cirrhosis: a randomized, double blind, placebo controlled clinical trial] *Zhonghua Ganzangbing Zazhi* 2004; **12**: 467-470
  - 12 **Wang BE**. Treatment of chronic liver diseases with traditional Chinese medicine. *J Gastroenterol Hepatol* 2000; **15** Suppl: E67-E70
  - 13 **Wang L**, Wang J, Wang BE, Xiao PG, Qiao YJ, Tan XH. Effects of herbal compound 861 on human hepatic stellate cell proliferation and activation. *World J Gastroenterol* 2004; **10**: 2831-2835
  - 14 **Xu L**, Hui AY, Albanis E, Arthur MJ, O'Byrne SM, Blaner WS, Mukherjee P, Friedman SL, Eng FJ. Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. *Gut* 2005; **54**: 142-151
  - 15 **Dooley S**, Delvoux B, Lahme B, Mangasser-Stephan K, Gressner AM. Modulation of transforming growth factor beta response and signaling during transdifferentiation of rat hepatic stellate cells to myofibroblasts. *Hepatology* 2000; **31**: 1094-1106
  - 16 **Abergel A**, Sapin V, Dif N, Chassard C, Darcha C, Marcand-Sauvant J, Gaillard-Martinie B, Rock E, Dechelotte P, Sauvant P. Growth arrest and decrease of alpha-SMA and type I collagen expression by palmitic acid in the rat hepatic stellate cell line PAV-1. *Dig Dis Sci* 2006; **51**: 986-995
  - 17 **Pan Q**, Li DG, Lu HM, Wang YQ, Zhang WZ, Xu QF. A new immortalized rat cell line, hepatic stellate cell-PQ, exhibiting characteristics of hepatic stellate cell. *Hepatobiliary Pancreat Dis Int* 2005; **4**: 281-284
  - 18 **Shen H**, Huang GJ, Gong YW. Effect of transforming growth factor beta and bone morphogenetic proteins on rat hepatic stellate cell proliferation and trans-differentiation. *World J Gastroenterol* 2003; **9**: 784-787
  - 19 **Shen H**, Huang G, Hadi M, Choy P, Zhang M, Minuk GY, Chen Y, Gong Y. Transforming growth factor-beta1 downregulation of Smad1 gene expression in rat hepatic stellate cells. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**: G539-G546
  - 20 **Wiercinska E**, Wickert L, Denecke B, Said HM, Hamzavi J, Gressner AM, Thorikay M, ten Dijke P, Mertens PR, Breitkopf K, Dooley S. Id1 is a critical mediator in TGF-beta-induced transdifferentiation of rat hepatic stellate cells. *Hepatology* 2006; **43**: 1032-1041
  - 21 **Ruzinova MB**, Benezra R. Id proteins in development, cell cycle and cancer. *Trends Cell Biol* 2003; **13**: 410-418
  - 22 **Damdinsuren B**, Nagano H, Kondo M, Yamamoto H, Hiraoka N, Yamamoto T, Marubashi S, Miyamoto A, Umeshita K, Dono K, Nakamori S, Wakasa K, Sakon M, Monden M. Expression of Id proteins in human hepatocellular carcinoma: relevance to tumor dedifferentiation. *Int J Oncol* 2005; **26**: 319-327
  - 23 **Miyazono K**, Kusanagi K, Inoue H. Divergence and convergence of TGF-beta/BMP signaling. *J Cell Physiol* 2001; **187**: 265-276
  - 24 **Moustakas A**, Heldin CH. Non-Smad TGF-beta signals. *J Cell Sci* 2005; **118**: 3573-3584
  - 25 **Abe H**, Matsubara T, Iehara N, Nagai K, Takahashi T, Arai H, Kita T, Doi T. Type IV collagen is transcriptionally regulated by Smad1 under advanced glycation end product (AGE) stimulation. *J Biol Chem* 2004; **279**: 14201-14206
  - 26 **Takahashi T**, Abe H, Arai H, Matsubara T, Nagai K, Matsuura M, Iehara N, Yokode M, Nishikawa S, Kita T, Doi T. Activation of STAT3/Smad1 is a key signaling pathway for progression to glomerulosclerosis in experimental glomerulonephritis. *J Biol Chem* 2005; **280**: 7100-7106
  - 27 **Matsubara T**, Abe H, Arai H, Nagai K, Mima A, Kanamori H, Sumi E, Takahashi T, Matsuura M, Iehara N, Fukatsu A, Kita T, Doi T. Expression of Smad1 is directly associated with mesangial matrix expansion in rat diabetic nephropathy. *Lab Invest* 2006; **86**: 357-368
  - 28 **Wang BE**, Wang TL, Jia JD, Ma H, Duan ZP, Li XM, Li J, Wang AM, Qian LX. Experiment and clinical study on inhibition and reversion of liver fibrosis with integrated Chinese and Western Medicine. *CJIM* 1999; **5**: 6-11
  - 29 **You H**, Wang B, Wang T. [Proliferation and apoptosis of hepatic stellate cells and effects of compound 861 on liver fibrosis] *Zhonghua Ganzangbing Zazhi* 2000; **8**: 78-80
  - 30 **Yin C**, Ma H, Wang A, Ma X, Jia J, Wang B. [Effect of compound 861 on tissue inhibitor of metalloprotenase 1 gene expression of HSC-T6 cells] *Zhonghua Ganzangbing Zazhi* 2002; **10**: 197-199

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RAPID COMMUNICATION

## Primary study of leptin and human hepatocellular carcinoma *in vitro*

Jing Zhou, Wei Lei, Lei Shen, He-Sheng Luo, Zhi-Xiang Shen

Jing Zhou, Lei Shen, He-Sheng Luo, Zhi-Xiang Shen, Department of Gastroenterology, Renmin Hospital of Wuhan University, Wuhan 430060, Hubei Province, China

Wei Lei, Department of Urology, Wuhan Children Hospital, Wuhan 430016, Hubei Province, China

Author contributions: Zhou J, Shen L, Luo HS, and Shen ZX designed research; Zhou J and Lei W performed research; Zhou J and Lei W analyzed data; and Zhou J, Lei W and Shen ZX wrote the paper.

Correspondence to: Zhi-Xiang Shen, Department of Gastroenterology, Renmin Hospital of Wuhan University, No. 238 Jiefang Road, Wuchang District, Wuhan 430060, Hubei Province, China. [nancy61317@163.com](mailto:nancy61317@163.com)

Telephone: +86-27-62530538 Fax: +86-27-88042292

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### Abstract

**AIM:** To investigate the expression level and effects of leptin in human hepatocellular carcinoma cells *in vitro* and to explore the correlation between them.

**METHODS:** Human hepatocellular carcinoma cell line HepG2 was cultured *in vitro*, and (the expression level) mRNA of leptin and leptin receptors in HepG2 were assessed using reverse transcription polymerase chain reaction (RT-PCR). Effects of different concentrations of leptin (50 ng/mL, 100 ng/mL, 200 ng/mL) on HepG2 were detected with colorimetric assay by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) after incubation periods of 24 h, 48 h, and 72 h. Flow cytometry was performed to assess cell cycle progression of different concentrations of leptin as stated above after each 24 h incubation period.

**RESULTS:** mRNA of leptin and leptin receptors (including short and long isoforms) were expressed in HepG2. The 72 h incubation of leptin at different concentrations (50 ng/mL, 100 ng/mL, 200 ng/mL) promoted proliferation of HepG2 in a concentration- and time-dependent manner. The experimental group shows significant statistical differences when compared to the controlled group which contained 0 ng/mL of leptin. As the concentration of leptin increases, significant fewer cells were detected in G<sub>0</sub>-G<sub>1</sub> phase and more cells in S and G<sub>2</sub>-M phases.

**CONCLUSION:** Leptin and leptin receptor are simultaneously expressed in human hepatocellular carcinoma cell line HepG2. Addition of leptin (0 ng/mL-

200 ng/mL) in 72 h periods indicated there is a concentration- and time-dependent correlation in the stimulation of HepG2 cell proliferation. The effect of proliferation by leptin is due to promotion of DNA synthesis and enhancement of mitotic activity. The relationship between leptin and human hepatocellular carcinoma cells might indicate that adipokine could be associated with the progression of human hepatocellular carcinoma.

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**Key words:** Leptin; Hepatocellular carcinoma; Proliferation

**Peer reviewers:** Frank J Burczynski, Faculty of Pharmacy, University of Manitoba, 50 Sifton Road, Winnipeg, Manitoba, R3T 2N2, Canada; Yukihiro Shimizu, Dr, Kyoto Katsura Hospital, 17 Yamada-Hirao, Nishikyo, Kyoto 615-8256, Japan; Byung Chul Yoo, Professor, Dr, Division of Gastroenterology, Samsung Medical Center, SungKyunKwan University School of Medicine, Seoul 135-710, Korea

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### INTRODUCTION

Leptin, a protein product encoded by the obese gene (ob gene)<sup>[1]</sup>, is primarily derived from adipocytes which plays an important role in the regulation of food intake and the control of body weight<sup>[2]</sup>. It takes part in various physical conditions such as lipid metabolism, immune defense, neuroendocrine regulation, pituitary hormone secretion, and pubertal development<sup>[3,4]</sup>. In rodents and humans, leptin is related with many pathological syndromes including obesity, hyperphagia, hyperinsulinemia, reduced fertility, and cholelithiasis (including gallstone and hepatolithiasis)<sup>[5-8]</sup>. Studies have shown that individuals who are obese have increased risk for most cancers compared to individuals who are not obese<sup>[9]</sup>. Since leptin is closely associated with obesity, it may be the bridge conjoining obesity and cancer.

The biological functions of leptin on target cells and tissues are carried out through interaction with its specific receptors (ob-R) which belong to class I cytokine receptor



family<sup>[10]</sup>. In rodents and humans, two leptin receptor isoforms predominate: the short leptin receptor isoform (ob-Ra) and the long leptin receptor isoform (ob-Rb)<sup>[11,12]</sup>. They share the same extracellular domain, but they differ in the length of the transmembrane/cytoplasmic coding regions<sup>[13]</sup>. The physiologic significance of each isoform in relationship to obesity and cancer is still unknown.

Hepatocellular carcinoma (HCC) is one of the most malignant tumors in the world. It causes proximately one hundred and ten thousand deaths annually in China. HCC is extremely difficult to detect in prognosis due to its early metastasis and distant transmission. It has been reported that obesity was related to HCC complicated with cryptogenic cirrhosis and alcoholic liver disease<sup>[14,15]</sup>. Therefore, we presume that leptin might be associated with HCC. In order to investigate the relationship between leptin and HCC, (the expression level) mRNA of leptin and its receptors in human HCC cell line HepG2 were detected by reverse transcription polymerase chain reaction (RT-PCR). In addition, the effects of leptin on HepG2 *in vitro* were also discussed in this study.

## MATERIALS AND METHODS

### Materials

Human hepatocellular carcinoma cell line HepG2 was obtained from Shanghai Cell Biology Institute of Chinese Academy of Sciences. Human recombinant leptin was the product from Sigma (St. Louis, Missouri, USA). Roswell Park Memorial Institute-1640 (RPMI-1640), fetal bovine serum (FBS) and Trizol reagent were the products of Invitrogen (Carlsbad, California, USA). Trypsin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), ethylene diamine tetraacetic acid (EDTA) and dimethyl sulfoxide (DMSO) were purchased from Amresco (Cleveland, Ohio, USA). Revert aid first strand cDNA synthesis kit and TaqDNA polymerase were the products of Fermentas (Burlington, Iowa, USA). All other reagents unless indicated were from Sigma (St. Louis, Missouri, USA).

### Methods

**Cell culture:** HepG2 were grown as a monolayer in RPMI-1640 medium supplemented with 10% heat-inactivated FBS, 100 kU/L penicillin, and 0.1 g/L streptomycin. They were cultured in T-75 cm<sup>2</sup> culture flasks and maintained at 37°C in 5% CO<sub>2</sub> humidified atmosphere. At the beginning of the experiment, cells in the exponential growth phase were detached from the flask with 0.25% trypsin and 0.02% EDTA solution.

**RT-PCR:** mRNA of leptin, ob-Ra and ob-Rb in cell line HepG2 were assessed using RT-PCR as described previously<sup>[16]</sup>. Total RNA was isolated from confluent cells using Trizol reagent according to the manufacture's instructions. The concentration of RNA was quantitated by absorbance change at 260 nm and 280 nm. Total RNA was suspended in DEPC-treated water and stored at -80°C.

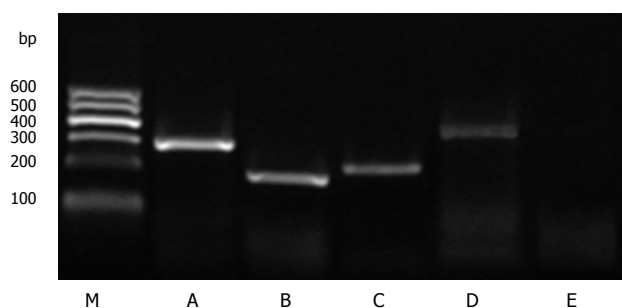
Single-stranded cDNA was synthesized from 1 µg of RNA using revert aid first strand cDNA synthesis kit

with random hexamer primer. The housekeeping gene β-actin was amplified to set a control for RNA loading and also to minimize variations in cDNA synthesis to ensure efficiency. Primer sequences for β-actin, leptin, ob-Ra and ob-Rb have been reported in the previous paper<sup>[17]</sup>. Following are the primers for: 1) β-actin: sense strand is 5'-ACCCACACTGTGCCCATCTA-3' and anti-sense strand is 5'-CGGAACCGCTCATTGCC-3' (encodes a 289-bp fragment); 2) leptin: sense strand is 5'-GTGCGGATTCTTGTGGCTTT-3' and anti-sense strand is 5'-GGAATGAAGTCCAAACCGGTG-3' (encodes a 174-bp fragment); 3) ob-Ra: sense strand is 5'-TTGTGCCAGTAATTATTTCTCTT-3' and anti-sense strand is 5'-AGTTGGCACATTGGGTTCAT-3' (encodes a 200-bp fragment); 4) ob-Rb: the sense strand is 5'-TTGTGCCAGTAATTATTTCTCTT-3' and antisense strand is 5'-CTGATCAGCGTGGCGTATTT-3' (encodes a 439-bp fragment).

Amplification of the resulting cDNA sequence was carried out using polymerase chain reaction (PCR). 3 µL cDNA was combined with 25 pmol oligonucleotide primers specific for β-actin, leptin, ob-Ra, ob-Rb and TaqDNA polymerase 1 U, PCR buffer (5 µL), 25 mmol/L MgCl<sub>2</sub> (4 µL), 10 mmol/L dNTP mixture (1 µL), and ddH<sub>2</sub>O (34 µL in a 50 µL solution). PCR was performed in a thermal cycler (Gene Amp PCR system 2400, Perkin-Elmer Corp., Uberlingen, Germany). The condition for the reaction were the following: 1 min at 94°C (predenaturation), followed by 30 cycles of 1 min (denaturation) at 52°C (for β-actin), 55°C (for leptin) or 50°C (for ob-Ra and ob-Rb); 1 min at 72°C (annealing), and then 5 min at 72°C (extension). PCR products were electrophoresed on 2% agarose gels stained with ethidium bromide, and the length were estimated to be 100-bp. Imaging was performed on JS-380 instrument (Peiqing Inc., Shanghai, China).

**MTT for cell proliferation:** MTT colorimetric assay was used to detect the effects of leptin on proliferation of cell line HepG2. Cells were washed extensively with filtered and sterilized phosphate-buffered saline (PBS) to remove the dead cells. HepG2 cells were suspended at a concentration of  $1.0 \times 10^4$ /mL, and then they were seeded into 96-well microplate at 150 µL/well and incubated to adhere overnight. RPMI-1640 medium containing different concentrations of human recombinant leptin were added in 0 ng/mL, 50 ng/mL, 100 ng/mL and 200 ng/mL to HepG2 cells with each concentration account for 6 wells. MTT assay was performed after incubation periods of 24 h, 48 h, and 72 h. MTT 20 µL (5 mg/mL) stock solution in PBS was added to each well. The microplate was incubated for 4 h and 100 µL DMSO was added (to each well). Optical density (A) value was measured by an ELISA plate reader (Digiscan Lab instruments, Austria) at wavelength of 492 nm. Each variant group was performed in triplicate wells for measurement accuracy.

**Flow cytometric analysis:** HepG2 cells in the exponential growth phase were detached by 0.25% trypsin and 0.02% EDTA. The cell suspension was then seeded uniformly



**Figure 1** Expression of  $\beta$ -actin, leptin, ob-Ra and ob-Rb in HepG2. M: DNA marker; A:  $\beta$ -actin 289 bp; B: Leptin 174 bp; C: Ob-Ra 200 bp; D: Ob-Rb 439 bp; E: Negative control.

into four T-75 cm<sup>2</sup> culture flasks and incubated to adhere overnight. RPMI-1640 medium containing different concentrations of human recombinant leptin were added in 0 ng/mL, 50 ng/mL, 100 ng/mL, and 200 ng/mL to HepG2 cells. Flow cytometric analysis was performed after incubated for 24 h. The cells were briefly washed twice with PBS, fixed in ice-cold ethanol (70% vol/vol in ddH<sub>2</sub>O) and stained with propidium iodide (PI) solution (25  $\mu$ g/mL PI, 180 U/ml RNase, 0.15% Triton X-100, and 30 mg/mL polyethylene glycol in 4 mmol/L citrate buffer w/pH = 7.8). DNA contents were detected using a FACScan flow cytometer (Becton Dickinson Co., San Jose, CA). The relative percentages of each phase of the cell cycle were analyzed using Cell Quest Software (Becton Dickinson Co., San Jose, CA). For the measurement accuracy, each variant group was tested in triplicate.

### Statistical analysis

The data were presented as mean  $\pm$  SD. ANOVA was used to analysis the results.  $P < 0.05$  were considered to indicate statistically significant differences. Entire data were analyzed with the statistical software SPSS 11.5.

## RESULTS

### Leptin, ob-Ra and ob-Rb mRNA assay by RT-PCR

Total RNA extracted from HepG2 cells were reversely transcribed to check whether mRNA of leptin, ob-Ra and ob-Rb were expressed. RT-PCR detection with  $\beta$ -actin primers revealed a 289-bp fragment in HepG2. The 174-bp cDNA band indicated that the cells expressed leptin mRNA. PCR products for both the short isoform (ob-Ra, 200-bp) and the long isoform (ob-Rb, 439-bp) of leptin receptor were also detectable. No fragment was detected in the negative control that PCR product without the cDNA (Figure 1).

### Effects of proliferation of leptin on HepG2

MTT assay indicated the cell proliferate conditions of HepG2. As shown in Table 1, leptin significantly stimulated HepG2 cells growth in a concentration- and time-dependent manner. Leptin caused significant growth potency on HepG2 within 72 h. There were significant statistical differences between concentration groups of 0 ng/mL *vs* 50 ng/mL ( $P < 0.01$ ), 0 ng/mL *vs* 100 ng/mL

**Table 1** Effect of leptin on proliferation of HepG2

Concentration (ng/mL)	A		
	24 h	48 h <sup>d</sup>	72 h <sup>d</sup>
0	0.29 $\pm$ 0.01	0.34 $\pm$ 0.01	0.37 $\pm$ 0.01
50 <sup>b</sup>	0.34 $\pm$ 0.02	0.42 $\pm$ 0.01	0.54 $\pm$ 0.02
100 <sup>b</sup>	0.41 $\pm$ 0.03	0.53 $\pm$ 0.05	0.62 $\pm$ 0.02
200 <sup>b</sup>	0.46 $\pm$ 0.04	0.63 $\pm$ 0.01	0.73 $\pm$ 0.03

<sup>b</sup> $P < 0.01$  *vs* group of 0 ng/mL leptin; <sup>d</sup> $P < 0.01$  *vs* group of incubation 24 h.

**Table 2** Effect of leptin on cell cycle of HepG2

Concentration (ng/mL)	Cell phase (%)		
	G <sub>0</sub> -G <sub>1</sub>	S	G <sub>2</sub> -M
0	61.8 $\pm$ 2.5	36.5 $\pm$ 1.0	1.6 $\pm$ 0.3
50	56.9 $\pm$ 2.2	38.5 $\pm$ 0.9	4.7 $\pm$ 1.4
100	50.2 $\pm$ 2.6 <sup>a</sup>	40.3 $\pm$ 1.2	9.4 $\pm$ 2.1 <sup>a</sup>
200	43.6 $\pm$ 2.7 <sup>b</sup>	42.0 $\pm$ 0.3 <sup>a</sup>	14.4 $\pm$ 2.5 <sup>a</sup>

<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  *vs* group of 0 ng/mL leptin.

( $P < 0.01$ ), 0 ng/mL *vs* 200 ng/mL ( $P < 0.01$ ), 50 ng/mL *vs* 100 ng/mL ( $P < 0.05$ ) and 50 ng/mL *vs* 200 ng/mL ( $P < 0.01$ ). Meanwhile, significant statistical differences also existed between duration groups of 24 h *vs* 48 h ( $P < 0.01$ ) and 24 h *vs* 72 h ( $P < 0.01$ ).

### Effects of leptin on cell cycle progressions of HepG2

The effects of leptin on cell cycle progressions of HepG2 as determined by flow cytometry analysis are shown in Table 2. In the 24 h frame, the results indicate that as the concentration of leptin increases (e.g. 50 ng/mL, 100 ng/mL and 200 ng/mL), the proportion of the HepG2 cells in G<sub>0</sub>-G<sub>1</sub> phase gradually reduces and the number of cells in S and G<sub>2</sub>-M phases gradually increases. In comparison with control group, the data indicates a significant reduction of cells in G<sub>0</sub>-G<sub>1</sub> phase ( $P < 0.01$ ) and a significant increase of cells in S phase ( $P < 0.01$ ) and G<sub>2</sub>-M phase ( $P < 0.01$ ) in response to treatment of leptin.

## DISCUSSION

The relationship between obesity and cancer has been excessively documented for cancers or adenocarcinoma in endometrium, breast, prostate, renal cells, pancreas, colon, and esophagus<sup>[18,19]</sup>. Epidemiological observations revealed that obesity was a risk factor of HCC complicated with cryptogenic cirrhosis and alcoholic liver disease<sup>[14,15]</sup>, and in addition, clinical investigations identified that the leptin level of serum had increased significantly in alcoholic and post-hepatitis liver cirrhosis patients with or without HCC as compared to control subjects w/o the complication<sup>[20-24]</sup>. Still, there were few studies on the involvement of leptin in HCC.

It has been reported that leptin participates in an auto/paracrine manner in the pituitary gland, and it plays regulations roles in the pig<sup>[25]</sup>. One study reported that the proteins of leptin and ob-R had been detected in 72.22% and 30.56% of HCC, respectively<sup>[26]</sup>. Present study

indicated that the mRNA of leptin, and as well as the short and the long leptin receptor isoforms were all expressed in HepG2. These findings suggested that leptin might act as an auto/paracrine growth factor towards hepatocytes, and together with its receptors, they could play role in HCC initiation and progression.

Serum leptin level fluctuations are also detected in some non-physiological conditions. For example, leptin levels up to 400 ng/mL have been reported in children with chronic renal failure<sup>[27]</sup>. When obese, but otherwise healthy subjects treated with leptin (1 mg/kg per day), serum leptin levels rose up to 736 ng/mL<sup>[28]</sup>. However, serum leptin level in normal physiological situation is less than 11.4 ng/mL. Thus, a relatively higher concentration of leptin (200 ng/mL) was used in our study. There are some divericate results among the studies of leptin and cancer. Leptin not only stimulated proliferation of some human cancer cell lines, including breast cancer cell lines (ZR75-1, MCF-7), esophageal cancer cell lines (KYSE 410) and prostate cancer cell lines (PC-3, DU 145), but also it inhibited the growth of other human cancer cell lines, such as pancreatic cancer cell lines (Mia-Paca, PANC-1)<sup>[18]</sup>. Data regarding leptin's effect on HCC cells are rarely reported, and they appear to be contradictory. In one *in vitro* study, leptin was found to have little effect on the proliferation of liver cancer cell line SMMC-7721<sup>[26]</sup>, while in another study, leptin had shown anti-tumor activity<sup>[29]</sup>. In our *in vitro* study, exogenous leptin had effects on the proliferation of HCC cell line HepG2 in a concentration- and time-dependent manner. We might conclude that human cancer cell lines exhibit differential responses to the leptin treatments, depending upon the biological characteristics of the cancer cells and the organ of derivation of the cell lines. Because our study was to interfere HCC cell line with exogenous leptin *in vitro*, results may differ in concentration threshold and activation initiation time with *in vivo* HCC cells in tumor tissues with endogenous leptin. Therefore, it will be necessary to continue the research on leptin's effects on HCC with targeted animal models.

By adopting the techniques of flow cytometry, we were able to significantly expand the analysis of cell cycle progression. With respect to the cell cycle, we were able to accurately determine the relative proportion of cells in each phase of the cycle progression. From our data, it was evident that as the concentration of exogenous leptin increased from 0 to 200 ng/mL, it gradually reduced the relative proportion of the HepG2 cells in G<sub>0</sub>-G<sub>1</sub> phase and gradually increased the number of the cells in S and G<sub>2</sub>-M phases. Since the S phase of the cell cycle is the synthetic phase of DNA, and G<sub>2</sub>-M phase represents a later synthetic phase of DNA and the splitting of the cells through mitotic phase, data from our study demonstrate that leptin increase cell proliferation of HepG2 by promoting of DNA synthesis and enhancing mitotic activity.

It has been reported that leptin replacement is a very promising therapeutic approach for managing the complications of lipodystrophy. In addition, leptin may have therapeutic potential in the treatment of epilepsy<sup>[30,31]</sup>. The primary finding of our study on leptin and HCC cell line indicates that adipokine could be associated with

the progression of human hepatocellular carcinoma. Administration of the leptin antagonists or receptors in the targeted cells may open a new way in HCC prevention and treatment. Further studies are warranted and ongoing.

## COMMENTS

### Background

Leptin is the protein product encoded by obese gene, which plays an important role in the regulation of food intake, and the control of body weight. It is related with many pathological syndrome including obesity, hyperphagia, hyperinsulinemia, reduced fertility, and cholelithiasis. There are considerable researches about the effects of leptin.

### Research frontiers

Recently, the researches of relationships between leptin and tumors are hotspots. But there are few studies on the involvement of leptin in human hepatocellular carcinoma.

### Innovations and breakthroughs

In this study, we found mRNA of leptin and leptin receptors (including short and long isoforms) were expressed in human hepatocellular carcinoma cell line HepG2. Leptin (0 ng/mL-200 ng/mL) in 72 h could stimulate proliferation of HepG2 *in vitro*, and that effect was due to promotion of DNA synthesis and enhancement of mitotic activity.

### Applications

The primary study of leptin and HepG2 may indicate that adipokine could be associated with the progression of human hepatocellular carcinoma, and thus may offer new aim for further understanding of the biological function of leptin and new therapeutic targets.

### Peer review

The manuscript written by Zhou J *et al* describes a possible role of leptin in hepatocarcinogenesis and promotion of the disease. The data are interesting and potentially important. It's recommended the additional experiments using another HCC cell lines which could make this study's conclusions more confidential.

## REFERENCES

- 1 **Zhang Y**, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994; **372**: 425-432
- 2 **Sierra-Honigsmann MR**, Nath AK, Murakami C, Garcia-Cardena G, Papapetropoulos A, Sessa WC, Madge LA, Schechner JS, Schwabb MB, Polverini PJ, Flores-Riveros JR. Biological action of leptin as an angiogenic factor. *Science* 1998; **281**: 1683-1686
- 3 **Janeckova R**. The role of leptin in human physiology and pathophysiology. *Physiol Res* 2001; **50**: 443-459
- 4 **Shimizu F**, Matsuzaki T, Iwasa T, Tanaka N, Minakuchi M, Kuwahara A, Yasui T, Furumoto H, Irahara M. Transition of leptin receptor expression during pubertal development in female rat pituitary. *Endocr J* 2008; **55**: 191-198
- 5 **Montague CT**, Farooqi IS, Whitehead JP, Soos MA, Rau H, Wareham NJ, Sewter CP, Digby JE, Mohammed SN, Hurst JA, Cheetham CH, Earley AR, Barnett AH, Prins JB, O'Rahilly S. Congenital leptin deficiency is associated with severe early-onset obesity in humans. *Nature* 1997; **387**: 903-908
- 6 **Lee GH**, Proenca R, Montez JM, Carroll KM, Darvishzadeh JG, Lee JL, Friedman JM. Abnormal splicing of the leptin receptor in diabetic mice. *Nature* 1996; **379**: 632-635
- 7 **Clement K**, Vaisse C, Lahlou N, Cabrol S, Pelloux V, Cassuto D, Goumelen M, Dina C, Chambaz J, Lacorte JM, Basdevant A, Bougneres P, Lehouc Y, Froguel P, Guy-Grand B. A mutation in the human leptin receptor gene causes obesity and pituitary dysfunction. *Nature* 1998; **392**: 398-401
- 8 **Lei ZM**, Ye MX, Fu WG, Chen Y, Fang C, Li J. Levels of serum leptin, cholecystokinin, plasma lipid and lipoprotein

- differ between patients with gallstone or/and those with hepatolithiasis. *Hepatobiliary Pancreat Dis Int* 2008; **7**: 65-69
- 9 **Calle EE**, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003; **348**: 1625-1638
  - 10 **Tartaglia LA**, Dembski M, Weng X, Deng N, Culpepper J, Devos R, Richards GJ, Campfield LA, Clark FT, Deeds J, Muir C, Sanker S, Moriarty A, Moore KJ, Smutko JS, Mays GG, Wool EA, Monroe CA, Tepper RI. Identification and expression cloning of a leptin receptor, OB-R. *Cell* 1995; **83**: 1263-1271
  - 11 **Fei H**, Okano HJ, Li C, Lee GH, Zhao C, Darnell R, Friedman JM. Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. *Proc Natl Acad Sci USA* 1997; **94**: 7001-7005
  - 12 **Friedman JM**. Leptin, leptin receptors, and the control of body weight. *Nutr Rev* 1998; **56**: s38-s46; discussion s54-s75
  - 13 **Ahima RS**, Flier JS. Leptin. *Annu Rev Physiol* 2000; **62**: 413-437
  - 14 **Nair S**, Mason A, Eason J, Loss G, Perrillo RP. Is obesity an independent risk factor for hepatocellular carcinoma in cirrhosis? *Hepatology* 2002; **36**: 150-155
  - 15 **Roth MJ**, Baer DJ, Albert PS, Castonguay TW, Dorgan JF, Dawsey SM, Brown ED, Hartman TJ, Campbell WS, Giffen CA, Judd JT, Taylor PR. Relationship between serum leptin levels and alcohol consumption in a controlled feeding and alcohol ingestion study. *J Natl Cancer Inst* 2003; **95**: 1722-1725
  - 16 **Zarkesh-Esfahani H**, Pockley G, Metcalfe RA, Bidlingmaier M, Wu Z, Ajami A, Weetman AP, Strasburger CJ, Ross RJ. High-dose leptin activates human leukocytes via receptor expression on monocytes. *J Immunol* 2001; **167**: 4593-4599
  - 17 **Yuan SS**, Chung YF, Chen HW, Tsai KB, Chang HL, Huang CH, Su JH. Aberrant expression and possible involvement of the leptin receptor in bladder cancer. *Urology* 2004; **63**: 408-413
  - 18 **Somasundar P**, Yu AK, Vona-Davis L, McFadden DW. Differential effects of leptin on cancer in vitro. *J Surg Res* 2003; **113**: 50-55
  - 19 **Somasundar P**, Riggs D, Jackson B, Vona-Davis L, McFadden DW. Leptin stimulates esophageal adenocarcinoma growth by nonapoptotic mechanisms. *Am J Surg* 2003; **186**: 575-578
  - 20 **Wang YY**, Lin SY. Leptin in relation to hepatocellular carcinoma in patients with liver cirrhosis. *Horm Res* 2003; **60**: 185-190
  - 21 **Lin SY**, Wang YY, Sheu WH. Increased serum leptin concentrations correlate with soluble tumour necrosis factor receptor levels in patients with cirrhosis. *Clin Endocrinol (Oxf)* 2002; **57**: 805-811
  - 22 **Testa R**, Franceschini R, Giannini E, Cataldi A, Botta F, Fasoli A, Tenerelli P, Rolandi E, Barreca T. Serum leptin levels in patients with viral chronic hepatitis or liver cirrhosis. *J Hepatol* 2000; **33**: 33-37
  - 23 **Comlekci A**, Akpinar H, Yesil S, Okan I, Ellidokuz E, Okan A, Ersoz G, Tankurt E, Batur Y. Serum leptin levels in patients with liver cirrhosis and chronic viral hepatitis. *Scand J Gastroenterol* 2003; **38**: 779-786
  - 24 **Greco AV**, Mingrone G, Favuzzi A, Capristo E, Gniuli D, Addolorato G, Brunani A, Cavagnin F, Gasbarrini G. Serum leptin levels in post-hepatitis liver cirrhosis. *J Hepatol* 2000; **33**: 38-42
  - 25 **Siawrys G**, Kaminski T, Smolinska N, Przala J. Expression of leptin and long form of leptin receptor genes and proteins in pituitary of cyclic and pregnant pigs. *J Physiol Pharmacol* 2007; **58**: 845-857
  - 26 **Wang XJ**, Yuan SL, Lu Q, Lu YR, Zhang J, Liu Y, Wang WD. Potential involvement of leptin in carcinogenesis of hepatocellular carcinoma. *World J Gastroenterol* 2004; **10**: 2478-2481
  - 27 **Daschner M**, Tonshoff B, Blum WF, Englaro P, Wingen AM, Schaefer F, Wuhl E, Rascher W, Mehls O. Inappropriate elevation of serum leptin levels in children with chronic renal failure. European Study Group for Nutritional Treatment of Chronic Renal Failure in Childhood. *J Am Soc Nephrol* 1998; **9**: 1074-1079
  - 28 **Fujioka K**, Patane J, Lubina J, Lau D. CSF leptin levels after exogenous administration of recombinant methionyl human leptin. *JAMA* 1999; **282**: 1517-1518
  - 29 **Elinav E**, Abd-Elnabi A, Pappo O, Bernstein I, Klein A, Engelhardt D, Rabbani E, Ilan Y. Suppression of hepatocellular carcinoma growth in mice via leptin, is associated with inhibition of tumor cell growth and natural killer cell activation. *J Hepatol* 2006; **44**: 529-536
  - 30 **Asterholm IW**, Halberg N, Scherer PE. Mouse Models of Lipodystrophy Key reagents for the understanding of the metabolic syndrome. *Drug Discov Today Dis Models* 2007; **4**: 17-24
  - 31 **Diano S**, Horvath TL. Anticonvulsant effects of leptin in epilepsy. *J Clin Invest* 2008; **118**: 26-28

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## Rosiglitazone prevents murine hepatic fibrosis induced by *Schistosoma japonicum*

Hui Chen, Yong-Wen He, Wen-Qi Liu, Jing-Hui Zhang

Hui Chen, Yong-Wen He, Department of Infectious Disease, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China  
Wen-Qi Liu, Department of Parasitology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China

Jing-Hui Zhang, Department of Surgical Laboratory, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China

Author contributions: Chen H contributed chiefly to this work; Chen H designed and performed the research and wrote the paper; Chen H, Liu WQ and Zhang JH performed the research; He YW gave some good suggestions.

Correspondence to: Hui Chen, Department of Infectious Disease, Union Hospital, Tongji Medical college, Huazhong University of Science and Technology, 1277# Jiefang Road, Wuhan 430022, Hubei Province, China. [chenhui0515@yahoo.com.cn](mailto:chenhui0515@yahoo.com.cn)

Telephone: +86-27-85726132 Fax: +86-27-85727851

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### Abstract

**AIM:** To evaluate the effect of rosiglitazone in a murine model of liver fibrosis induced by *Schistosoma japonicum* infection.

**METHODS:** A total of 50 mice were randomly and averagely divided into groups A, B, C, D and E. The mice in group A served as normal controls, while those in the other four groups were infected with *Schistosoma japonicum* to induce the model of liver fibrosis. Besides, the mice in groups C, D and E were treated with praziquantel, rosiglitazone and praziquantel plus rosiglitazone, respectively. NF- $\kappa$ B binding activity and expression of PPAR $\gamma$ -mRNA were determined by Western blot assay and real-time quantitative PCR. Radioimmunoassay technique was used to detect the serum content changes of TNF- $\alpha$  and IL-6. Histological specimens were stained with HE. Expression of TGF- $\beta$ 1,  $\alpha$ -smooth muscle actin and type I and type III collagen was detected by immunohistochemistry and multimedia color pathographic analysis system.

**RESULTS:** Inflammation and fibrosis in the rosiglitazone plus praziquantel treatment group (group E) were lightest among the mice infected with *Schistosoma* ( $P < 0.05$ ). To further explore the mechanism of rosiglitazone action, we found that rosiglitazone can significantly increase the expression of PPAR $\gamma$  [E:  $-18.212 \pm (-3.909)$  vs B:  $-27.315 \pm (-6.348)$  and C:  $-25.647 \pm (-5.694)$ ,  $P < 0.05$ ],

reduce the NF- $\kappa$ B binding activity (E:  $88.89 \pm 19.34$  vs B:  $141.11 \pm 15.37$ , C:  $112.89 \pm 20.17$  and D:  $108.89 \pm 20.47$ ,  $P < 0.05$ ), and lower the serum level of TNF- $\alpha$  (E:  $1.613 \pm 0.420$  ng/mL vs B:  $2.892 \pm 0.587$  ng/mL, C:  $2.346 \pm 0.371$  ng/mL and D:  $2.160 \pm 0.395$  ng/mL,  $P < 0.05$ ) and IL-6 (E:  $0.106 \pm 0.021$  ng/mL vs B:  $0.140 \pm 0.031$  ng/mL and C:  $0.137 \pm 0.027$  ng/mL,  $P < 0.05$ ) in mice with liver fibrosis. Rosiglitazone can also substantially reduce the hepatic expression of TGF- $\beta$ 1,  $\alpha$ -SMA type I and type III collagen in mice with liver fibrosis.

**CONCLUSION:** The activation of PPAR $\gamma$  by its ligand can retard liver fibrosis and suggest the use of rosiglitazone for the treatment of liver fibrosis due to *Schistosoma japonicum* infection.

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**Key words:** Peroxisome proliferators-activated receptor $\gamma$ ; Rosiglitazone; Liver fibrosis; Schistosomiasis; Hepatic stellate cell

**Peer reviewer:** Ana Cristina Simões e Silva, Professor, Pediatrics Department, Federal University of Minas Gerais Institution, Avenida Professor Alfredo Balena, 190, Belo Horizonte 30130-100, Brazil

Chen H, He YW, Liu WQ, Zhang JH. Rosiglitazone prevents murine hepatic fibrosis induced by *Schistosoma japonicum*. *World J Gastroenterol* 2008; 14(18): 2905-2911 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2905.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2905>

### INTRODUCTION

Hepatic schistosomiasis is one of the most prevalent forms of chronic liver diseases in the world, resulting in the morbidity from infection due to its complications of liver fibrosis. However, there are few medicines or means available to control and treat fibrosis in schistosomiasis.

The key pathogenic event in liver fibrosis is the activation of hepatic stellate cells (HSC) and their transformation into myofibroblasts<sup>[1,2]</sup>. The HSC (formally called the Ito cell) is the primary cell-type in the liver responsible for excessive collagen synthesis during hepatic fibrosis<sup>[3,4]</sup>. Following liver injury, the HSC undergoes a complex transformation or activation process in which the cell changes from a quiescent, vitamin A-storing cell to an

activated myofibroblast, resulting in considerable changes such as the appearance of the cytoskeletal protein smooth muscle  $\alpha$ -actin ( $\alpha$ -SMA), the loss in the cellular vitamin A stores<sup>[5,6]</sup>, and the up-regulation of type I and III collagen genes. In addition, transforming growth factor- $\beta$  (TGF- $\beta$ ), as the most potent fibrogenic cytokine described in HSC<sup>[7]</sup>, accompanying its receptors are increased following HSC activation as well. These pathogenic alterations cause excessive depositions of extracellular matrix (ECM) proteins including three large families of protein-glycoproteins, collagens and proteoglycans, and disrupt the balance in ECM integrity to induce hepatic fibrosis<sup>[8]</sup>.

Peroxisome proliferator-activated receptors (PPARs) are a family of ligand-activated nuclear transcription factors, members of the nuclear hormone receptor super-family<sup>[9]</sup>. There are 3 mammalian subtypes identified as PPAR- $\alpha$ , - $\beta$  (or - $\delta$ ), and - $\gamma$ <sup>[10]</sup>; however, current studies demonstrate that only PPAR $\gamma$  is mainly expressed in human HSC, which contributes to the process of liver fibrogenesis, and both its translational and transcriptional levels are significantly decreased in *in vitro* and/or *in vivo* activated human HSC cells. The decreased PPAR $\gamma$  at the early stage of HSC activation and the amelioration of stimulated PPAR $\gamma$  by its ligands to some deterioration resulting from activated HSCs, suggest that PPAR $\gamma$  may be involved in the maintenance of a quiescent phenotype of HSC<sup>[11]</sup>, although the identification of PPAR $\gamma$  as a novel modulator to liver fibrosis remains controversial.

PPAR $\gamma$  ligands, 15-deoxy-triangle up (1214) prostaglandin J (2) (15d-PGJ (2) and rosiglitazone, significantly decreased the expression of  $\alpha$ -SMA and proliferation in activated human HSCs induced by platelet-derived growth factor<sup>[12]</sup>. Oral administration of rosiglitazone is found able to diminish extracellular matrix deposition and HSC activation in liver fibrosis of rat models which are created by bile duct ligation<sup>[13]</sup>. They found PPAR $\gamma$ -specific DNA binding activities in nuclear extracts of HSCs isolated from liver fibrotic rat models are impaired significantly, although they can return, through rosiglitazone administration, to the normal levels as controls. Intriguingly, rosiglitazone induces PPAR $\gamma$  activation to inhibit collagen and fibronectin synthesis in human HSCs initiated by transforming growth factor (TGF)- $\beta$ 1 *in vitro*. These findings implicate that the PPAR $\gamma$  activation in HSC retards fibrosis, suggesting the use of PPAR $\gamma$  ligands for the treatment of fibrosis following liver injury.

To determine amelioration of PPAR $\gamma$  and its ligands to liver fibrosis, we studied several different treatments of ligands, rosiglitazone, with/out the additives and praziquantel in liver fibrosis model mice created by infection of schistosome.

## MATERIALS AND METHODS

### Animal preparation and treatment

Fifty 4-5-wk-old Kunming mice, weighing 16-22 g (obtained from the experimental animal center of Tongji Medical College, Huazhong University of Science and Technology) were used. All animals were housed in a temperature and humidity controlled environment, and all

animal experiments were carried out in accordance with the Chinese Council on Animal Care Guide for the Care and Use of Laboratory Animals.

The mice were randomly divided into five groups (10 per group) as follows: group A, B, C, D and E. The mice in group A served as normal controls, while those in the other four groups were infected with 40 *Schistosoma japonicum cercariae* through skin to create a liver fibrosis model. The mice in groups C, D and E were treated with praziquantel, rosiglitazone, and praziquantel plus rosiglitazone, 4 wk after infection, respectively. Praziquantel (500 mg/kg) was given daily for 2 d by intragastric administration in groups C and E. Rosiglitazone (4 mg/kg) was given daily for 6 wk by intragastric administration in groups D and E. Meanwhile, the mice in the normal control group (group A) and the model control group (group B) were given normal saline daily for 6 wk. All mice were sacrificed at the end of the study period. Vein blood was centrifuged at 1500 r/min for 15 min, and the serum was stored at -20°C for use. Parts of liver tissues were fixed by 40 g/L formaldehyde, and embedded in paraffin. The remaining liver was stored at -70°C.

### Histopathological examination

The sections of the liver were stained with hematoxylin-eosin (HE) staining. Criteria used in histopathological analysis strictly obeyed WHO category and nomenclature for liver fibrosis<sup>[14]</sup>. Mean degrees of liver fibrosis examined from ten randomized scopes under optical microscopy in each slice were used for statistical analysis in current study. Pathological phases are graded as “-”, “+”, “++” and “+++” respectively: “-”, no fibrosis in liver lobules marked phase “0”; “+”, limit fibrosis in lobules, phase “1”; “++”, typical fibrosis spacing around liver lobules, phase “2”; and “+++”, early cirrhosis, fibrosis enveloping liver lobules and spacing into central vein area, phase “3”.

### Serum assay

Serum TNF- $\alpha$  and IL-6 were detected using radioimmunoassay kit (Institute of Radioimmunoassay, Science and Technology Empolder Center, General Hospital of PLA) according to the manufacturer's instructions.

### Immunohistochemistry for TGF- $\beta$ , $\alpha$ -SMA and collagens

TGF- $\beta$ ,  $\alpha$ -SMA and collagens were detected using the three step streptavidin-biotin immunoperoxidase method. Briefly, tissue sections were de-paraffinized and rehydrated, and then were heated in microwave oven for 10 min to enhance antigen retrieval. For minimizing endogenous peroxidase, activity slides were incubated with 3% H<sub>2</sub>O<sub>2</sub>. After blocking with 5% normal goat serum in 0.01% PBS, the primary antibodies (mice raised against  $\alpha$ -SMA, rabbit raised against TGF- $\beta$ , and types I and III collagens, Bosider, Wuhan, China) were applied and incubated at 37°C in a moisture chamber for 1 h. Sections were then washed with PBS 3 times. After reacted with biotinylated hircine anti-mouse (or rabbit) IgG and then avidin at 37°C for 20 min each, sections were washed with PBS 3 times. Then diaminobenzidine solution (1 mg/mL in PBS

containing 0.03% hydrogen peroxide) was applied as the chromogen. Sections were counterstained with hematoxylin for 15 s before checked under microscope. As a negative control, PBS was used instead of primary antibody. The cytoplasm or membrane of the positive cell was stained brown and yellow. The sections were observed under microscope. For quantification, 10 random fields of intralobular and periportal areas were evaluated under microscope at 40 × magnification. The integral light density was determined by multimedia color pathographic analysis system.

### Protein preparation and western blotting

One hundred mg of frozen tissues was homogenized and centrifuged at 5000 r/min at 4°C for 10 min. The sediment was resuspended in 200 µL of ice-cold extraction buffer A (10 mmol/L HEPES pH 7.9, 1.5 mmol/L MgCl<sub>2</sub>, 10 mmol/L KCl, 0.5 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 µg/mL Aprotinin), and spin down at 5000 r/min at 4°C for 10 min. The sediment was resuspended in 100 µL of extraction buffer C (25% glycerol, 420 mmol/L NaCl, 20 mmol/L HEPES pH 7.9, 1.5 mmol/L MgCl<sub>2</sub>, 1% NP40, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.5 mmol/L phenylmethylsulfonyl fluoride, 1 µg/mL each of aprotinin) and kept on ice for 10 min. Finally, it was transferred to a microdosis centrifuge tube and centrifuged at 15000 r/min at 4°C for 20 min. The supernatant was stored at -70°C for use.

Protein concentration was determined using the Coomassie brilliant blue method with G-250 as a standard. Nuclear or cytoplasmic proteins were electrophoresed through 12% SDS-PAGE in Tris-glycine electrophoresis buffer and transferred onto nitrocellulose membrane. The blot was pre-incubated in blocking buffer [Tris buffered saline (TBS) containing Tween and 7% non-fat dried milk powder] at room temperature for 2 h and probed with a primary antibody [rabbit anti-mouse NF-κB polyclonal antibody (Santa Cruz, CA, USA)] at 4°C overnight. After washed three times with 0.1% Tween-TBS, it was incubated with 1:2000 goat anti-rabbit conjugated with horseradish peroxidase (The Eastman Company, Beijing, China) at room temperature for 2 h. Immunoreactive bands were detected by epiluminescence and quantified in arbitrary units [optical density (A) × band area using Vilber Lourmat image analysis system].

### RNA isolation, reverse transcription and quantitative PCR

Total RNA was extracted from the frozen liver tissue with Trizol reagent (GIBCO, USA). Complementary DNA (cDNA) was synthesized from total RNA using the reverse transcriptase Superscript II (GIBCO, USA) according to the manufacturer's instructions.

Relative quantification of target gene expression was performed using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control. The threshold cycle and the standard curve method were used for calculating the relative amount of the target mRNA. For *PPARγ* mRNA detection, the forward and reverse primers were: 5'-TTTCAAGGGTGCCAGTTTCG-3', and 5'-TCTTTATTCATCAGGGAGGC-3'; for GAPDH, the

primers were: 5'-GATGGTGAAGGTCGGTGTG-3', and 5'-GAGGTCAATGAAGGGGTCG-3'. Real-time PCR was carried out in 50 µL of PCR reaction mixture which contained 1.5 µL of the extracted cDNA, 25 mmol/L MgCl<sub>2</sub>, 20 µmol/L each primer, and 1 µL of SYBR Green I (Biotium, USA). A "no template" control was added, which consisted of all the reagents listed above for real-time PCR, except the cDNA template was replaced with water. The following thermal profile was used: 10 min at 94°C, followed by 45 cycles of 94°C for 30 s, 53°C for 30 s, and 72°C for 30 s. Dissociation curve was run to prove the purity of the product.

Data analysis: The relative *PPARγ* mRNA expression levels were calculated by the  $\Delta\Delta C_t$  (threshold cycle) method in relation to *PPARγ* expression in the liver tissues.  $\Delta\Delta C_t = \Delta C_t$  (specimen) -  $\Delta C_t$  (GAPDH),  $\Delta C_t = C_t$  (negative control) -  $C_t$  (specimen).  $\Delta C_t$  is the relative gene expression ( $C_t$ -the number of fractional cycle at which the reporter fluorescence was generated by cleavage of the probe passes a fixed threshold above baseline). The relative difference in expression of the gene of interest and of the internal reference gene is represented by  $\Delta C_t$ . Changes of gene expression in relation to the calibrator are represented by  $2^{\Delta\Delta C_t}$  [relative quantification (RQ)].

### Statistical analysis

Means of triplicates were used for statistical analysis by one-way analysis of variance (ANOVA) with post hoc Tukey test for pairwise group comparisons (SPSS 15, SPSS Inc, Chicago, IL, USA). The level of statistical significance was set at 0.05 (two-sided).

## RESULTS

### Histopathological analysis

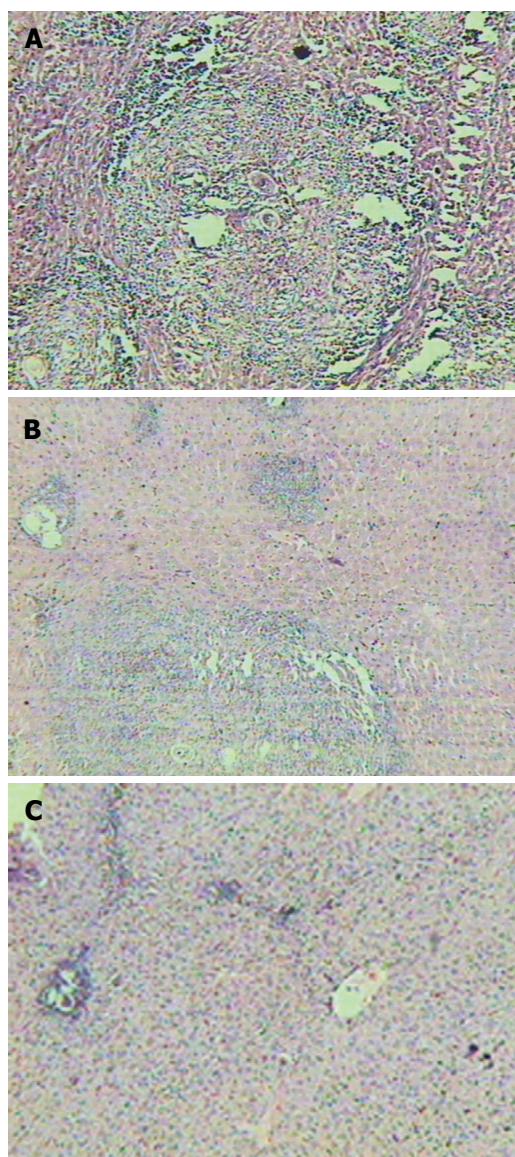
Compared with normal mice, slice of the liver from fibrosis model (group B) showed typical damage in the liver lobules: the segregation of liver by collagen fibers, necrosis lesions in the granulomas and inflammatory cells extensively in the periphery of granulomas (Figure 1A). Compared with group B, the decreases were not significant in the fibroplasias, hepatocellular necrosis and inflammatory cell infiltration in the liver slice from groups C and D (fibrosis models treated only with praziquantel or rosiglitazone respectively) (Figure 1B). However, the severity of hepatocellular necrosis and fibroplasias was significantly decreased in the liver of mice treated with praziquantel plus rosiglitazone (group E) compared with group B (Figure 1C). Group E mice also showed thin fibroseptal and decreased inflammatory cell infiltrations in the livers, demonstrating a markedly improved or normal architecture of hepatic lobule.

The pathological phase quantities of groups B, C, D and E were  $2.31 \pm 0.63$ ,  $1.49 \pm 0.77$ ,  $1.38 \pm 0.60$  and  $0.78 \pm 0.53$ , respectively. Fibrosis in the rosiglitazone plus praziquantel treatment group (group E) was lightest among those with *Schistosoma* infection ( $P < 0.05$ ).

### Immunohistochemical findings

TGF-β<sub>1</sub>-positive cells were concentrated in the portal



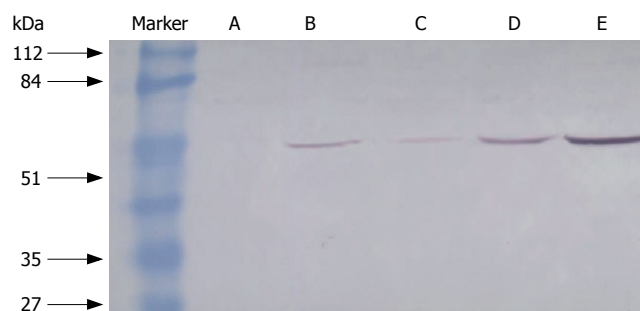


**Figure 1** Liver histopathology of mice (HE staining,  $\times 10$ ). **A:** Represents the model group; **B:** Represents the group treated with praziquantel; **C:** Represents the group treated with rosiglitazone plus praziquantel.

venule pericytes with perisinusoidal distribution in the normal mouse liver. Similarly distributed and decreased TGF- $\beta_1$ -positive cells were found in group E mice (treated with praziquantel plus rosiglitazone). However, there was a significantly large number of TGF- $\beta_1$ -positive cells in groups B, C and D, which distributed mainly at the fibro-septa band, the area of necrosis and inflammatory cell infiltration, few hepatic cells and lipid-containing vacuoles.

There were few expressions of  $\alpha$ -SMA positive cells in venule pericytes and periphery of granulomas in the mouse livers of normal control and group E. Nevertheless, the positive cells of  $\alpha$ -SMA were substantially distributed at venule pericytes, inflammatory cell infiltration, periphery of granulomas and fibrous proliferation in groups B, C and D.

Moreover, few expressions of type I & III collagen were only found in the central venule pericytes and the linkage region of lobules from the mouse liver of group A (normal control), group D or E. In contrast, there



**Figure 2** NF- $\kappa$ B binding activity in mouse livers with Western blot assay. **A:** Normal group; **B:** Rosiglitazone treatment; **C:** Praziquantel plus rosiglitazone treatment; **D:** Praziquantel treatment; **E:** Model group.

was a significant larger number of positive collagen cells distributed not only in these areas but also in fibrous proliferation and Disec cavities of mouse livers from groups B (fibrosis model) and C treated with praziquantel alone.

TGF- $\beta_1$ ,  $\alpha$ -SMA, type I & III collagen positive cells were quantified with multimedia color pathographic analysis. Quantities of TGF- $\beta_1$  and type I collagen in the mouse livers of praziquantel treatment group (group C) were reduced much more than in group B ( $P < 0.05$ ). No significant difference was found in the volumes of  $\alpha$ -SMA and type III collagen in the mouse livers of groups C and group B ( $P > 0.05$ ). Additionally, volumes of  $\alpha$ -SMA and type III collagen in the mouse livers of rosiglitazone treatment group (group D) decreased dramatically compared with groups B and C ( $P < 0.05$ ). TGF- $\beta_1$  and type I collagen in mouse livers of group D decreased more significantly than in group B ( $P < 0.05$ ). Notably, volumes of TGF- $\beta_1$ ,  $\alpha$ -SMA, type I & III collagen in the mouse livers of group E (praziquantel plus rosiglitazone treatment) decreased substantially compared to groups B and C as well ( $P < 0.05$ ). All data are summarized in Table 1.

### Serum assays

The serum level of TNF- $\alpha$  was markedly lower in group E ( $1.613 \pm 0.420$  ng/mL) than that in groups B ( $2.892 \pm 0.587$  ng/mL), C ( $2.346 \pm 0.371$  ng/mL) and D ( $2.160 \pm 0.395$  ng/mL) ( $P < 0.05$ ). However, the serum level of IL-6 was markedly higher in groups B ( $0.140 \pm 0.031$  ng/mL), and/or C ( $0.137 \pm 0.027$  ng/mL) than that in groups D ( $0.108 \pm 0.021$  ng/mL) and/or E ( $0.106 \pm 0.021$  ng/mL) ( $P < 0.05$ ) as summarized in Table 2.

### NF- $\kappa$ B binding activity

Less NF- $\kappa$ B activity was found in groups A, C, D or E than in group B (without any treatment) ( $P < 0.05$ ). Moreover, the activity of NF- $\kappa$ B decreased much more in group A and E than in either group C or D. The activity of NF- $\kappa$ B in groups C and D ( $112.89 \pm 20.17$  and  $108.89 \pm 20.47$ ) was higher than in groups A and E ( $78.89 \pm 18.12$ ,  $88.89 \pm 19.34$ ,  $P < 0.05$ ), but lower than in group B ( $141.11 \pm 15.37$ ,  $P < 0.05$ ). There was no significant difference between groups C and D ( $P > 0.05$ ) as seen in Table 3 and Figure 2.



**Table 1** Quantitative analysis of TGF- $\beta$ 1,  $\alpha$ -SMA, type I and type III collagen in mouse livers (mean  $\pm$  SD)

Groups	n	TGF- $\beta$ 1	$\alpha$ -SMA	Type I collagen	Type III collagen
A	10	0.356 $\pm$ 0.145	0.602 $\pm$ 0.116	0.303 $\pm$ 0.117	0.317 $\pm$ 0.133
B	10	0.829 $\pm$ 0.154 <sup>a</sup>	0.915 $\pm$ 0.172 <sup>a</sup>	0.654 $\pm$ 0.186 <sup>a</sup>	0.735 $\pm$ 0.192 <sup>a</sup>
C	10	0.655 $\pm$ 0.117 <sup>a,c</sup>	0.902 $\pm$ 0.155 <sup>a</sup>	0.505 $\pm$ 0.103 <sup>a,c</sup>	0.701 $\pm$ 0.174 <sup>a</sup>
D	10	0.603 $\pm$ 0.126 <sup>a,c</sup>	0.732 $\pm$ 0.109 <sup>a,c,e</sup>	0.477 $\pm$ 0.132 <sup>a,c</sup>	0.508 $\pm$ 0.127 <sup>a,c,e</sup>
E	10	0.459 $\pm$ 0.107 <sup>a,c,e,g</sup>	0.689 $\pm$ 0.132 <sup>c,e</sup>	0.382 $\pm$ 0.125 <sup>c,e,g</sup>	0.436 $\pm$ 0.112 <sup>a,c,e</sup>

Compared with group A, <sup>a</sup>*P* < 0.05; Compared with group B, <sup>c</sup>*P* < 0.05; Compared with group C, <sup>e</sup>*P* < 0.05; Compared with group D, <sup>g</sup>*P* < 0.05.

**Table 2** Serum level of TNF- $\alpha$  and IL-6 in mice (mean  $\pm$  SD)

Groups	n	TNF- $\alpha$	IL-6
A	10	1.530 $\pm$ 0.380	0.094 $\pm$ 0.026
B	10	2.892 $\pm$ 0.587 <sup>a</sup>	0.140 $\pm$ 0.031 <sup>a</sup>
C	10	2.346 $\pm$ 0.371 <sup>a,c</sup>	0.137 $\pm$ 0.027 <sup>a</sup>
D	10	2.160 $\pm$ 0.395 <sup>a,c</sup>	0.108 $\pm$ 0.021 <sup>c,e</sup>
E	10	1.613 $\pm$ 0.420 <sup>c,e,g</sup>	0.106 $\pm$ 0.021 <sup>c,e</sup>

Compared with group A, <sup>a</sup>*P* < 0.05; Compared with group B, <sup>c</sup>*P* < 0.05; Compared with group C, <sup>e</sup>*P* < 0.05; Compared with group D, <sup>g</sup>*P* < 0.05.

**Table 3** NF- $\kappa$ B binding activity and the expression of PPAR- $\gamma$  mRNA in mouse livers (mean  $\pm$  SD)

Groups	n	NF- $\kappa$ B	PPAR- $\gamma$ mRNA
A	10	78.89 $\pm$ 18.12	-16.557 $\pm$ (-3.022)
B	10	141.11 $\pm$ 15.37 <sup>a</sup>	-27.315 $\pm$ (-6.348) <sup>a</sup>
C	10	112.89 $\pm$ 20.17 <sup>a,c</sup>	-25.647 $\pm$ (-5.694) <sup>a</sup>
D	10	108.89 $\pm$ 20.47 <sup>a,c</sup>	-18.217 $\pm$ (-4.498) <sup>c,e</sup>
E	10	88.89 $\pm$ 19.34 <sup>c,e,g</sup>	-18.212 $\pm$ (-3.909) <sup>c,e</sup>

Compared with group A, <sup>a</sup>*P* < 0.05; Compared with group B, <sup>c</sup>*P* < 0.05; Compared with group C, <sup>e</sup>*P* < 0.05; Compared with group D, <sup>g</sup>*P* < 0.05.

### Expression of PPAR- $\gamma$ -mRNA

Levels of PPAR $\gamma$  mRNAs were markedly higher in groups A [-16.557  $\pm$  (-3.022)], D [-18.217  $\pm$  (-4.498)] and E [-18.212  $\pm$  (-3.909)] than that in either group B [-27.315  $\pm$  (-6.348)] or C [-25.647  $\pm$  (-5.694), *P* < 0.05] as summarized in Table 3.

## DISCUSSION

PPAR $\gamma$  is a ligand-activated nuclear transcription factor which belongs to the nuclear hormone receptor superfamily. Normally, PPAR $\gamma$  expresses in quiescent human liver HSC, but significantly lowers both its translational and transcriptional activities after HSC activation in culture<sup>[15,16]</sup>. Some recent findings support a role of PPAR $\gamma$  in the development of liver fibrosis and suggest that PPAR $\gamma$  ligands can be therapeutically used to inhibit HSC activation and the progression to liver fibrosis<sup>[17-19]</sup>.

A mouse model of liver fibrosis through *Japonicum cercariae* infection was generated, and then treated with PPAR $\gamma$  ligand, rosiglitazone and its additive, and praziquantel to diminish liver fibrosis in the current study. Our results demonstrated that the level of hepatic PPAR $\gamma$  mRNA markedly decreased in the model mice and praziquantel treated group as compared with the normal mice. However, rosiglitazone significantly increased the expression of PPAR $\gamma$  and decreased HSC activation and liver fibrosis progression.

Our mouse model successfully showed the key pathogenic events in liver fibrosis: the activation of HSCs and transformation of HSCs into myofibroblasts. Consequently, livers of model mice exhibited cellular changes including the appearance of cytoskeletal protein  $\alpha$ -SMA and substantial expression of TGF- $\beta$ , both fibrogenic cytokines which are activated accompanying

liver HSC activation<sup>[20,21]</sup>.

Based on the results from histopathological and immunohistochemical analyses, current fibrosis model clearly confirmed that an imbalance between the synthesis and degradation of ECM in chronic liver injury is the indispensable process developing into liver fibrosis. Although there are three large families of ECM proteins, glycoproteins, collagens and proteoglycans<sup>[22,23]</sup>, excessive depositions of types I and III collagens are mainly pathologic characteristics of liver fibrosis due to *Schistosoma japonicum* infection. Our results further demonstrated that rosiglitazone alone or plus praziquantel can reduce the inflammation and liver fibrosis with *Schistosoma* infection by reducing the hepatic expression of TGF- $\beta$ 1,  $\alpha$ -SMA, types I & III collagens. These results also suggest that PPAR $\gamma$  ligand, rosiglitazone can impede liver fibrosis after *Schistosoma* infection.

To seek possible regulatory mechanism in liver fibrosis, we further measured some cell factors such as TNF- $\alpha$  and IL-6, and the nuclear factors NF- $\kappa$ B in our model mice with/out treatment. TNF- $\alpha$ , a pro-inflammatory cytokine, plays a key role in a wide variety of physiological processes, including inflammation, proliferation and programmed cell death<sup>[24]</sup> which lead to regeneration of ECM and fibrogenesis<sup>[25]</sup>. Our results showed that the serum level of TNF- $\alpha$  and IL-6 increased in liver tissues. These data also provided a further evidence for the role of these cytokines in inducing hepatic fibrosis. Rosiglitazone alone or rosiglitazone plus praziquantel significantly reduced the serum level of TNF- $\alpha$  and IL-6, suggesting that rosiglitazone could be an alternative treatment for liver fibrosis after liver injury or infection.

NF- $\kappa$ B, a heterodimer, is a functional protein which

is regulated by interaction with a family of regulatory proteins, the inhibitor of nuclear factor  $\kappa$ B (I $\kappa$ B) proteins may be stimulated by many factors, such as TNF- $\alpha$  and lipopolysaccharide, *via* the phosphorylation and degradation of I $\kappa$ B. Activated NF- $\kappa$ B then transports into the cell nucleus and combines with gene promoters to induce the transcription of many cytokines and adhesion molecules<sup>[26,27]</sup>. Physical stress, oxidative stress, and exposure to certain chemicals can also activate NF- $\kappa$ B, suggesting its critical functions in mediating stress responses as well<sup>[28]</sup>. Recent studies show that the activation of NF- $\kappa$ B has a great impact on the pathogenesis of liver fibrosis through regulating hepatocyte, HSC and Kupffer cells<sup>[29,30]</sup>. Some studies demonstrate that NF- $\kappa$ B is also associated with the development of the activated phenotype of HSC and promotes survival of activated HSC<sup>[31]</sup> by protecting activated HSCs against TNF-induced apoptosis<sup>[32]</sup>. Notably, NF- $\kappa$ B binding activity increased in our mouse fibrosis model, while rosiglitazone significantly decreased the activity of, suggesting further that rosiglitazone could partially inhibit liver fibrosis by down-regulating NF- $\kappa$ B as well.

As indicated, our study showed a significant reduction of liver fibrosis following the treatment with rosiglitazone, a PPAR- $\gamma$  ligand, in a murine model of hepatic fibrosis induced by *Schistosoma japonicum*. The effect of this compound on preventing liver fibrosis may be through down-regulation of liver TGF- $\beta$ 1 and collagens, and reduction of inflammatory mediators (IL-6, TNF- $\alpha$ , NF- $\kappa$ B). It suggested the use of rosiglitazone for the treatment of liver fibrosis.

Interestingly, our results showed that praziquantel alone reduced markedly the serum level of TNF- $\alpha$ , NF- $\kappa$ B binding activity, and volumes of TGF- $\beta$ 1 and type I collagen in liver fibrosis due to *Schistosoma* infection. These data may suggest an antifibrotic effect of praziquantel in the early stage of liver fibrosis. Praziquantel might be able to block liver fibrosis through killing parasite to alleviate liver inflammation. Further studies on mechanisms of rosiglitazone and praziquantel during liver injury or infection may shed lights on developing therapeutic methods in clinical practice.

## COMMENTS

### Background

Hepatic schistosomiasis is one of the most prevalent forms of chronic liver diseases in the world, resulting in the morbidity due to its complications of liver fibrosis from infection. However, there are currently few medicines or means available to control and treat fibrosis in schistosomiasis. Many studies demonstrate that PPAR $\gamma$  is mainly expressed in human HSC and contributes to the process of liver fibrogenesis, but the identification of PPAR $\gamma$  as a novel modulator to liver fibrosis remains controversial.

### Research frontiers

Current findings further support the role of PPAR $\gamma$  in the development of liver fibrosis and suggest that PPAR $\gamma$  ligands can be therapeutically used to inhibit HSC activation and the progression of liver fibrosis.

### Innovations and breakthroughs

This is a first report about PPAR $\gamma$  and liver fibrosis due to *Schistosoma* infection. This study evaluates the relationship of PPAR $\gamma$  ligand and hepatic fibrosis due to *Schistosoma* infection.

## Applications

This study provides a perspective for a new therapeutic approach to prevent liver fibrosis following *Schistosoma japonicum* infection.

## Peer review

The study reported a significant reduction of liver fibrosis following the treatment with rosiglitazone, a PPAR- $\gamma$  ligand, in a murine model of hepatic fibrosis induced by *Schistosoma japonicum*. The effect of this compound on preventing liver fibrosis may be through down-regulation of liver TGF- $\beta$ 1 and collagens, and reduction of inflammatory mediators (IL-6, TNF- $\alpha$ , NF- $\kappa$ B). The study is interesting and may herald a new therapeutic approach to prevent liver fibrosis following *Schistosoma japonicum* infection.

## REFERENCES

- Bartley PB, Ramm GA, Jones MK, Ruddell RG, Li Y, McManus DP. A contributory role for activated hepatic stellate cells in the dynamics of *Schistosoma japonicum* egg-induced fibrosis. *Int J Parasitol* 2006; **36**: 993-1001
- Gutierrez-Ruiz MC, Gomez-Quiroz LE. Liver fibrosis: searching for cell model answers. *Liver Int* 2007; **27**: 434-439
- Parsons CJ, Takashima M, Rippe RA. Molecular mechanisms of hepatic fibrogenesis. *J Gastroenterol Hepatol* 2007; **22** Suppl 1: S79-S84
- Galli A, Svegliati-Baroni G, Ceni E, Milani S, Ridolfi F, Salzano R, Tarocchi M, Grappone C, Pellegrini G, Benedetti A, Surrenti C, Casini A. Oxidative stress stimulates proliferation and invasiveness of hepatic stellate cells via a MMP2-mediated mechanism. *Hepatology* 2005; **41**: 1074-1084
- Zhang XL, Liu JM, Yang CC, Zheng YL, Liu L, Wang ZK, Jiang HQ. Dynamic expression of extracellular signal-regulated kinase in rat liver tissue during hepatic fibrogenesis. *World J Gastroenterol* 2006; **12**: 6376-6381
- Reeves HL, Friedman SL. Activation of hepatic stellate cells-a key issue in liver fibrosis. *Front Biosci* 2002; **7**: d808-d826
- Moreira RK. Hepatic stellate cells and liver fibrosis. *Arch Pathol Lab Med* 2007; **131**: 1728-1734
- Yang C, Zeisberg M, Mosterman B, Sudhakar A, Yerramalla U, Holthaus K, Xu L, Eng F, Afdhal N, Kalluri R. Liver fibrosis: insights into migration of hepatic stellate cells in response to extracellular matrix and growth factors. *Gastroenterology* 2003; **124**: 147-159
- Shearer BG, Hoekstra WJ. Recent advances in peroxisome proliferator-activated receptor science. *Curr Med Chem* 2003; **10**: 267-280
- Berkenstam A, Gustafsson JA. Nuclear receptors and their relevance to diseases related to lipid metabolism. *Curr Opin Pharmacol* 2005; **5**: 171-176
- Marra F, Efsen E, Romanelli RG, Caligiuri A, Pastacaldi S, Batignani G, Bonacchi A, Caporale R, Laffi G, Pinzani M, Gentilini P. Ligands of peroxisome proliferator-activated receptor gamma modulate profibrogenic and proinflammatory actions in hepatic stellate cells. *Gastroenterology* 2000; **119**: 466-478
- Galli A, Crabb D, Price D, Ceni E, Salzano R, Surrenti C, Casini A. Peroxisome proliferator-activated receptor gamma transcriptional regulation is involved in platelet-derived growth factor-induced proliferation of human hepatic stellate cells. *Hepatology* 2000; **31**: 101-108
- Galli A, Crabb DW, Ceni E, Salzano R, Mello T, Svegliati-Baroni G, Ridolfi F, Trozzi L, Surrenti C, Casini A. Antidiabetic thiazolidinediones inhibit collagen synthesis and hepatic stellate cell activation in vivo and in vitro. *Gastroenterology* 2002; **122**: 1924-1940
- Anthony PP, Ishak KG, Nayak NC, Poulsen HE, Scheuer PJ, Sobin LH. The morphology of cirrhosis. Recommendations on definition, nomenclature, and classification by a working group sponsored by the World Health Organization. *J Clin Pathol* 1978; **31**: 395-414
- Yang L, Chan CC, Kwon OS, Liu S, McGhee J, Stimpson SA, Chen LZ, Harrington WW, Symonds WT, Rockey DC. Regulation of peroxisome proliferator-activated receptor-

- gamma in liver fibrosis. *Am J Physiol Gastrointest Liver Physiol* 2006; **291**: G902-G911
- 16 **Guo YT**, Leng XS, Li T, Peng JR, Song SH, Xiong LF, Qin ZZ. Effect of ligand of peroxisome proliferator-activated receptor gamma on the biological characters of hepatic stellate cells. *World J Gastroenterol* 2005; **11**: 4735-4739
  - 17 **Kawaguchi K**, Sakaida I, Tsuchiya M, Omori K, Takami T, Okita K. Pioglitazone prevents hepatic steatosis, fibrosis, and enzyme-altered lesions in rat liver cirrhosis induced by a choline-deficient L-amino acid-defined diet. *Biochem Biophys Res Commun* 2004; **315**: 187-195
  - 18 **Marra F**, DeFranco R, Robino G, Novo E, Efsen E, Pastacaldi S, Zamara E, Vercelli A, Lottini B, Spirli C, Strazzabosco M, Pinzani M, Parola M. Thiazolidinedione treatment inhibits bile duct proliferation and fibrosis in a rat model of chronic cholestasis. *World J Gastroenterol* 2005; **11**: 4931-4938
  - 19 **Zhao C**, Chen W, Yang L, Chen L, Stimpson SA, Diehl AM. PPARgamma agonists prevent TGFbeta1/Smad3-signaling in human hepatic stellate cells. *Biochem Biophys Res Commun* 2006; **350**: 385-391
  - 20 **Jiang W**, Yang CQ, Liu WB, Wang YQ, He BM, Wang JY. Blockage of transforming growth factor beta receptors prevents progression of pig serum-induced rat liver fibrosis. *World J Gastroenterol* 2004; **10**: 1634-1638
  - 21 **Kershenovich Stalnikowitz D**, Weissbrod AB. Liver fibrosis and inflammation. A review. *Ann Hepatol* 2003; **2**: 159-163
  - 22 **Tsukada S**, Parsons CJ, Rippe RA. Mechanisms of liver fibrosis. *Clin Chim Acta* 2006; **364**: 33-60
  - 23 **Benyon RC**, Arthur MJ. Extracellular matrix degradation and the role of hepatic stellate cells. *Semin Liver Dis* 2001; **21**: 373-384
  - 24 **Theiss AL**, Simmons JG, Jobin C, Lund PK. Tumor necrosis factor (TNF) alpha increases collagen accumulation and proliferation in intestinal myofibroblasts via TNF receptor 2. *J Biol Chem* 2005; **280**: 36099-36109
  - 25 **Simeonova PP**, Gallucci RM, Hulderman T, Wilson R, Kommineni C, Rao M, Luster MI. The role of tumor necrosis factor-alpha in liver toxicity, inflammation, and fibrosis induced by carbon tetrachloride. *Toxicol Appl Pharmacol* 2001; **177**: 112-120
  - 26 **Cogswell PC**, Kashatus DF, Keifer JA, Guttridge DC, Reuther JY, Bristow C, Roy S, Nicholson DW, Baldwin AS Jr. NF-kappa B and I kappa B alpha are found in the mitochondria. Evidence for regulation of mitochondrial gene expression by NF-kappa B. *J Biol Chem* 2003; **278**: 2963-2968
  - 27 **Lawrence T**, Bebie M, Liu GY, Nizet V, Karin M. IKKalpha limits macrophage NF-kappaB activation and contributes to the resolution of inflammation. *Nature* 2005; **434**: 1138-1143
  - 28 **Li X**, Stark GR. NFkappaB-dependent signaling pathways. *Exp Hematol* 2002; **30**: 285-296
  - 29 **Mann DA**, Smart DE. Transcriptional regulation of hepatic stellate cell activation. *Gut* 2002; **50**: 891-896
  - 30 **Smart DE**, Vincent KJ, Arthur MJ, Eickelberg O, Castellazzi M, Mann J, Mann DA. JunD regulates transcription of the tissue inhibitor of metalloproteinases-1 and interleukin-6 genes in activated hepatic stellate cells. *J Biol Chem* 2001; **276**: 24414-24421
  - 31 **Muhlbauer M**, Weiss TS, Thasler WE, Gelbmann CM, Schnabl B, Scholmerich J, Hellerbrand C. LPS-mediated NFkappaB activation varies between activated human hepatic stellate cells from different donors. *Biochem Biophys Res Commun* 2004; **325**: 191-197
  - 32 **Elsharkawy AM**, Oakley F, Mann DA. The role and regulation of hepatic stellate cell apoptosis in reversal of liver fibrosis. *Apoptosis* 2005; **10**: 927-939

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## CASE REPORT

# Cytomegalovirus colitis in a patient with Behcet's disease receiving tumor necrosis factor alpha inhibitory treatment

Ismail Sari, Merih Birlik, Can Gonen, Servet Akar, Duygu Gurel, Fatos Onen, Nurullah Akkoc

Ismail Sari, Merih Birlik, Servet Akar, Fatos Onen, Nurullah Akkoc, Division of Rheumatology, Department of Internal Medicine, Dokuz Eylul University School of Medicine, Izmir 35340, Turkey

Can Gonen, Division of Gastroenterology, Department of Internal Medicine, Dokuz Eylul University School of Medicine, Izmir 35340, Turkey

Duygu Gurel, Department of Pathology, Dokuz Eylul University School of Medicine, Izmir 35340, Turkey

**Author contributions:** Sari I and Birlik M wrote the paper; Sari I and Akar S drafted the article; Sari I, Gonen C and Gurel D collected and assembled the data; Onen F and Akkoc N critically revised the article for important intellectual content; Akkoc N finally approved the article.

**Correspondence to:** Professor Nurullah Akkoc, MD, Dokuz Eylul Universitesi Tip Fakultesi Ic hastaliklari ABD Immunoloji-Romatoloji BD 35340 Inciralti, Izmir 35340, Turkey. [nurullah.akkoc@deu.edu.tr](mailto:nurullah.akkoc@deu.edu.tr)

Telephone: +90-232-4123781 Fax: +90-232-2792739

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## INTRODUCTION

Anti-tumor necrosis factor alpha (TNF- $\alpha$ ) inhibitors are relatively new drugs in the rheumatic arena which are effective in the treatment of various inflammatory rheumatic conditions. Increased risk of infections and possible risk of lymphoma with the use of TNF targeting therapies are the major issues concerning the long-term safety of these agents<sup>[1]</sup>. The association of anti-TNF- $\alpha$  therapy and serious infections has generally been attributed to an increased risk of bacterial (especially microbacteria) and opportunistic fungal microorganisms<sup>[2]</sup>. In recent years, an increasing number of studies, mainly based on case reports, have reported an increased risk of serious viral infections in patients treated with anti-TNF agents.

In this report, we describe a case of severe, biopsy proven cytomegalovirus (CMV) colitis in a patient with Behcet's disease (BD) whose symptoms started 10 d after the third Infliximab (INF; Remicade®, Schering-Plough) infusion.

## CASE REPORT

A 25-year-old man presented with diarrhea and weight loss for two months. He was diagnosed as neuro BD 4 years ago according to recurrent oral and genital ulcerations, uveitis, urinary incontinence and seizures. He was treated with various immunosuppressive drugs including monthly cyclophosphamide infusions, interferon and a combination of cyclosporine and azathioprine. Although these medications were used, his eye involvement could not be controlled and visual acuity decreased to no light perception in the left eye and 10/100 in the right eye. Because of this, INF at a dose of 5 mg/kg was started. Remission of ocular symptoms was observed after two infusions. Ten days after the third dose of INF (52 d after the first infusion), the patient developed abdominal discomfort, anorexia, epigastric pain and diarrhea occurring seven to ten times per day with watery stools, but without any associated blood or mucus. He had no history of chronic diarrhea before INF administration. His symptoms continued to worsen and he lost six kilograms during the past two months. He did not use any medications other than INF, colchicine (1.5 mg/d) and

## Abstract

Anti-tumor necrosis factor alpha (TNF- $\alpha$ ) inhibitors are effective in the treatment of various inflammatory rheumatic conditions. Increased risks of serious infections are the major issues concerning the long-term safety of these agents. We present a case of a young male Behcet's patient whose disease was complicated by cytomegalovirus (CMV) colitis. Colitis started 10 d after the third Infliximab dose and responded to the cessation of TNF blocking treatment and administration of ganciclovir. Tumor necrosis factor alpha and interferon gamma act at several levels in combating viral infections. CMV infections should be kept in mind and included in the differential diagnosis of severe gastrointestinal symptoms in patients receiving anti-TNF agents.

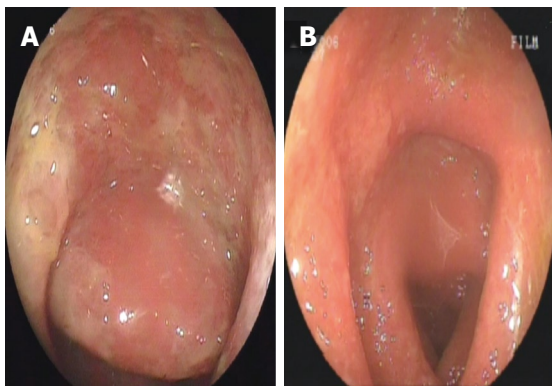
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**Key words:** Tumor necrosis factor alpha inhibitors; Adverse effects; Virus diseases

**Peer reviewer:** Burton I Korelitz, MD, Department of Gastroenterology, Lenox Hill Hospital, 100 East 77th Street, 3 Achelis, New York 10021, United States

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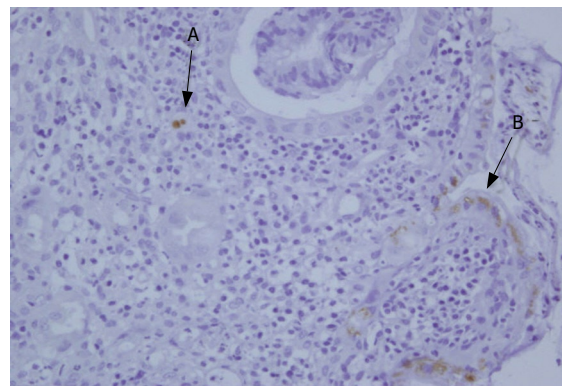
**Figure 1** Colonoscopic appearance of the mucosa before (A) and during the 14 d of anti-viral treatment (B). The presence of edema and redness is seen in both images. Note the ulcers in the sigmoid colon before the treatment as shown in A.

carbamazepine (600 mg/d). Physical examination showed epigastric tenderness on palpation, with hyperactive bowel sounds. Admission laboratory studies were significant for increased C-reactive protein (CRP = 14.4 mg/L). Stool examination was negative for parasites and *Clostridium difficile* toxin. Repeated stool cultures were also negative for enteric pathogens. Colonoscopic examination revealed diffuse redness and edematous mucosa and multiple ulcers throughout the colon including rectum and terminal ileum (Figure 1). Histological examination of the biopsy specimen identified cytomegalovirus (CMV) by specific immunohistochemistry (Figure 2). Serological testing revealed a positive CMV immunoglobulin (Ig) G and a negative IgM antibody. CMV pp65 antigenemia as determined by immunofluorescence was also negative. Infliximab was stopped and the patient was given ganciclovir (600 mg/d IV). On the 14th d of treatment, stool frequency decreased to 4-5 per day and repeated colonoscopy revealed edematous mucosa without ulcers. Immunohistochemical staining for CMV turned negative on histopathological examination of the repeat biopsy specimen. Ganciclovir was continued for an additional 14 d. The patient gained 5 kg and his stool frequency decreased to two per day. In a follow-up period of 30 mo, he did not reveal any recurrence of colitis symptoms. TNF targeting treatments were not considered during this period.

## DISCUSSION

We present a case of a young male Behcet's patient whose disease was complicated by CMV colitis. Colitis started 10 d after the third INF dose and responded to the cessation of TNF blocking treatment and administration of ganciclovir.

CMV is a member of the herpesviridae family which is prevalent among the general population with an overall seroprevalence of 30%-70% in developed countries<sup>[3]</sup>. CMV causes a variety of clinical syndromes in immunocompromised patients. Pneumonitis, retinitis and gastrointestinal (GI) CMV disease are the mostly encountered clinical manifestations<sup>[3]</sup>. GI tract may be affected anywhere from the mouth to the anus. Esophagus



**Figure 2** Colonic endothelial cells showing positive nuclear (A) and cytoplasmic (B) immunostaining of the CMV antigen.

and colon are the frequently involved sites. Ulcerations, erosions, and mucosal hemorrhage are the primary macroscopic findings<sup>[4]</sup>. The clinical signs and symptoms vary depending upon the involved areas. Patients with colonic CMV disease may present with diarrhea with or without blood, abdominal pain, urgency and tenesmus accompanied with systemic symptoms such as fever, malaise anorexia and weight loss<sup>[4]</sup>. Diagnosis of CMV infection is based upon the presence of CMV in clinical specimens demonstrated by conventional tissue culture or rapid culture with confirmation by specific monoclonal antibodies, or by detection of the pp65 CMV antigen in peripheral blood leukocytes<sup>[5]</sup>.

Severe CMV infections during TNF targeting treatments have been reported including retinitis<sup>[6]</sup>, hepatitis<sup>[7]</sup>, duodenitis<sup>[8]</sup>, ileitis<sup>[9]</sup>, colitis<sup>[10]</sup> and disseminated CMV infection<sup>[11]</sup>. All these cases received concomitant immunosuppressive treatment in addition to INF. Except for our patient and patients who developed retinitis, all other reported CMV infections developed in subjects who had primary GI problems, such as inflammatory bowel disease, common variable immunodeficiency sprue and indeterminate colitis.

BD may affect the intestine. Intestinal involvement commonly accompanies ulcerative lesions in the small and large bowel. The lesions are most commonly found in the terminal ileum and the cecum and less frequently in the colon. Rectal and anal involvement is quite rare<sup>[12]</sup>. In our patient, clinical symptomatology mimicked intestinal BD. However, diffuse involvement of the colon including the rectum and development of symptoms after the initiation of TNF inhibitory treatment suggested an infectious etiology. Following histopathological examination of the biopsies, which revealed the causative microorganism, colonic CMV disease was diagnosed. Reactivation of the virus was thought since CMV serology was positive for IgG and negative for IgM.

Tumor necrosis factor alpha and interferon gamma act at several levels in combating viral infections. TNF exhibits its antiviral activities against both DNA and RNA viruses by enhancing the induction of antiviral state in uninfected cells and by selectively killing virus-infected cells<sup>[13]</sup>. CD8+ T cells are the major defense against viruses by direct cytolysis of target cells mediated by perforin release and

Fas, or by secreting cytokines such as TNF and interferon- $\gamma$  and expressing chemokines that attract inflammatory cells to the sites of infection<sup>[14]</sup>. It has been shown that treatment with monoclonal antibodies directing against TNF- $\alpha$  is associated with a progressive loss of CD4+ and CD8+ T cells<sup>[15]</sup>. In addition, Infliximab treatment has been reported to be associated with decreased levels of TNF- $\alpha$  and interferon- $\gamma$ <sup>[16]</sup>. On the other hand, TNF levels are increased during CMV infection and CD4+ T cells specific for the human CMV result in early gene products<sup>[17]</sup>. Up-regulation of TNF gene expression by CMV<sup>[18]</sup> is the major cause for the increased TNF. It has been shown that increased TNF production in response to CMV infection displays anti-CMV activity *in vitro*<sup>[19]</sup>. Furthermore, recombinant TNF- $\alpha$  possesses anti-CMV activity<sup>[17]</sup>.

In conclusion, TNF- $\alpha$  in line with interferon- $\gamma$  acts at several levels in combating viral infections. Blocking the action of TNF- $\alpha$  with novel medications may predispose these patients to serious viral diseases. CMV infections should be kept in mind and included in the differential diagnosis of severe gastrointestinal symptoms in patients receiving Infliximab.

## REFERENCES

- 1 **Cush JJ**. Unusual toxicities with TNF inhibition: heart failure and drug-induced lupus. *Clin Exp Rheumatol* 2004; **22**: S141-S147
- 2 **Ellerin T**, Rubin RH, Weinblatt ME. Infections and anti-tumor necrosis factor alpha therapy. *Arthritis Rheum* 2003; **48**: 3013-3022
- 3 **Gandhi MK**, Khanna R. Human cytomegalovirus: clinical aspects, immune regulation, and emerging treatments. *Lancet Infect Dis* 2004; **4**: 725-738
- 4 **Goodgame RW**. Gastrointestinal cytomegalovirus disease. *Ann Intern Med* 1993; **119**: 924-935
- 5 **Reusser P**. Cytomegalovirus Infection. In: Cohen J, Powderly WG, editors. *Cohen & Powderly: Infectious Diseases*. 2nd: Mosby, An Imprint of Elsevier, 2004: 1175-1177
- 6 **Haerter G**, Manfras BJ, de Jong-Hesse Y, Wilts H, Mertens T, Kern P, Schmitt M. Cytomegalovirus retinitis in a patient treated with anti-tumor necrosis factor alpha antibody therapy for rheumatoid arthritis. *Clin Infect Dis* 2004; **39**: e88-e94
- 7 **Mizuta M**, Schuster MG. Cytomegalovirus hepatitis associated with use of anti-tumor necrosis factor-alpha antibody. *Clin Infect Dis* 2005; **40**: 1071-1072
- 8 **Medlicott SA**, Coderre S, Horne G, Panaccione R. Multimodal immunosuppressant therapy in steroid-refractory common variable immunodeficiency sprue: a case report complicating cytomegalovirus infection. *Int J Surg Pathol* 2006; **14**: 101-106
- 9 **Kohara MM**, Blum RN. Cytomegalovirus ileitis and hemophagocytic syndrome associated with use of anti-tumor necrosis factor-alpha antibody. *Clin Infect Dis* 2006; **42**: 733-734
- 10 **Papadakis KA**, Tung JK, Binder SW, Kam LY, Abreu MT, Targan SR, Vasilias EA. Outcome of cytomegalovirus infections in patients with inflammatory bowel disease. *Am J Gastroenterol* 2001; **96**: 2137-2142
- 11 **Helbling D**, Breitbach TH, Krause M. Disseminated cytomegalovirus infection in Crohn's disease following anti-tumour necrosis factor therapy. *Eur J Gastroenterol Hepatol* 2002; **14**: 1393-1395
- 12 **Bayraktar Y**, Ozaslan E, Van Thiel DH. Gastrointestinal manifestations of Behcet's disease. *J Clin Gastroenterol* 2000; **30**: 144-154
- 13 **Wong GH**, Goeddel DV. Tumour necrosis factors alpha and beta inhibit virus replication and synergize with interferons. *Nature* 1986; **323**: 819-822
- 14 **Wong P**, Pamer EG. CD8 T cell responses to infectious pathogens. *Annu Rev Immunol* 2003; **21**: 29-70
- 15 **Baert FJ**, D'Haens GR, Peeters M, Hiele MI, Schaible TF, Shealy D, Geboes K, Rutgeerts PJ. Tumor necrosis factor alpha antibody (infliximab) therapy profoundly down-regulates the inflammation in Crohn's ileocolitis. *Gastroenterology* 1999; **116**: 22-28
- 16 **Zou J**, Rudwaleit M, Brandt J, Thiel A, Braun J, Sieper J. Down-regulation of the nonspecific and antigen-specific T cell cytokine response in ankylosing spondylitis during treatment with infliximab. *Arthritis Rheum* 2003; **48**: 780-790
- 17 **Davignon JL**, Castanie P, Yorke JA, Gautier N, Clement D, Davrinche C. Anti-human cytomegalovirus activity of cytokines produced by CD4+ T-cell clones specifically activated by IE1 peptides in vitro. *J Virol* 1996; **70**: 2162-2169
- 18 **Geist LJ**, Monick MM, Stinski MF, Hunninghake GW. The immediate early genes of human cytomegalovirus upregulate tumor necrosis factor-alpha gene expression. *J Clin Invest* 1994; **93**: 474-478
- 19 **Herbein G**, O'Brien WA. Tumor necrosis factor (TNF)-alpha and TNF receptors in viral pathogenesis. *Proc Soc Exp Biol Med* 2000; **223**: 241-257

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## Agenesis of the dorsal pancreas

Lale Pasaoglu, Murat Vural, Hatice Gul Hatipoglu, Gokce Tereklioglu, Suha Koparal

Lale Pasaoglu, Murat Vural, Hatice Gul Hatipoglu, Gokce Tereklioglu, Suha Koparal, Department of Radiology, Ankara Numune State Hospital, Ankara 06100, Turkey

**Author contributions:** Pasaoglu L, Vural M contributed equally to this case; Hatipoglu HG dealt with MR images; and Tereklioglu G and Koparal S wrote the paper.

**Correspondence to:** Dr. Lale Pasaoglu, Ankara Numune State Hospital, Ankara 06100, Turkey. [ldamgaci@hotmail.com](mailto:ldamgaci@hotmail.com)

**Telephone:** +90-535-7687688 **Fax:** +90-312-4338666

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### Abstract

Developmental anomalies of the pancreas have been reported but dorsal pancreatic agenesis is an extremely rare entity. We report an asymptomatic 62-year-old woman with complete agenesis of the dorsal pancreas. Abdominal computed tomography (CT) revealed a normal pancreatic head, but pancreatic body and tail were not visualized. Magnetic resonance imaging (MRI) findings were similar to CT. At magnetic resonance cholangiopancreatography (MRCP), the major pancreatic duct was short and the dorsal pancreatic duct was not visualized. The final diagnosis was dorsal pancreatic agenesis.

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**Key words:** Agenesis; Pancreatic anomaly; Computed tomography; Magnetic resonance cholangiopancreatography

**Peer reviewers:** Jia-Yu Xu, Professor, Shanghai Second Medical University, Rui Jin Hospital, 197 Rui Jin Er Road, Shanghai 200025, China; Aydin Karabacakoglu, MD, Assistant Professor, Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

Pasaoglu L, Vural M, Hatipoglu HG, Tereklioglu G, Koparal S. Agenesis of the dorsal pancreas. *World J Gastroenterol* 2008; 14(18): 2915-2916 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2915.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2915>

### INTRODUCTION

Agenesis of the dorsal pancreas is an extremely rare anomaly which results from defective pancreas formation. A few case reports have been published in the literature about this anomaly. Agenesis of the dorsal pancreas is mostly asymptomatic but abdominal pain, pancreatitis and

diabetes mellitus may be associated<sup>[1]</sup>. We present a case of asymptomatic dorsal pancreatic agenesis.

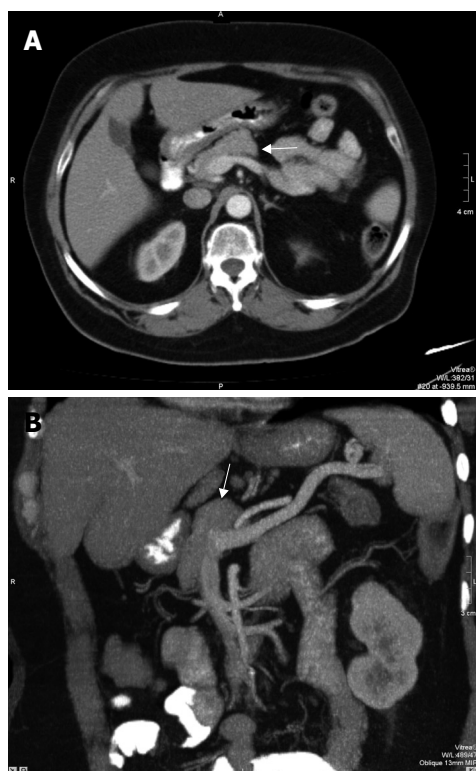
### CASE REPORT

A 62-year-old female patient was admitted to our hospital with a prediagnosis of gastric lymphoma. The patient has a suspicion of lymphoma with gastroscopy. The biochemical evaluation of the patient revealed mild elevation of fasting plasma glucose (126 mg/dL; reference range: 70-115 mg/dL<sup>[1]</sup>). Pancreatic amylase and lipase levels in serum were within normal limits. On abdominal computed tomography (CT), perigastric lymph nodes were demonstrated ranging between 10 mm and 20 mm. At this examination pancreas body and tail were not seen. Pancreatic head and neck were normal in size and shape (Figure 1A and B). Upper abdominal Magnetic resonance imaging (MRI) and magnetic resonance cholangiopancreatography (MRCP) were carried out with the suspicion of dorsal pancreatic agenesis. At MRCP, the major pancreatic duct was short and the dorsal pancreatic duct was not visualized (Figure 2A and B).

### DISCUSSION

The pancreas develops by ventral and dorsal endodermal buds. The dorsal bud forms the upper part of the head, body and tail of the pancreas which drains through the Santorini duct. The ventral bud gives rise to the major part of the head and uncinate process which drains through Wirsung duct<sup>[1]</sup>. Agenesis of the ventral pancreas and complete agenesis of the pancreas are lethal conditions<sup>[2]</sup>. Dorsal pancreatic agenesis is an extremely rare congenital anomaly. In the literature, about 23 cases of partial agenesis of dorsal pancreas were reported between 1913 and 2007. Pancreas divisum and autodigestion secondary to chronic pancreatitis must be considered in the differential diagnosis of the dorsal pancreatic agenesis. Failure of the ventral and dorsal pancreatic ducts to fuse is called pancreas divisum. In this entity, the ventral duct drains into major papilla, while the dorsal duct drains into separate minor papilla<sup>[1]</sup>. Atrophy of the body and the tail of the pancreas secondary to chronic pancreatitis and sparing of the pancreatic head is called pseudo-agenesis<sup>[3]</sup>. This situation may mimic dorsal pancreatic agenesis. In the differential diagnosis of pseudo-agenesis, histories of previous abdominal pain, pancreatitis, CT scanning and the serum amylase level may be helpful. The complete absence of the dorsal duct and demonstration of short ventral duct is important in the diagnosis of the dorsal bud agenesis. Abdominal CT may not evaluate the pancreatic duct in a

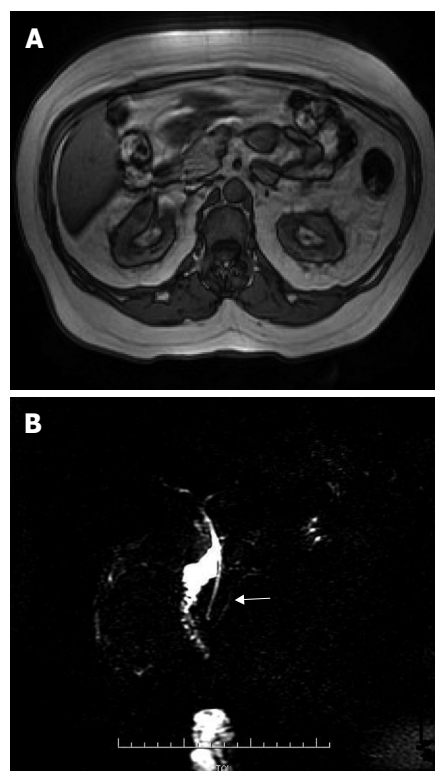




**Figure 1** Axial CT (A) and thick-slice coronal oblique MIP CT (B) images show the pancreatic head (arrow) and the absence of the corpus and tail of the pancreas.

detailed fashion. Therefore ERCP or MRCP is necessary for revealing the major and the accessory duct systems. ERCP is an invasive technique. It might challenge the cannulation of the minor papilla in selected cases. There is also radiation risk to the patient. MRCP can help the diagnosis of the dorsal pancreatic agenesis noninvasively with no radiation risk<sup>[4-6]</sup>.

In our patient, distal pancreas was absent on CT and MRI images. On MRCP, dorsal duct system was not visualized and a short ventral duct was present which supports the diagnosis of dorsal pancreatic agenesis. In summary, this extremely rare congenital anomaly must be kept in mind when the corpus and tail of the pancreas are not seen at routine examinations or as in our case an incidental finding at the examinations for different pathologies.



**Figure 2** Abdominal axial T1-weighted MR image (A) reveals pancreatic head; MRCP demonstrates a short major pancreatic duct (arrow) and dorsal duct is not visualized (B).

## REFERENCES

- 1 **Fukuoka K**, Ajiki T, Yamamoto M, Fujiwara H, Onoyama H, Fujita T, Katayama N, Mizuguchi K, Ikuta H, Kuroda Y, Hanioka K. Complete agenesis of the dorsal pancreas. *J Hepatobiliary Pancreat Surg* 1999; **6**: 94-97
- 2 **Voldsgaard P**, Kryger-Baggesen N, Lisse I. Agenesis of pancreas. *Acta Paediatr* 1994; **83**: 791-793
- 3 **Gold RP**. Agenesis and pseudo-agenesis of the dorsal pancreas. *Abdom Imaging* 1993; **18**: 141-144
- 4 **Balakrishnan V**, Narayanan VA, Siyad I, Radhakrishnan L, Nair P. Agenesis of the dorsal pancreas with chronic calcific pancreatitis. case report, review of the literature and genetic basis. *JOP* 2006; **7**: 651-659
- 5 **Uygur-Bayramicli O**, Dabak R, Kilicoglu G, Dolapcioglu C, Oztas D. Dorsal pancreatic agenesis. *JOP* 2007; **8**: 450-452
- 6 **DU J**, Xu GQ, Xu P, Jin EY, Liu Q, Li YM. Congenital short pancreas. *Chin Med J (Engl)* 2007; **120**: 259-262

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## A rare etiology of post-endoscopic retrograde cholangiopancreatography pneumoperitoneum

Stelios F Assimakopoulos, Konstantinos C Thomopoulos, Sofia Giali, Christos Triantos, Dimitrios Siagris, Charalambos Gogos

Stelios F Assimakopoulos, Sofia Giali, Dimitrios Siagris, Charalambos Gogos, Department of Internal Medicine, School of Medicine, University of Patras, Patras 26110, Greece  
Konstantinos C Thomopoulos, Christos Triantos, Division of Gastroenterology, Department of Internal Medicine, School of Medicine, University of Patras, Patras 26110, Greece  
Author contributions: Assimakopoulos SF, Giali S and Siagris D were the patient's doctors in the clinic; Thomopoulos KC and Triantos C performed the ERCP and gastroenterological consultation; Assimakopoulos SF and Gogos C had the idea for case presentation; all authors acquired the data; Assimakopoulos SF wrote the manuscript; Thomopoulos KC and Gogos C revised the manuscript.

Correspondence to: Dr. Stelios F Assimakopoulos, Department of Internal Medicine, School of Medicine, University of Patras, Vironos 18, Patras 26224, Greece. [sassim@upatras.gr](mailto:sassim@upatras.gr)  
Telephone: +30-2610-346946 Fax: +30-2610-993982  
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### Abstract

Major complications of endoscopic retrograde cholangiopancreatography (ERCP) include pancreatitis, hemorrhage, cholangitis, and duodenal perforation. The occurrence of free air in the peritoneal cavity post-ERCP is a rare event (< 1%), which is usually the result of duodenal or ductal perforation related to therapeutic ERCP with sphincterotomy. We describe for the first time a different aetiology of pneumoperitoneum, in an 84-year-old woman with pancreatic cancer and a large hepatic metastasis, after ERCP with common bile duct stent deployment. Our patient developed pneumoperitoneum due to air leakage from rupture of intrahepatic bile ducts and Glisson's capsule in the area of a peripheral large hepatic metastasis. The potential mechanism underlying this complication might be post-ERCP pneumobilia and increased pressure of intrahepatic bile ducts leading to rupture of intrahepatic bile ducts in the liver metastatic mass owing to neoplastic tissue friability. This case indicates the need for close clinical and radiological observation of patients with hepatic masses (primary or metastatic) subjected to ERCP. In such patients, avoidance of excessive air insufflation during ERCP and/or placement of a nasogastric tube for bowel decompression immediately after ERCP might be a reasonable strategy to prevent such unusual complications.

**Key words:** Endoscopic retrograde cholangiopancreatography; Pneumoperitoneum; Complications; Pneumobilia; Hepatic metastases

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### INTRODUCTION

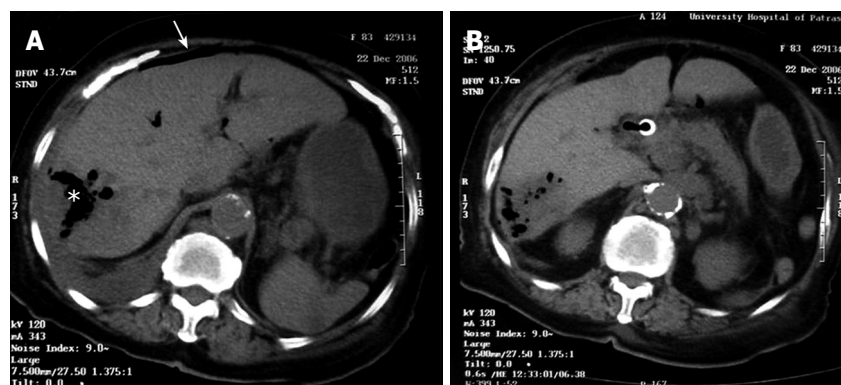
Endoscopic retrograde cholangiopancreatography (ERCP) and sphincterotomy are increasingly used in the diagnosis and management of patients with pancreaticobiliary diseases, carrying a lower morbidity and mortality rate than surgery<sup>[1]</sup>. This invasive procedure has been proven to be a safe and effective method for diagnosis and treatment of biliary and pancreatic disorders, with a very low rate of complications even in the very old (> 80 years old) patients despite the higher prevalence of co-morbidities<sup>[2,3]</sup>.

Major complications of ERCP include pancreatitis, hemorrhage, cholangitis, and duodenal perforation<sup>[4,5]</sup>. Pneumoperitoneum occurring after ERCP is usually a sinister sign of bowel or ductal perforation<sup>[6,7]</sup>. We describe for the first time a case of post-ERCP pneumoperitoneum in an 84-year-old woman due to air leakage from rupture of intrahepatic bile ducts and Glisson's capsule in the area of a large hepatic metastasis.

### CASE REPORT

An 84-year-old woman with a past medical history of a carcinoma of the head of the pancreas with hepatic and lung metastases, who underwent an ERCP with stent placement because of obstructive jaundice at the time of diagnosis two months ago, was admitted again to our department because of icterus.

At admission, physical examination was unremarkable except for the presence of skin and conjunctival icterus. Initial laboratory evaluation confirmed the cholestatic



**Figure 1** Contrast-enhanced abdominal CT showing an extensive necrotic air-containing lesion touching Glisson's capsule in the posterior right hepatic lobe within a metastatic hepatic mass (asterisk) and free air (arrow) (A). The metallic stent in the common bile duct is at the proper position and there is pneumobilia of the common bile duct and intrahepatic bile ducts of the metastatic mass in the posterior right hepatic lobe (B).

syndrome [total bilirubin = 8.13 mg/dL (reference range 0.1-1.3 mg/dL), direct bilirubin = 5.6 mg/dL (reference range < 0.4 mg/dL),  $\gamma$ -glutamyl-transaminase = 386 IU/L (reference range 10-50 IU/L), alkaline phosphatase = 541 IU/L (reference range 34-104 IU/L), aspartate aminotransferase = 510 IU/L (reference range 5-40 IU/L) and alanine aminotransferase = 413 IU/L (reference range 5-40 IU/L)]. An upper abdominal ultrasound showed a significant dilatation of the common bile duct and a hypoechoic mass of the right lobe of the liver (known metastasis). The patient underwent a second ERCP to check the stent's patency, function and position. The stent was found to be partially obstructed by tumor ingrowth and reopened by placing a new stent through the previous one. Cholangiography after stent deployment showed that the endoprosthesis was at a correct position and fully expanded, whilst there was a free contrast medium flowing to the common bile duct without any leakage. On the next day, the patient complained of distending abdominal discomfort. Physical examination showed that the patient was afebrile, hemodynamically stable, with abdominal distension but no signs of acute abdomen, such as guarding or rebound tenderness. The peripheral blood cell count showed leukocytosis ( $27\,000/\text{mm}^3$ , with 94% of neutrophils). An urgent contrast-enhanced abdominal computed tomography (CT) scan was performed, demonstrating the presence of free air in the peritoneal cavity, mainly perihepatically (Figure 1). There was no evidence of pneumoretroperitoneum, extraluminal contrast medium leakage or intraperitoneal fluid collection on computed tomography. Beyond the known tumour of the head of the pancreas, the abdominal CT showed that the metallic stent was at the proper position within the common bile duct and that there was pneumobilia of the common bile duct and intrahepatic bile ducts. At the position of the known large metastatic mass of the right hepatic lobe, there was an extensive necrotic air-containing lesion touching Glisson's capsule. The patient was treated conservatively, under close clinical surveillance, with no oral intake of, analgesics and systemic broad-spectrum antibiotics (meropenem, 1 g, three times a day and teicoplanin, 12 mg/kg per day), nasogastric drainage and intravenous fluid replacement. The patient's condition was gradually improved, oral feeding was reinstituted on post-ERCP day 10 without problems and she was discharged in a good condition on post-ERCP d 14.

## DISCUSSION

Major complications of ERCP include pancreatitis, hemorrhage, cholangitis, and duodenal perforation<sup>[4,5]</sup>. The occurrence of free air in the peritoneal cavity post-ERCP is usually the result of duodenal or ductal perforation related to therapeutic ERCP and sphincterotomy<sup>[4,6-8]</sup>. Given that the overall incidence of duodenal and common bile duct perforations is about 1% and most of these cases (80%) have retroperitoneal perforations causing pneumoretroperitoneum, it becomes apparent that post-ERCP pneumoperitoneum is a very rare complication<sup>[4]</sup>.

In our patient, the developed post-ERCP pneumoperitoneum was considered benign in nature as it was not accompanied with peritonitis. The use of contrast-enhanced abdominal CT was helpful in excluding ERCP-related retroperitoneal and bowel perforation, as demonstrated by the absence of pneumoretroperitoneum, extraluminal contrast medium leakage or abnormal intra-abdominal fluid collection. Also the cholangiogram obtained during ERCP after endoprosthesis deployment did not demonstrate any contrast medium leakage secondary to common bile duct injury. Surprisingly, the abdominal CT revealed a large necrotic air-containing lesion in a metastatic mass of the right hepatic lobe, touching Glisson's capsule, suggesting that air from this lesion can pass into the peritoneal cavity through rupture of Glisson's capsule. Analytically, the development of pneumoperitoneum in our patient may result from (1) air insufflation during ERCP leading to bowel distension, (2) retrograde airflow through the widely patent biliary tract after metallic stent deployment, (3) increased pressure of intrahepatic bile ducts and pneumobilia due to ERCP manipulations and retrograde airflow, (4) rupture of intrahepatic bile ducts of the liver metastatic mass owing to neoplastic tissue friability, (5) development of a necrotic air containing cavity in the liver metastatic mass touching Glisson's capsule, and (6) rupture of Glisson's capsule with subsequent air leakage in the peritoneal cavity.

Although there are data on the conservative or surgical management of duodenal perforations complicating ERCP, no previous experience with such an unusual complication is available. There are only scarce reports on benign pneumoperitoneum successfully managed with conservative treatment after endoscopic biliary procedures<sup>[9]</sup>. Regarding the management of ERCP-related duodenal perforations, arguments have been made about

both surgical<sup>[10-12]</sup> and non-surgical management<sup>[13]</sup>. Stapfer and colleagues<sup>[14]</sup> analyzed fourteen cases of ERCP-related perforations and concluded that patients with no peritonitis, sepsis, significant contrast medium leak during ERCP or follow-up upper gastrointestinal study, and retro- or intraperitoneal fluid collections during computed tomography, can be successfully managed conservatively. Based on these criteria, we decided to treat our patient conservatively under frequent clinical re-evaluation for the early recognition of the potential need for surgical treatment. Our patient's rare post-ERCP complication was successfully treated conservatively without need for surgical intervention.

In conclusion, this is the first report of post-ERCP pneumoperitoneum caused by rupture of intrahepatic bile ducts and Glisson's capsule in the area of a large peripheral hepatic metastasis. This case indicates the need for close clinical and radiological observation of patients with hepatic masses (primary or metastatic) subjected to ERCP. In such patients, avoidance of excessive air insufflation during ERCP and/or placement of a nasogastric tube for bowel decompression immediately after ERCP might be a reasonable strategy to prevent such unusual complications.

## REFERENCES

- 1 Carr-Locke DL. Therapeutic role of ERCP in the management of suspected common bile duct stones. *Gastrointest Endosc* 2002; **56**: S170-S174
- 2 Koklu S, Parlak E, Yuksel O, Sahin B. Endoscopic retrograde cholangiopancreatography in the elderly: a prospective and comparative study. *Age Ageing* 2005; **34**: 572-577
- 3 Thomopoulos KC, Vagenas K, Assimakopoulos SF, Giannikoulis C, Arvaniti V, Pagoni N, Nikolopoulou VN. Endoscopic retrograde cholangiopancreatography is safe and effective method for diagnosis and treatment of biliary and pancreatic disorders in octogenarians. *Acta Gastroenterol Belg* 2007; **70**: 199-202
- 4 Loperfido S, Angelini G, Benedetti G, Chilovi F, Costan F, De Berardinis F, De Bernardin M, Ederle A, Fina P, Fratton A. Major early complications from diagnostic and therapeutic ERCP: a prospective multicenter study. *Gastrointest Endosc* 1998; **48**: 1-10
- 5 Salminen P, Laine S, Gullichsen R. Severe and fatal complications after ERCP: Analysis of 2555 procedures in a single experienced center. *Surg Endosc* 2008; **22**: 1965-1970
- 6 Baron TH. Complications of endoscopic biliary sphincterotomy: the first cut is the deepest. *Clin Gastroenterol Hepatol* 2004; **2**: 968-970
- 7 Menuck L, Siemers PT. Pneumoperitoneum: importance of right upper quadrant features. *AJR Am J Roentgenol* 1976; **127**: 753-756
- 8 Pannu HK, Fishman EK. Complications of endoscopic retrograde cholangiopancreatography: spectrum of abnormalities demonstrated with CT. *Radiographics* 2001; **21**: 1441-1453
- 9 Hui YT, Lam WM, Lam TW, Cheung WC, Sze SF, Wong CT. Benign pneumoperitoneum developed after endoscopic biliary metallic stent placement with the rendezvous procedure. *Gastrointest Endosc* 2008; **67**: 179-180
- 10 Sarr MG, Fishman EK, Milligan FD, Siegelman SS, Cameron JL. Pancreatitis or duodenal perforation after peri-Vaterian therapeutic endoscopic procedures: diagnosis, differentiation, and management. *Surgery* 1986; **100**: 461-466
- 11 Bell RC, Van Stiegmann G, Goff J, Reveille M, Norton L, Pearlman NW. Decision for surgical management of perforation following endoscopic sphincterotomy. *Am Surg* 1991; **57**: 237-240
- 12 Martin DF, Tweedle DE. Retroperitoneal perforation during ERCP and endoscopic sphincterotomy: causes, clinical features and management. *Endoscopy* 1990; **22**: 174-175
- 13 Scarlett PY, Falk GL. The management of perforation of the duodenum following endoscopic sphincterotomy: a proposal for selective therapy. *Aust N Z J Surg* 1994; **64**: 843-846
- 14 Stapfer M, Selby RR, Stain SC, Katkhouda N, Parekh D, Jabbour N, Garry D. Management of duodenal perforation after endoscopic retrograde cholangiopancreatography and sphincterotomy. *Ann Surg* 2000; **232**: 191-198

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## CASE REPORT

# A case of biliary stones and anastomotic biliary stricture after liver transplant treated with the rendez - vous technique and electrokinetic lithotritor

Marta Di Pisa, Mario Traina, Roberto Miraglia, Luigi Maruzzelli, Riccardo Volpes, Salvatore Piazza, Angelo Luca, Bruno Gridelli

Marta Di Pisa, Mario Traina, Department of Gastroenterology, IsMeTT, UPMC, Via Tricomi, Palermo 90100, Italy  
Roberto Miraglia, Luigi Maruzzelli, Department of Radiology, IsMeTT, UPMC, Via Tricomi 1, Palermo 90100, Italy  
Riccardo Volpes, Department of Hepatology, IsMeTT, UPMC, Palermo 90100, Italy

Salvatore Piazza, Civico Hospital, Via Tricomi, Palermo 90100, Italy

Angelo Luca, Department of Radiology, IsMeTT, UPMC, Palermo 90100, Italy

Bruno Gridelli, Department of Surgery, IsMeTT, UPMC, Palermo 90100, Italy

**Author contributions:** Di Pisa M and Traina M designed the work; Di Pisa M wrote the paper with a contribution from Volpes R and Miraglia R; The rest of the authors contributed equally to this work.

**Correspondence to:** Marta Di Pisa, Dr, IsMeTT, UPMC, Via Tricomi 1, Palermo 90100, Italy. [mdipisa@ismett.edu](mailto:mdipisa@ismett.edu)

Telephone: +39-91-2192111 Fax: +39-91-2192400

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**Peer reviewer:** Dario Conte, Professor, GI Unit - IRCCS Osp. Maggiore, Chair of Gastroenterology, Via F. Sforza, 35, Milano 20122, Italy

Di Pisa M, Traina M, Miraglia R, Maruzzelli L, Volpes R, Piazza S, Luca A, Gridelli B. A case of biliary stones and anastomotic biliary stricture after liver transplant treated with the rendez - vous technique and electrokinetic lithotritor. *World J Gastroenterol* 2008; 14(18): 2920-2923 Available from: URL: <http://www.wjg-net.com/1007-9327/14/2920.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2920>

## Abstract

The paper studies the combined radiologic and endoscopic approach (rendez vous technique) to the treatment of the biliary complications following liver transplant. The "rendez-vous" technique was used with an electrokinetic lithotripter, in the treatment of a biliary anastomotic stricture with multiple biliary stones in a patient who underwent orthotopic liver transplant. In this patient, endoscopic or percutaneous transhepatic management of the biliary complication failed. The combined approach, percutaneous transhepatic and endoscopic treatment (rendez-vous technique) with the use of an electrokinetic lithotritor, was used to solve the biliary stenosis and to remove the stones. Technical success, defined as disappearance of the biliary stenosis and stone removal, was obtained in just one session, which definitively solved the complications. The combined approach of percutaneous transhepatic and endoscopic (rendez-vous technique) treatment, in association with an electrokinetic lithotritor, is a safe and feasible alternative treatment, especially after the failure of endoscopic and/or percutaneous trans-hepatic isolated procedures.

## INTRODUCTION

Biliary complications after liver transplant are a very common and important problem (8%-50%)<sup>[1,2]</sup>. Untreated biliary complications are associated with high morbidity and mortality rates. Magnetic resonance is an effective technique in the diagnosis of the biliary complications<sup>[3]</sup>, whereas Endoscopic retrograde cholangiopancreatography (ERCP)<sup>[4,5]</sup>, and Percutaneous Transhepatic Cholangiography (PTC) remain the gold standard therapeutic options (with successful results in between 70% and 80% of cases)<sup>[6,7]</sup>. Common biliary complications after liver transplant are leaks and strictures, which are divided into anastomotic and non-anastomotic groups. Other less frequent complications include stones, cuts, and oddities<sup>[8,9]</sup>.

We report a case of biliary anastomotic stenosis with multiple biliary stones in a patient after orthotopic liver transplantation, treated with a combined percutaneous radiologic and endoscopic approach (rendez - vous technique) and with the use of an electrokinetic lithotritor.

## CASE REPORT

A 67-year-old man underwent orthotopic liver transplantation for HCV related cirrhosis in 2000, with a choledocho - choledocho anastomosis. The surgical procedure was complicated by renal failure which





**Figure 1** A CT scan showing severe obesity and umbilical laparocoele.

was treated with hemodialysis for 1 mo. No vascular abnormalities were present at the Doppler ultrasound examination. Five years later, after an unsuccessful antiviral treatment for HCV recurrence, medium size esophageal varices were diagnosed. Other co-morbidities present at that time were severe obesity and hypertension.

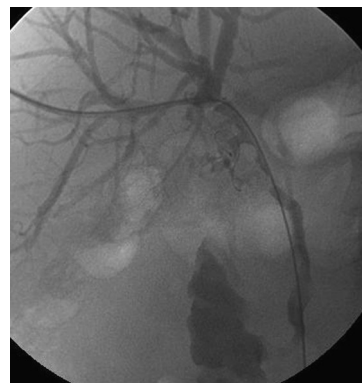
The patient was recently admitted to our facility because of the onset of severe cholestasis with episodes of cholangitis, and for a general reassessment of the liver disease.

At admission, physical examination showed severe obesity (BMI > 30) and umbilical laparocoele (Figure 1). Laboratory data were the following: AST/ALT 130/187 U/L (normal, 5/65 U/L-40/65 U/L), bilirubin tot/dir 26.46/19.69 mg/dL (0-1.5 mg/dL), alkaline phosphates 357 U/L (40 U/L-134 U/L), gamma-GT 1161 mg/L (5 U/L-85 U/L). A Magnetic Resonance Cholangiography was not performed because of the severe obesity; ERCP was therefore planned. Under general anesthesia, the choledochus was cannulated, showing a mild anastomotic stricture with a very tight angle, just above the stricture, multiple stones above the stenosis and at the confluence, and mild intrahepatic biliary dilation (Figure 2). A guide wire was passed through the stenosis, but because of the stones and the kinking, it was impossible to cross, by the guide wire, either Dormia basket, Fogarty or other catheters. Because of ERCP failure, a PTC was planned. In the angiographic room, under sonographic and fluoroscopic control, a peripheral right biliary duct was punctured with a 20 G needle. A cholangiogram showed a mild biliary dilation and multiple filling defects. It was impossible to put a guide wire into the choledochus, and thus a 6.6 F Ring external catheter (Boston Scientific, MA, USA) was placed for bile drainage. One week later, a second cholangiogram was performed and a 6.6 Fr external - internal biliary catheter (Boston Scientific) was successfully placed (Figure 3).

In the following days, because of worsening jaundice (bilirubin tot/dir 34.22/28.78 mg/dL), a combined radiologic and endoscopic technique (rendez vous technique) was performed. In the angiographic suite under general anesthesia, the patient was monitored continuously with electrocardiography, pulse oximeter and automatic recording of blood pressure and pulse. Intravenous



**Figure 2** A first attempt of ERCP showing multiple stones and anastomotic stricture.



**Figure 3** PTC with external - internal catheter placement.

antibiotic prophylaxis was given before the procedure. After visualizing the biliary tree, and under fluoroscopic control, on an Amplatz guide wire, the biliary drainage was changed with a 7 Fr vascular introducer (St. Jude Medical, Minnetonka, USA).

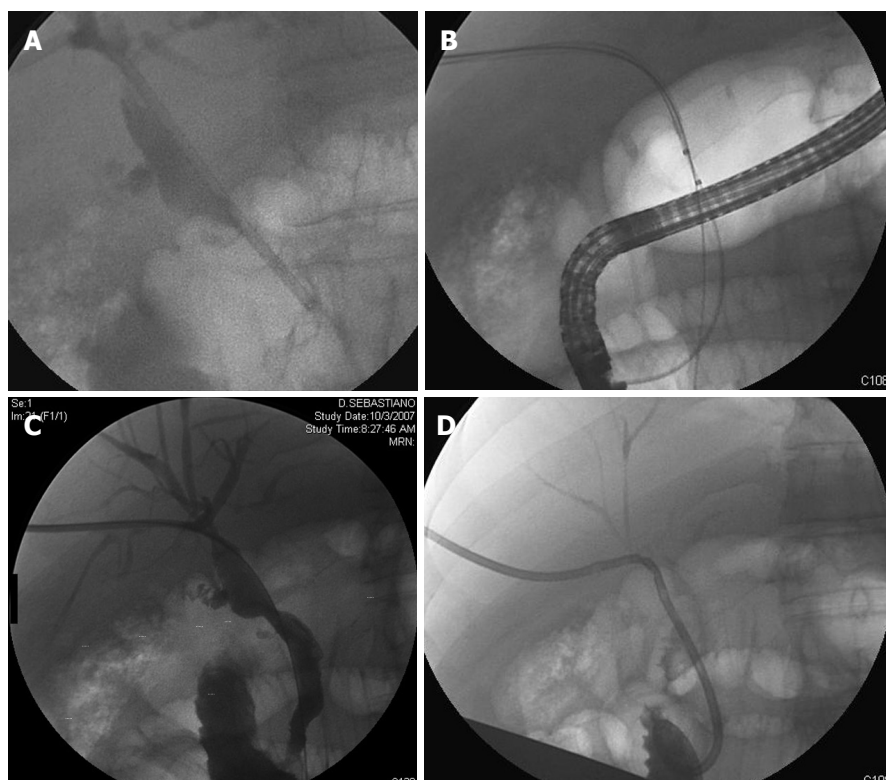
With an electro - kinetic lithotripter, a ballistic lithotripter which uses high-energy magnetic fields (Electro Medical Supplies, LTD), placed through the introducer, percutaneous lithotripsy was performed (Figure 4A). A percutaneous balloon dilatation of the anastomotic stricture was then performed using an 8 mm balloon catheter (Blumax, Boston Scientific, MA, USA) and, at the same time, under endoscopic control, the stones were completely removed with a Fogarty balloon and a Dormia basket (Figure 4B). A final cholangiogram showed no filling defects or stricture (Figure 4C) and, at the end of the procedure, a 12 Fr external - internal biliary drainage was finally placed (Boston Scientific, MA, USA) (Figure 4D).

Two days later, laboratory data showed: AST/ALT 28/29 U/L (normal, 5/65-40/65 U/L), bilirubin tot/dir 6.5/5.3 mg/dL (0-1.5 mg/dL), alkaline phosphates 104 U/L (40-134 U/L), gamma-GT 107 mg/L (5-85 U/L).

Two months after the procedure, the patient has a biliary catheter in place, is asymptomatic, in good general condition, without signs of cholestasis and in follow up for biliary catheter removal.

## DISCUSSION

Biliary complications after liver transplant are common and biliary stones represent a small part of these<sup>[1,2]</sup>. In



**Figure 4** **A:** With a ballistic lithotripter, placed through the introductory, percutaneous lithotripsy was performed; **B:** Fragmented stones were removed with a Fogarty balloon; **C:** Normal Cholangiography after lithotripsy and stenosis dilation; **D:** An external - internal catheter was placed.

the majority of cases, ERCP alone is not only the best diagnostic and therapeutic treatment with a successful result in 70%-80% of cases<sup>[4,5,10]</sup> but is also considered the least invasive procedure, although with some important complications<sup>[9]</sup>. Western studies showed the efficacy of percutaneous transhepatic cholangioscopy (PTHC) and holmium:yttrium-aluminum-garnet (YAG) laser to remove biliary stones in patients unable or unwilling to undergo endoscopic or surgical removal, but this technique requires much time and prolonged biliary access<sup>[11]</sup>.

In our patient, endoscopic management was attempted first, but we were unable to place a stent in a correct position or to remove the stones, because of the severe biliary stenosis. By a percutaneous transhepatic approach however, we were able to place an external - internal biliary catheter, though not to remove the stones or solve the stenosis.

By a combined technique - radiologic and endoscopic procedure (i.e. the “rendez-vous” technique), associated with the use of a ballistic lithotripter which usually fragments nearly all urinary calculi, we were able to remove the stones and to solve the stenosis.

The rendez-vous technique has been described in the treatment of benign or malignant biliary obstruction, in traumatic bile duct injury repair and in the treatment of large biliary leaks in a liver transplant recipient<sup>[12-15]</sup>. But, to our knowledge, this is the first report of the use of this technique in treatment of biliary stones and anastomotic stenosis, with the use of a ballistic lithotripter in a patient after liver transplantation.

In conclusion, the radiological/endoscopic rendez-vous technique, associated with an electrokinetic lithotritor, is a feasible alternative for stone removal and biliary stricture treatment, especially after the failure of endoscopic

and/or percutaneous trans-hepatic isolated procedures. Also, this combined approach improve costs, timing and effectiveness in the therapy of biliary complications.

## REFERENCES

- 1 **Stratta RJ**, Wood RP, Langnas AN, Hollins RR, Bruder KJ, Donovan JP, Burnett DA, Lieberman RP, Lund GB, Pillel TJ. Diagnosis and treatment of biliary tract complications after orthotopic liver transplantation. *Surgery* 1989; **106**: 675-683; discussion 683-684
- 2 **Greif F**, Bronsther OL, Van Thiel DH, Casavilla A, Iwatsuki S, Tzakis A, Todo S, Fung JJ, Starzl TE. The incidence, timing, and management of biliary tract complications after orthotopic liver transplantation. *Ann Surg* 1994; **219**: 40-45
- 3 **Boraschi P**, Braccini G, Gigoni R, Sartoni G, Neri E, Filippini F, Mosca F, Bartolozzi C. Detection of biliary complications after orthotopic liver transplantation with MR cholangiography. *Magn Reson Imaging* 2001; **19**: 1097-1105
- 4 **Pfau PR**, Kochman ML, Lewis JD, Long WB, Lucey MR, Olthoff K, Shaked A, Ginsberg GG. Endoscopic management of postoperative biliary complications in orthotopic liver transplantation. *Gastrointest Endosc* 2000; **52**: 55-63
- 5 **Thuluvath PJ**, Atassi T, Lee J. An endoscopic approach to biliary complications following orthotopic liver transplantation. *Liver Int* 2003; **23**: 156-162
- 6 **Ernst O**, Sergent G, Mizrahi D, Delemazure O, L'Hermine C. Biliary leaks: treatment by means of percutaneous transhepatic biliary drainage. *Radiology* 1999; **211**: 345-348
- 7 **Cozzi G**, Severini A, Civelli E, Milella M, Pulvirenti A, Salvetti M, Romito R, Suman L, Chiaraviglio F, Mazzaferro V. Percutaneous transhepatic biliary drainage in the management of postsurgical biliary leaks in patients with nondilated intrahepatic bile ducts. *Cardiovasc Intervent Radiol* 2006; **29**: 380-388
- 8 **Pascher A**, Neuhaus P. Bile duct complications after liver transplantation. *Transpl Int* 2005; **18**: 627-642
- 9 **Khuroo MS**, Al Ashgar H, Khuroo NS, Khan MQ, Khalaf HA, Al-Sebayel M, El Din Hassan MG. Biliary disease after liver transplantation: the experience of the King Faisal Specialist

- Hospital and Research Center, Riyadh. *J Gastroenterol Hepatol* 2005; **20**: 217-228
- 10 **Loperfido S**, Angelini G, Benedetti G, Chilovi F, Costan F, De Berardinis F, De Bernardin M, Ederle A, Fina P, Fratton A. Major early complications from diagnostic and therapeutic ERCP: a prospective multicenter study. *Gastrointest Endosc* 1998; **48**: 1-10
- 11 **Piraka C**, Shah RJ, Awadallah NS, Langer DA, Chen YK. Transpapillary cholangioscopy-directed lithotripsy in patients with difficult bile duct stones. *Clin Gastroenterol Hepatol* 2007; **5**: 1333-1338
- 12 **Hazey JW**, McCreary M, Guy G, Melvin WS. Efficacy of percutaneous treatment of biliary tract calculi using the holmium:YAG laser. *Surg Endosc* 2007; **21**: 1180-1183
- 13 **Wayman J**, Mansfield JC, Matthewson K, Richardson DL, Griffin SM. Combined percutaneous and endoscopic procedures for bile duct obstruction: simultaneous and delayed techniques compared. *Hepatogastroenterology* 2003; **50**: 915-918
- 14 **Aytekinc C**, Boyvat F, Yimaz U, Harman A, Haberal M. Use of the rendezvous technique in the treatment of biliary anastomotic disruption in a liver transplant recipient. *Liver Transpl* 2006; **12**: 1423-1426
- 15 **Miraglia R**, Traina M, Maruzzelli L, Caruso S, Di Pisa M, Gruttadauria S, Luca A, Gridelli B. Usefulness of the "Rendezvous" Technique in Living Related Right Liver Donors with Postoperative Biliary Leakage from Bile Duct Anastomosis. *Cardiovasc Intervent Radiol* 2008; **31**: 999-1002

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## CASE REPORT

# An autopsy case of granulocyte-colony-stimulating-factor-producing extrahepatic bile duct carcinoma

Satoru Matsuyama, Tomonori Shimonishi, Hirofumi Yoshimura, Kensaku Higaki, Kenji Nasu, Mariko Toyooka, Shigehisa Aoki, Keiko Watanabe, Hajime Sugihara

Satoru Matsuyama, Tomonori Shimonishi, Hirofumi Yoshimura, Kensuke Higaki, Kenji Nasu, Department of Surgery, Takagi Hospital, 141-11, Sakemi, Ohkawa-City, Fukuoka 831-0016, Japan

Mariko Toyooka, Keiko Watanabe, Hajime Sugihara, Department of Pathology, Takagi Hospital, 141-11, Sakemi, Ohkawa-City, Fukuoka 831-0016, Japan

Shigehisa Aoki, Department of Pathology and Biodefence, Faculty of Medicine, Saga University, 5-1-1, Nabeshima, Saga-City, Saga 849-8501, Japan

**Author contributions:** Matsuyama S, Shimonishi T, Yoshimura H, Higaki K and Nasu K treated the patient; Toyooka M, Watanabe K and Sugihara H performed cytological and pathological diagnosis, as well as autopsy, in cooperation with Aoki S.

**Correspondence to:** Dr. Satoru Matsuyama, Department of Surgery, Takagi Hospital, 141-11, Sakemi, Ohkawa-City, Fukuoka 831-0016, Japan. [matsuyama@kouhoukai.org](mailto:matsuyama@kouhoukai.org)

Telephone: +81-944-870001 Fax: +81-944-87 9310

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scintigraphy and chest CT should also be considered for distant metastasis.

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**Key words:** Bile duct carcinoma; Granulocyte colony-stimulating factor; Multiple metastases; Autopsy

**Peer reviewer:** Tom H Karlsen, MD, Institute of Immunology, Rikshospitalet University Hospital, N-0027 Oslo, Norway

Matsuyama S, Shimonishi T, Yoshimura H, Higaki K, Nasu K, Toyooka M, Aoki S, Watanabe K, Sugihara H. An autopsy case of granulocyte-colony-stimulating-factor-producing extrahepatic bile duct carcinoma. *World J Gastroenterol* 2008; 14(18): 2924-2927 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2924.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2924>

## Abstract

A 79-year-old man was referred to this department due to the presence of extrahepatic bile duct carcinoma with a tumor at the left chest wall. The lesion was suspected to be a metastasis of bile duct carcinoma to the left wall, however, computed tomography (CT) revealed no regional lymph node or liver metastases. In addition, cytological and pathological examinations did not show malignancy. At the time of admission, the white blood cell count was 21 460 cells/ $\mu$ L (neutrophils, 18 240 cells/ $\mu$ L) and this elevated to 106 040 before death. In addition, serum granulocyte colony-stimulating factor (G-CSF) was elevated. At 28 d after admission, the patient died. An autopsy showed a poorly differentiated adenocarcinoma with sarcomatous change, which had slightly invaded into the pancreas around the bile duct, and was found in the distal bile duct with multiple metastases to the chest wall, lung, kidney, adrenal body, liver, mesentery, vertebra and mediastinal and para-aortic lymph nodes, without locoregional lymph node and liver metastasis. The cancer cells showed positive immunohistochemical staining for anti-G-CSF antibody. This is believed to be the first report of an extrahepatic bile duct carcinoma that produces G-CSF. Since G-CSF-producing carcinoma and sarcomatous change of the biliary tract leads to poor prognosis, early diagnosis and treatment are needed. When infection is ruled out, the G-CSF in serum should be examined. In addition, examinations such as bone

## INTRODUCTION

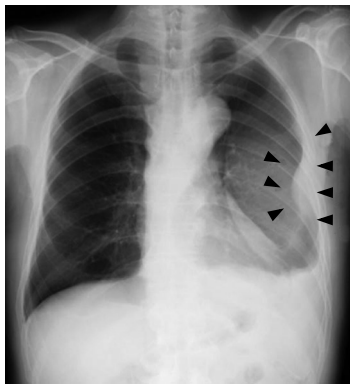
Granulocyte colony-stimulating factor (G-CSF) produced in some cancers is thought to be an autocrine growth factor<sup>[1,2]</sup>. Therefore, patients with G-CSF-producing cancer generally have a poor prognosis, and early diagnosis and treatment are required. Biliary cancer, such as cholangiocellular and gallbladder carcinoma are reported to produce G-CSF in some cases<sup>[3-10]</sup>, however, extrahepatic bile duct cancer that produces G-CSF has not yet been reported.

This report describes a case of extrahepatic bile duct cancer that produced G-CSF, in which neither regional lymph node metastasis nor massive progression was observed by radiological examination, but autopsy showed multiple organ metastasis.

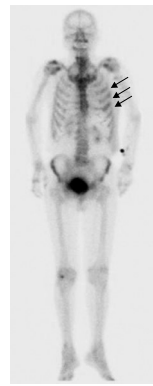
## CASE REPORT

A 79-year-old man who suffered from left lateral chest pain for one month was referred to our department for treatment of a tumor on the left chest wall and extrahepatic bile duct cancer found during an annual medical examination. His body temperature was 36.9°C. From the left axillar to anterior axillary line of the left chest wall, many nodules, 5-10 mm in diameter, were palpated, without tenderness. He had no history of chest trauma. Chest radiography showed massive pleural effusion

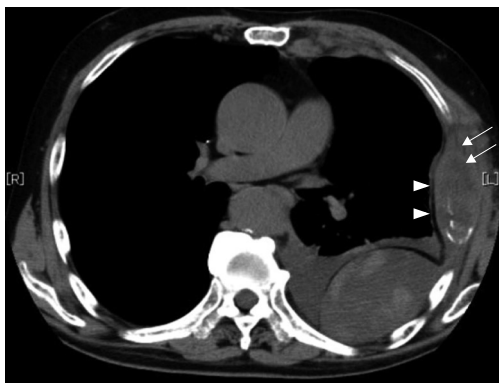




**Figure 1** Chest radiography revealed massive pleural effusion and a tumor elevating smoothly from the left chest wall (arrowheads), near which the fifth rib could not be traced.



**Figure 3** Bone scintigraphy showed an abnormal opaque uptake in the left third to fifth ribs (arrows).

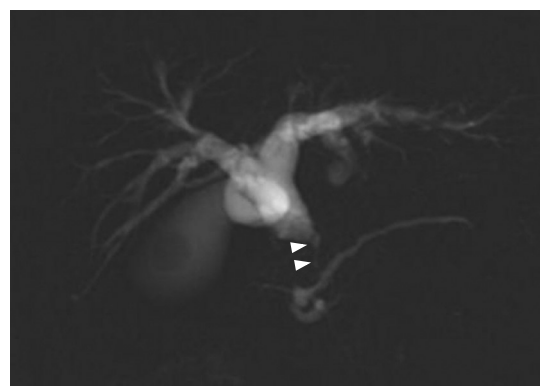


**Figure 2** Chest CCT depicted a mass with necrosis inside the tumor (arrows) and enhancement of its border (arrowheads), and extrapleural tumor and pleural effusion.



**Figure 4** Abdominal CT revealed a gradually enhanced tumor in the distal bile duct (arrows), without any regional lymph node or liver metastasis.

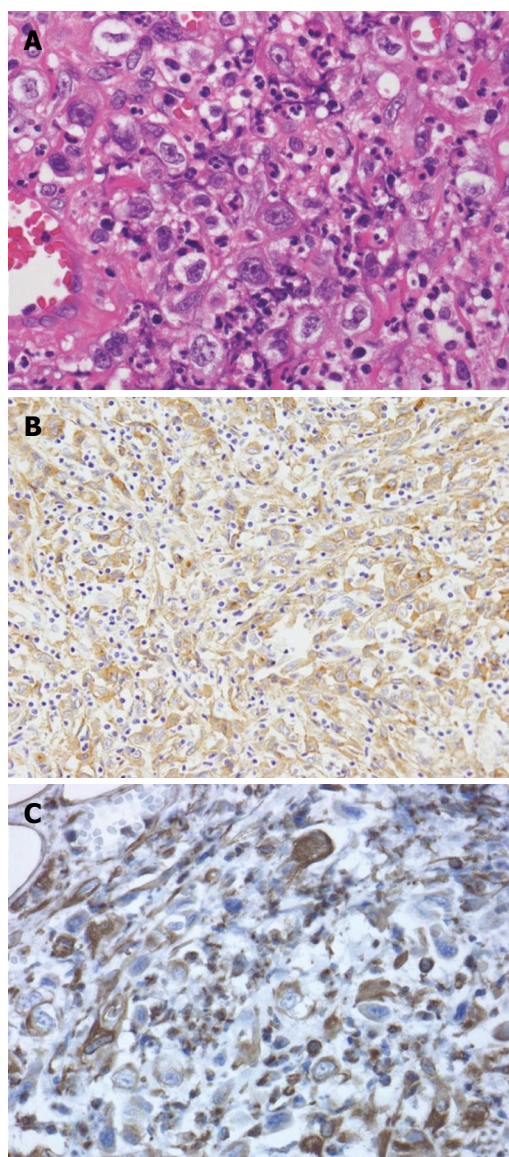
and a tumor that elevated smoothly from the left chest wall, near which the fifth rib could not be traced (Figure 1). Enhanced chest computed tomography (CT) depicted a mass with necrosis inside the tumor and enhancement on its border, as well as two osteolytic lesions on the seventh thoracic vertebra, swelling of many anterior mediastinal and left axillary lymph nodes, and pleural effusion around extrapleural fluid collection (Figure 2). Enhanced magnetic resonance imaging (MRI) of the chest showed extensive enhancement of the muscle and connective tissue around the ribs, as well as a cavity within the muscle. Bone scintigraphy showed an abnormal opaque uptake in the left third to fifth ribs and vertebrae (Figure 3). Cytopathology of the pleural and extrapleural effusion showed solitary atypical cells, regarded as histiocytes, scattered in numerous inflammatory cells. Those atypical cells had swelling nuclei and a distinct eosinophilic nucleolus; however, it was difficult to diagnose the lesion as an adenocarcinoma from the chromatin pattern. Abdominal CT demonstrated a gradually enhanced tumor in the distal bile duct, without any regional lymph node and liver metastasis (Figure 4), and magnetic resonance cholangiopancreatography (MRCP) revealed stenosis of the distal common bile duct (Figure 5). Laboratory data showed an increase in the following: white blood cell count (WBC) 21 460 cells/ $\mu$ L (neutrophils, 18 240 cells/ $\mu$ L), ALP 536 IU/L,  $\gamma$ -GTP 300 IU/L, LDH 251 IU/L, CRP 4.33 mg/dL, but carcinoembryonic antigen (CEA), carbohydrate antigen 19-9 (CA19-9) and DU-PAN-2, a tumor marker which is especially elevated in



**Figure 5** MRCP revealed stenosis (arrow heads) of the distal common bile duct.

pancreato-biliary cancer patients, were within the normal limit. The neutrophil alkaline phosphatase (NAP) score was elevated to 381 to exclude chronic myeloblastoid leukemia.

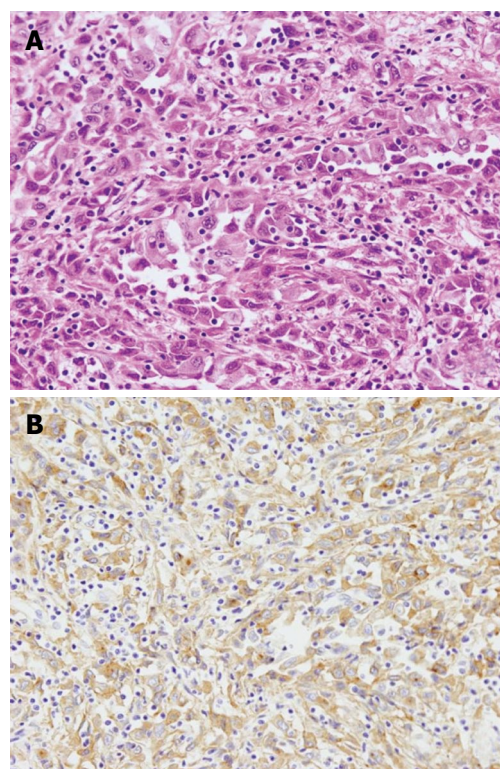
Histological examination of the nodule on the chest wall that was partially resected for diagnosis revealed scattered atypical cells among numerous fibroblasts, macrophages and neutrophils in the skeletal muscle and adipose tissue. These atypical cells showed positive immunohistochemical staining for pan-cytokeratin and vimentin, but not for desmin, which excluded leiomyosarcoma (Figure 6). Since such atypical cells had no epithelial connection or basement membrane, they



**Figure 6** Histological examination of the resected chest wall showed scattered atypical cells among numerous inflammatory cells (A). Immunohistochemistry for pan-cytokeratin as an epithelial cell marker (B) and vimentin as a mesenchymal cell marker (C) both showed positivity in these atypical cells.

were thought to be reactive mesothelial and not carcinoma cells. Therefore, the pathological diagnosis was considered to be either proliferative myositis or nodular fasciitis-like granuloma.

Eighteen days after admission the patient's appetite, consciousness and daily activity deteriorated, and WBC was elevated to 86 050 cells/ $\mu$ L (neutrophils, 80 880 cells/ $\mu$ L) without pyrexia. Until then, no bacterium was seen in the sputum, pleural effusion and fluid in the chest wall cavity. Expecting an improvement of his general condition and inflammation of myositis on the left chest wall, we drained pleural effusion fluid and the patient received an injection of dexamethasone. However, his general condition and laboratory data did not improve. WBC increased to 106 040 cells/ $\mu$ L (neutrophils, 91 900 cells/ $\mu$ L) before death. Twenty-seven days after admission, a multiple cerebral infarction was found, which had not been observed 7 d earlier. The patient died the following day and



**Figure 7** Histological examination revealed a poorly differentiated adenocarcinoma with sarcomatous change in the distal bile duct, which slightly invaded into the pancreas (A), and cancer cells showed positive immunohistochemical staining for G-CSF (B).

underwent an autopsy.

The autopsy revealed a poorly differentiated adenocarcinoma with sarcomatous change, which had slightly invaded into the pancreas in the distal bile duct (Figure 7A). In addition, no other primary cancer was observed macroscopically in the chest and abdomen. The cancer cells showed positive immunohistochemical staining for G-CSF (Figure 7B). The G-CSF concentration in serum samples archived by the hospital was 53, 38 and 134 pg/mL on 21, 23 and 26 d after admission, respectively (normal < 18 pg/mL). This result was obtained 9 d after death. In a specimen of the left chest wall, solitary cancer cells, numerous surrounding inflammatory cells such as neutrophils and macrophages, had invaded into the skeletal muscles, similar to the biopsy tissue. Therefore, the pathological diagnosis of the left chest wall was a metastasis of the bile duct carcinoma with sarcomatous change, and the anterior mediastinal lymph nodes showed the same histological findings. Moreover, under the serosa of the mesentery and liver, some white nodules, measuring 5 mm in diameter, were macroscopically discovered and their histology showed them to be metastases. In addition, histological examination of all the organs in the chest and abdomen demonstrated multiple metastases in the lung, kidney, adrenal gland, mesentery, vertebral bone marrow and mediastinal and para-aortic lymph nodes. In contrast, no metastasis was found in the regional lymph nodes. These findings proved the presence of extensive systemic dissemination of cancer cells.



## DISCUSSION

This is believed to be the first report of an extrahepatic bile duct carcinoma that produces G-CSF. A search of PubMed by the key words CSF and biliary cancer, revealed three and five reports, respectively, of intrahepatic cholangiocarcinoma and gallbladder carcinoma<sup>[3-10]</sup>, but there have so far been no reports of extrahepatic bile duct malignancy. While two of these patients underwent curative surgery and survived without recurrence for 18 mo and 27 mo, respectively<sup>[6,8]</sup>, three patients died within 6 mo after palliative or exploratory surgery<sup>[3,5,9]</sup>. In one case, the details of the surgery were unknown<sup>[4]</sup>, and the other was a report of archival histological examination<sup>[7]</sup>. In a case of rapid progression, the patient died 5 d after admission<sup>[10]</sup>. As with other organs such as the lung, thyroid and kidney, the prognosis of G-CSF-producing carcinoma in the biliary tract is considered to be poor, and early diagnosis and surgical treatment are needed. In the present case, surgery could not have been performed, even if the diagnosis had been done correctly and rapidly. However, an early examination of G-CSF in the serum would have identified the lesions as multiple metastasis of a common bile duct cancer, thereby leading to more appropriate treatment, such as chemotherapy. To avoid wasting time with additional radiological examination and inefficient treatment such as antibiotics for leukocytosis, G-CSF in serum should be examined when bacterial infection is ruled out for leukocytosis by a routine preoperative examination.

In this case, it was not possible to diagnose the correct extent of the carcinoma, in spite of various radiological and pathological data. This was because the accumulation of inflammatory cells induced by G-CSF produced by the cancer cells in the metastatic lesions mimicked inflammation of the chest wall and vertebra in the radiological and pathological examinations. Furthermore, the lack of loco-regional lymph node and liver metastases in the radiological examination led us to consider other lesions in the differential diagnosis, such as mediastinal lymphadenopathy and chest wall nodules, as an inflammatory reaction from the left chest wall lesion. Although endoscopic ultrasonography was not performed in this case, the loco-regional stage of cancer was diagnosed as stage I a or I b. This type of dissociation between the loco-regional and whole body extent of carcinoma has occasionally been reported with gastric cancer<sup>[11-13]</sup>, however, no report has been found regarding extrahepatic bile duct carcinoma. Scattered carcinoma cells in many inflammatory cells, thus indicating sarcomatous change, could not be correctly diagnosed due to these changes, such as the loss of basal membrane and intercellular connection between cancer cells. Shimada *et al.*<sup>[14]</sup> have reported cholangiocarcinomas with two types of sarcomatous change, spindle and pleomorphic type, which have very poor prognosis, especially the latter type. Moreover, Nakajima *et al.*<sup>[15]</sup> have shown aggressive intrahepatic spreading and widespread metastasis of the sarcomatous cells of an intrahepatic cholangiocarcinoma.

In the present case, dissociation between the findings of the loco-regional and whole body examination might have been due to sarcomatous change and G-CSF, which is thought to be a growth factor. For a case of extrahepatic bile duct carcinoma that produces G-CSF, an examination for distant metastasis, such as bone scintigraphy and chest CT, should be considered.

## REFERENCES

- 1 Kyo S, Kanaya T, Takakura M, Inoue M. A case of cervical cancer with aggressive tumor growth: possible autocrine growth stimulation by G-CSF and Il-6. *Gynecol Oncol* 2000; **78**: 383-387
- 2 Tachibana M, Miyakawa A, Nakashima J, Murai M, Nakamura K, Kubo A, Hata J. Autocrine growth promotion by multiple hematopoietic growth factors in the established renal cell carcinoma line KU-19-20. *Cell Tissue Res* 2000; **301**: 353-367
- 3 Takahashi M, Fujiwara M, Kishi K, Sakai C, Sanada M, Moriyama Y, Shibata A. CSF producing gall bladder cancer: case report and characteristics of the CSF produced by tumor cells. *Int J Cell Cloning* 1985; **3**: 294-303
- 4 Sakamoto K, Egami H, Yoshimura R, Nakamura S, Ikei S, Mori K, Matsumoto M, Akagi M. Colony-stimulating factor producing carcinoma of the gallbladder. *Jpn J Clin Oncol* 1986; **16**: 87-96
- 5 Takeda T, Ichianagi A, Sano K, Yoshida J, Tsutsumi Y, Miyaji T. A case of gallbladder cancer producing granulocyte-colony-stimulating factor. *Gastroenterol Jpn* 1990; **25**: 762-767
- 6 Omura N, Abe S, Hirai K, Aoki T. A case of granulocyte-colony stimulating factor producing gallbladder cancer. *Am J Gastroenterol* 1999; **94**: 273-275
- 7 Sasaki M, Tsuneyama K, Ishikawa A, Nakanuma Y. Intrahepatic cholangiocarcinoma in cirrhosis presents granulocyte and granulocyte-macrophage colony-stimulating factor. *Hum Pathol* 2003; **34**: 1337-1344
- 8 Ikeda T, Ohgaki K, Miura M, Aishima S, Shimizu T, Maehara Y. Granulocyte-colony stimulating factor-producing gallbladder cancer without recurrence more than 2 years after resection: report of a case. *Surg Today* 2005; **35**: 590-593
- 9 Amano H, Itamoto T, Emoto K, Hino H, Asahara T, Shimamoto F. Granulocyte colony-stimulating factor-producing combined hepatocellular/cholangiocellular carcinoma with sarcomatous change. *J Gastroenterol* 2005; **40**: 1158-1159
- 10 Sohma T, Shiga H, Nakane H, Watanabe H, Takeshita M, Sakisaka S. Cholangiocellular carcinoma that produced both granulocyte-colony-stimulating factor and parathyroid hormone-related protein. *Int J Clin Oncol* 2006; **11**: 246-249
- 11 Suzuki S, Kosugi S, Kuwabara S, Ueki K, Oka Y, Nishimaki T, Aizawa K, Suzuki T, Soga J, Hatakeyama K. Tumor recurrence in patients with early gastric cancer: a clinicopathologic evaluation. *J Exp Clin Cancer Res* 1998; **17**: 187-191
- 12 Shiomi M, Kamisako T, Yutani I, Kudo M, Shigeoka H, Tanaka A, Okuno K, Yasutomi M. Two cases of histopathologically advanced (stage IV) early gastric cancers. *Tumori* 2001; **87**: 191-195
- 13 Kobayashi M, Araki K, Matsuura K, Kawai S, Moriki T. Early gastric cancer giving rise to bone and brain metastases--a review of the Japanese literature. *Hepatogastroenterology* 2002; **49**: 1751-1754
- 14 Shimada M, Takenaka K, Rikimaru T, Hamatsu T, Yamashita Y, Kajiyama K, Taguchi K, Shirabe K, Sugimachi K. Characteristics of sarcomatous cholangiocarcinoma of the liver. *Hepatogastroenterology* 2000; **47**: 956-961
- 15 Nakajima T, Tajima Y, Sugano I, Nagao K, Kondo Y, Wada K. Intrahepatic cholangiocarcinoma with sarcomatous change. Clinicopathologic and immunohistochemical evaluation of seven cases. *Cancer* 1993; **72**: 1872-1877



## CASE REPORT

# A ruptured large extraluminal ileal gastrointestinal stromal tumor causing hemoperitoneum

Shoji Hirasaki, Kohei Fujita, Minoru Matsubara, Hiromitsu Kanzaki, Hiromichi Yamane, Masato Okuda, Seiyuu Suzuki, Atsuko Shirakawa, Hideyuki Saeki

Shoji Hirasaki, Kohei Fujita, Minoru Matsubara, Hiromitsu Kanzaki, Hiromichi Yamane, Masato Okuda, Seiyuu Suzuki, Department of Internal Medicine, Sumitomo Besshi Hospital, Niihama 7928543, Japan

Atsuko Shirakawa, Department of Pathology, Sumitomo Besshi Hospital, Niihama 7928543, Japan

Hideyuki Saeki, Department of Surgery, Sumitomo Besshi Hospital, Niihama 7928543, Japan

Author contributions: Hirasaki S, Fujita K, Matsubara M, Kanzaki H, Yamane H, Okuda M and Suzuki S were involved in the care of the patient; Shirakawa A studied the specimen; Saeki H performed the surgery; Hirasaki S wrote the paper.

Correspondence to: Shoji Hirasaki, Department of Internal Medicine, Sumitomo Besshi Hospital, 3-1 Ohji-cho, Niihama 7928543, Japan. [shoji\\_hirasaki@ni.sbh.gr.jp](mailto:shoji_hirasaki@ni.sbh.gr.jp)

Telephone: +81-897-377111 Fax: +81-897-377121

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## INTRODUCTION

Gastrointestinal stromal tumor (GIST) is the designation for a major subset of mesenchymal tumors of the gastrointestinal (GI) tract<sup>[1-5]</sup>. GIST arising in the digestive tract is most commonly located in the stomach and small intestine<sup>[6,7]</sup>. GIST originating from the small intestine rarely causes hemoperitoneum<sup>[8]</sup>. Herein, we describe a relatively rare case of extraluminal ileal GIST causing hemoperitoneum.

## Abstract

We describe an 87-year-old woman with a large ileal gastrointestinal stromal tumor (GIST) causing hemoperitoneum. A CT scan demonstrated a large heterogeneous mass measuring about 13 cm × 11 cm in the pelvis and hemoperitoneum, with a non-uniform enhancement pattern. The mass was diagnosed as a GIST originating from the gastrointestinal tract. She underwent an urgent laparotomy and an ileal GIST with a rupture was found 130 cm from the anal to the Treitz's ligament. Hemoperitoneum caused by ileal GIST rupture is a rare condition. Bleeding in the large tumor leading to rupture of the capsule might cause hemoperitoneum in the present case.

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**Key words:** Intestinal neoplasm; Small intestine; Extraluminal growth; Laparotomy

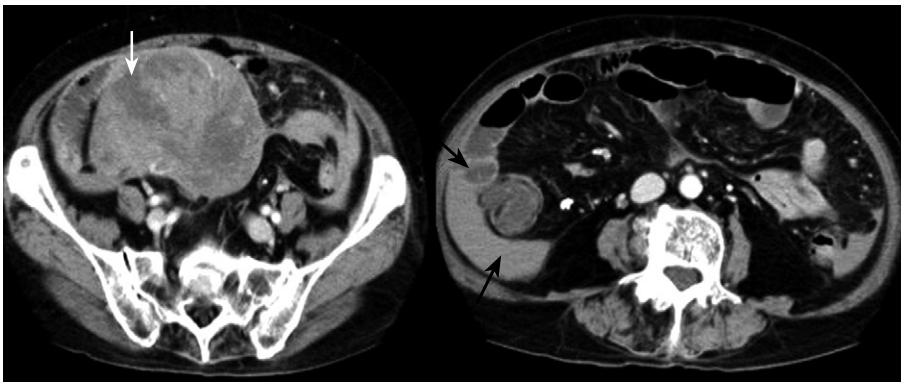
**Peer reviewers:** Ton Lisan, PhD, Thrombosis and Haemostasis Laboratory, Department of Haematology G.03.550, University Medical Centre, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands; Vincent W Yang, Professor and Director, 201 Whitehead Research Building, 615 Michael Street, Atlanta, GA 30322, United States; Jayaram Menon, Head, Department of Medicine, Queen Elizabeth Hospital, Department of Medicine, Queen Elizabeth Hospital, Kota Kinabalu, Sabah, Malaysia

Hirasaki S, Fujita K, Matsubara M, Kanzaki H, Yamane H, Okuda M, Suzuki S, Shirakawa A, Saeki H. A ruptured large extraluminal

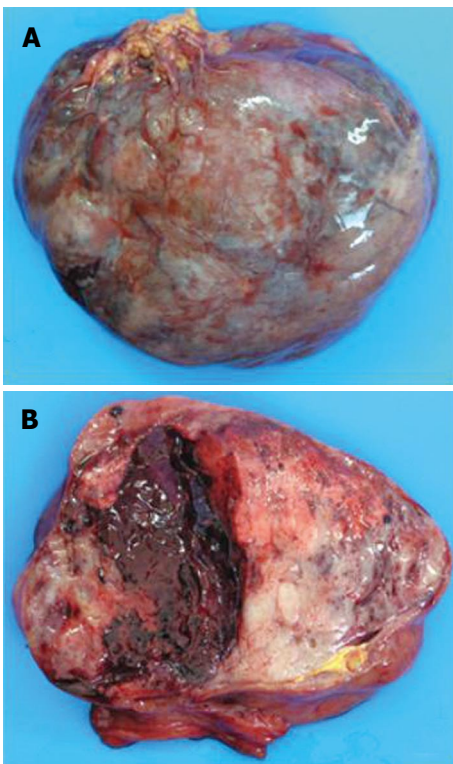
## CASE REPORT

An 87-year-old woman presented with the symptom of a short loss of consciousness. She was in good health with no specific family or past medical history. Her body temperature was 36.7°C, blood pressure was 148/82 mmHg, radial pulse rate was 72 beats/min and regular. She had slight anemia, but no jaundice. Neurological examination revealed no abnormal findings and lymphadenopathy. Abdominal palpation revealed tenderness in the right lower quadrant. Laboratory tests showed a red blood cell count of  $315 \times 10^4/\mu\text{L}$  [normal range (NR),  $380-500 \times 10^4/\mu\text{L}$ ], a white blood cell count of  $10\,500/\mu\text{L}$  (NR,  $4000-9000/\mu\text{L}$ ), a platelet count of  $29.8 \times 10^4/\mu\text{L}$ , and a hemoglobin concentration of 10.1 g/dL (NR, 12-16 g/dL). The levels of hepatic and biliary enzymes, such as aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), leucin aminopeptidase (LAP), and  $\gamma$ -glutamyltranspeptidase ( $\gamma$ -GTP) were normal except for lactate dehydrogenase (LDH) which was 336 IU/L (NR, 106-211 IU/L). A test for C reactive protein revealed a level of 30.3 mg/dL (NR, < 0.5 mg/dL). Renal function tests showed that the blood urea nitrogen level was 38.0 mg/dL (NR, 8-20 mg/dL) and the creatinine level was normal. Serological studies for hepatitis B and C viruses were negative. Urinary protein and sugar were negative. A computed tomography (CT) scan demonstrated a large heterogeneous mass measuring about 13 cm × 11 cm in the pelvis and hemoperitoneum, with a non-

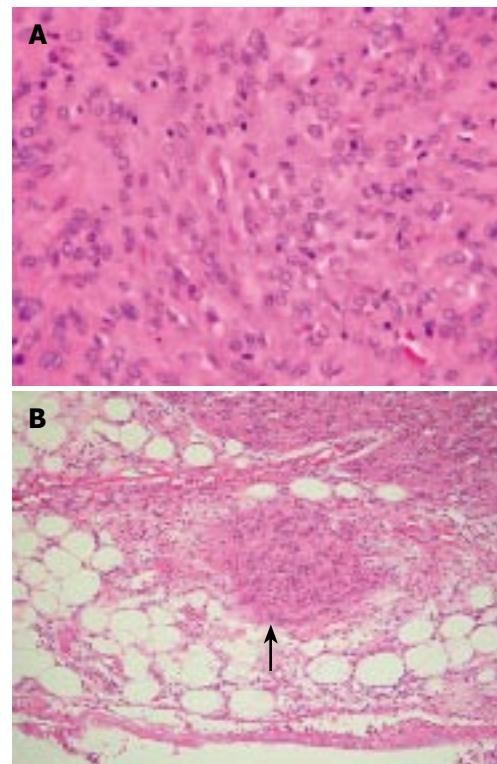




**Figure 1** CT scan demonstrating a large heterogeneous mass with a non-uniform enhancement pattern (white arrow) in the pelvis and hemoperitoneum (black arrows).



**Figure 2** Macroscopic finding of the tumor. **A:** A large tumor (measuring 13 cm × 11 cm) arising from the ileum with extraluminal growth; **B:** The cut surface showing bleeding blood clots in the tumor.



**Figure 3** Microscopic findings of the tumor. **A:** Histological examination demonstrating interlaced bundles of large Bizarre spindle cells without mitotic figures (HE, × 100); **B:** Tumor cells present in the subserosa (black arrow) (HE, × 20).

uniform enhancement pattern (Figure 1). Based on the imaging examination, this tumor was diagnosed as a GIST originating from the GI tract or omentum, although it should be distinguished from an ovarian tumor or an adenocarcinoma of the small bowel. The patient underwent an urgent laparotomy.

At laparotomy, a 13 cm × 11 cm semipedunculated solid tumor that was 130 cm from the anal to the Treitz's ligament, showed extraluminal growth (Figure 2A). The tumor was ruptured with no peritoneal metastasis, and partial resection of the ileum was carried out. The resected tumor was brown-red in color, and had bleeding blood clots (Figure 2B). Histological examination of the resected specimen revealed interlaced bundles of large Bizarre spindle-like tumor cells without mitotic figures (Figure 3A). No fission images were evident. Tumor cells were present in the subserosa (Figure 3B). Immunohistological findings

were negative for CD34,  $\alpha$ -smooth muscle actin (SMA), desmin and S-100 protein, but positive for CD117. Based on the above findings, this tumor was diagnosed as a malignant GIST. The postoperative course was uneventful. The patient has been followed up for 16 mo with no evidence of recurrence.

## DISCUSSION

GIST is the most common mesenchymal tumor of the GI tract and expresses c-kit protein, also known as CD117, which is considered a highly specific marker differentiating GIST from other mesenchymal tumors, such as leiomyomas<sup>[9-11]</sup>. The majority of GISTs occur in the stomach (60%-70%) and small intestine (20%-30%)<sup>[10]</sup>. Approximately, 10%-30% of patients with GIST may be asymptomatic<sup>[11]</sup>. Gastric and small intestinal stromal tumors

Table 1 Summary of 10 cases of small bowel GIST causing hemoperitoneum in Japan (since 2000)

	Author <sup>Ref</sup>	Age	Sex	Year	Tumor location	Tumor size (cm)	Symptoms
1	Ri	45	Male	2000	Ileum	5	Abdominal pain
2	Yanaginuma	58	Male	2001	Ileum	13 × 10 × 8	Abdominal pain
3	Sugawara <sup>[8]</sup>	37	Male	2001	Ileum	4 × 4 × 3	Abdominal pain
4	Okita	79	Male	2003	Ileum	18.5 × 15.7 × 6.5	Abdominal pain
5	Hirose	71	Female	2003	Ileum	7	Vomiting
6	Kinoshita	70	Male	2005	Jejunum	11 × 7	Abdominal pain
7	Saito	62	Male	2006	Ileum	5.0 × 4.5 × 3	Abdominal pain
8	Goto	71	Female	2006	Ileum	9 × 4 × 5	Consciousness loss, vomiting
9	Hisaoka <sup>[13]</sup>	73	Female	2007	Ileum	1	Abdominal pain
10	Present case	87	Female	2007	Ileum	13 × 11	Consciousness loss, abdominal pain

are usually associated with abdominal pain, GI bleeding or palpable mass<sup>[12]</sup>. However, GIST in the small intestine rarely causes hemoperitoneum. A MEDLINE search of the literature has revealed only 10 cases of GIST in the small intestine with hemoperitoneum since 2000, including the present case in Japan<sup>[8,13]</sup> (Table 1). The tumor size was over 5 cm in 9 of the 10 cases. However, Hisaoka *et al*<sup>[13]</sup> have reported a case of small intestinal GIST, measuring about 1 cm in size, with intraperitoneal hemorrhage. Thus, a small GIST does not necessarily have a low risk of bleeding. We found that 7 of the 10 patients were in their sixties or over and GIST was predominant in older patients. The male to female ratio was 6:4. The major symptom was abdominal pain. Two patients had consciousness loss, including our case, which might be related to bleeding.

The mechanism underlying hemoperitoneum might be due to bleeding in the tumor leading to hematoma and rupture of the capsule, or transudation of blood components from the tumor. In the present case, bleeding in the tumor leading to rupture of the capsule might have caused hemoperitoneum. Currently, there is no single prognostic factor that can be used alone to predict tumor behavior. The biological behavior of tumors depends on the location (GIST arising from the small bowel is generally associated with a less favorable outcome than that arising in the stomach)<sup>[14]</sup>. Radiological and surgical factors that have been used to determine malignancy include invasion to adjacent organs, omental or peritoneal seeding, tumor recurrence after surgical resection, and distant metastasis<sup>[9,15]</sup>. Pathological factors that determine malignancy are tumor size, mitotic activity, pleomorphism of nuclei, degree of cellularity, nucleus/cytoplasm ratio, and mucosal invasion<sup>[16]</sup>. The present patient was diagnosed with malignant GIST because of the tumor size and rupture. Although peritoneal metastasis was not seen in the present patient, we should pay attention to tumor recurrence because the tumor was ruptured. This patient remains alive without disease 16 mo after surgery. We should carefully follow up with CT or MRI images and this patient is going to undergo imatinib mesilate therapy if tumor recurrence is identified.

Computed tomography (CT) and MR imaging are useful for the diagnosis of GIST and demonstration of the tumor extent<sup>[17-19]</sup>. Because of the high soft-tissue contrast, MR imaging shows a tendency of GIST toward necrosis and hemorrhage<sup>[20]</sup>. In particular, hemorrhage observed in large tumors is associated with large necrosis.

Because GIST can rupture and result in hemoperitoneum, as in the present case, the presence of hemorrhage inside and outside the tumor should be detected. It is impossible to differentiate benign from malignant GIST confidently based on imaging findings alone. It was recently reported that a markedly enhanced GIST on MRI might demonstrate a higher mitosis index even if it is relatively small<sup>[20]</sup>. To clarify the relationship between MRI and pathological findings, we should accumulate and analyze many cases of GIST. Because of hemoperitoneum, our patient underwent urgent laparotomy but not MRI. In addition, previous reports of contrast-enhanced CT indicate that tumor density and heterogeneity after contrast enhancement may reflect the poor prognosis and tumor response to chemotherapy, respectively<sup>[21,22]</sup>. Further studies on the correlation between imaging diagnosis and pathological findings of GIST are certainly required.

In conclusion, we reported the case of a woman with a large ileal GIST causing hemoperitoneum. It is necessary to know that a large GIST may cause hemoperitoneum, although GIST in the small intestine rarely causes hemoperitoneum.

## REFERENCES

- Miettinen M, Lasota J. Gastrointestinal stromal tumors--definition, clinical, histological, immunohistochemical, and molecular genetic features and differential diagnosis. *Virchows Arch* 2001; **438**: 1-12
- Fletcher CD, Berman JJ, Corless C, Gorstein F, Lasota J, Longley BJ, Miettinen M, O'Leary TJ, Remotti H, Rubin BP, Shmookler B, Sobin LH, Weiss SW. Diagnosis of gastrointestinal stromal tumors: A consensus approach. *Hum Pathol* 2002; **33**: 459-465
- Joensuu H, Fletcher C, Dimitrijevic S, Silberman S, Roberts P, Demetri G. Management of malignant gastrointestinal stromal tumours. *Lancet Oncol* 2002; **3**: 655-664
- Suzuki K, Kaneko G, Kubota K, Horigome N, Hikita H, Senga O, Miyakawa M, Shimojo H, Uehara T, Itoh N. Malignant tumor, of the gastrointestinal stromal tumor type, in the greater omentum. *J Gastroenterol* 2003; **38**: 985-988
- Miettinen M, Lasota J. Gastrointestinal stromal tumors (GISTs): definition, occurrence, pathology, differential diagnosis and molecular genetics. *Pol J Pathol* 2003; **54**: 3-24
- DeMatteo RP, Lewis JJ, Leung D, Mudan SS, Woodruff JM, Brennan MF. Two hundred gastrointestinal stromal tumors: recurrence patterns and prognostic factors for survival. *Ann Surg* 2000; **231**: 51-58
- Grassi N, Cipolla C, Torcivia A, Mandala S, Graceffa G, Bottino A, Latteri F. Gastrointestinal stromal tumour of the rectum: Report of a case and review of literature. *World J*

- Gastroenterol* 2008; **14**: 1302-1304
- 8 **Sugawara G**, Yamaguchi A, Isogai M, Kaneoka Y, Suzuki M. A case of gastrointestinal stromal tumor of the ileum with intraabdominal hemorrhage (in Japanese). *J Jpn Soc Clin Surg* 2003; **64**: 3092-3096
  - 9 **Miettinen M**, Sarlomo-Rikala M, Lasota J. Gastrointestinal stromal tumors: recent advances in understanding of their biology. *Hum Pathol* 1999; **30**: 1213-1220
  - 10 **Fletcher CD**, Berman JJ, Corless C, Gorstein F, Lasota J, Longley BJ, Miettinen M, O'Leary TJ, Remotti H, Rubin BP, Shmookler B, Sobin LH, Weiss SW. Diagnosis of gastrointestinal stromal tumors: A consensus approach. *Hum Pathol* 2002; **33**: 459-465
  - 11 **Miettinen M**, Sobin LH, Sarlomo-Rikala M. Immunohistochemical spectrum of GISTs at different sites and their differential diagnosis with a reference to CD117 (KIT). *Mod Pathol* 2000; **13**: 1134-1142
  - 12 **Mehta RM**, Sudheer VO, John AK, Nandakumar RR, Dhar PS, Sudhindran S, Balakrishnan V. Spontaneous rupture of giant gastric stromal tumor into gastric lumen. *World J Surg Oncol* 2005; **3**: 11
  - 13 **Hisaoka S**, Wakata T, Hirose C, Kakehisa M, kajikawa A, Yoneda A, Hirokawa M. A case of small intestinal GIST with intraperitoneal hemorrhage (in Japanese). *Rinsho Hoshasen* 2007; **52**: 820-824
  - 14 **Emory TS**, Sobin LH, Lukes L, Lee DH, O'Leary TJ. Prognosis of gastrointestinal smooth-muscle (stromal) tumors: dependence on anatomic site. *Am J Surg Pathol* 1999; **23**: 82-87
  - 15 **Miettinen M**, Sarlomo-Rikala M, Lasota J. Gastrointestinal stromal tumours. *Ann Chir Gynaecol* 1998; **87**: 278-281
  - 16 **Franquemont DW**. Differentiation and risk assessment of gastrointestinal stromal tumors. *Am J Clin Pathol* 1995; **103**: 41-47
  - 17 **Levy AD**, Remotti HE, Thompson WM, Sobin LH, Miettinen M. Anorectal gastrointestinal stromal tumors: CT and MR imaging features with clinical and pathologic correlation. *AJR Am J Roentgenol* 2003; **180**: 1607-1612
  - 18 **Kim HC**, Lee JM, Kim SH, Kim KW, Lee M, Kim YJ, Han JK, Choi BI. Primary gastrointestinal stromal tumors in the omentum and mesentery: CT findings and pathologic correlations. *AJR Am J Roentgenol* 2004; **182**: 1463-1467
  - 19 **Lau S**, Tam KF, Kam CK, Lui CY, Siu CW, Lam HS, Mak KL. Imaging of gastrointestinal stromal tumour (GIST). *Clin Radiol* 2004; **59**: 487-498
  - 20 **Amano M**, Okuda T, Amano Y, Tajiri T, Kumazaki T. Magnetic resonance imaging of gastrointestinal stromal tumor in the abdomen and pelvis. *Clin Imaging* 2006; **30**: 127-131
  - 21 **Tateishi U**, Hasegawa T, Satake M, Moriyama N. Gastrointestinal stromal tumor. Correlation of computed tomography findings with tumor grade and mortality. *J Comput Assist Tomogr* 2003; **27**: 792-798
  - 22 **Choi H**, Charnsangavej C, de Castro Faria S, Tamm EP, Benjamin RS, Johnson MM, Macapinlac HA, Podoloff DA. CT evaluation of the response of gastrointestinal stromal tumors after imatinib mesylate treatment: a quantitative analysis correlated with FDG PET findings. *AJR Am J Roentgenol* 2004; **183**: 1619-1628

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## CASE REPORT

# A case of invasive hemolymphangioma of the pancreas

Yoshikazu Toyoki, Kenichi Hakamada, Shunji Narumi, Masaki Nara, Daisuke Kudoh, Keinosuke Ishido, Mutsuo Sasaki

Yoshikazu Toyoki, Kenichi Hakamada, Shunji Narumi, Masaki Nara, Daisuke Kudoh, Keinosuke Ishido, Mutsuo Sasaki, Department of Gastroenterological Surgery, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 0368652, Japan

Author contributions: Toyoki Y, Hakamada K, Narumi S, Nara M, Kudoh D, Ishido K and Sasaki M contributed in this case, images and wrote the paper.

Correspondence to: Yoshikazu Toyoki, Dr, Department of Gastroenterological Surgery, Hirosaki University School of Medicine, 5 Zaifu-cho, Hirosaki, Aomori 0368652, Japan. [ytoyoki@cc.hirosaki-u.ac.jp](mailto:ytoyoki@cc.hirosaki-u.ac.jp)

Telephone: +81-172-395079 Fax: +81-172-395080

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**Peer reviewers:** Massimo Conio, Professor, Department of Gastroenterology, Sanremo Hospital, Via Borea, 56, Sanremo 18038, Italy; Gisela Sparmann, MD, Division of Gastroenterology, Department of Internal Medicine, University of Rostock, Ernst-Heydemann-Str. 6, Rostock D-18057, Germany

Toyoki Y, Hakamada K, Narumi S, Nara M, Kudoh D, Ishido K, Sasaki M. A case of invasive hemolymphangioma of the pancreas. *World J Gastroenterol* 2008; 14(18): 2932-2934 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2932.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2932>

## Abstract

Hemolymphangioma of the pancreas is a very rare benign tumor. There were only five reports of this disease until March 2008. Herein, we report a case of hemolymphangioma of the pancreas with gastrointestinal bleeding due to duodenal invasion. A 53-year-old man had been admitted a referral hospital because of severe anemia due to gastrointestinal bleeding in December 2005. He was then transferred to our institute with a diagnosis of a tumor of the head of the pancreas with duodenal invasion in January 2006. No abnormalities were revealed except for anemia in laboratory data including CEA and CA19-9. Gastrointestinal endoscopy revealed bleeding at the duodenum. Computed tomography also demonstrated a heterogenous mass at the pancreatic head and suspected invasion to the duodenal wall. Ultrasonography showed a huge mass at the pancreatic head with a mixture of high and low echoic areas. Pylorus-preserving pancreatoduodenectomy was performed. The pancreatic tumor was soft and had invaded to the duodenum. The pathological diagnosis was a hemolymphangioma of the pancreas invaded to the duodenum. His postoperative course was uneventful and he was discharged on the 26th d after surgery. Hemolymphangioma of the pancreas is a very rare benign tumor. In a literature review until March 2008, we found five case reports. Major symptoms are abdominal pain and distension due to the enlarged tumor. However, we experienced a case of hemolymphangioma of the pancreas with gastrointestinal bleeding due to invasion to the duodenum. This disease is a very rare entity, but should be considered when patients have gastrointestinal bleeding.

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## INTRODUCTION

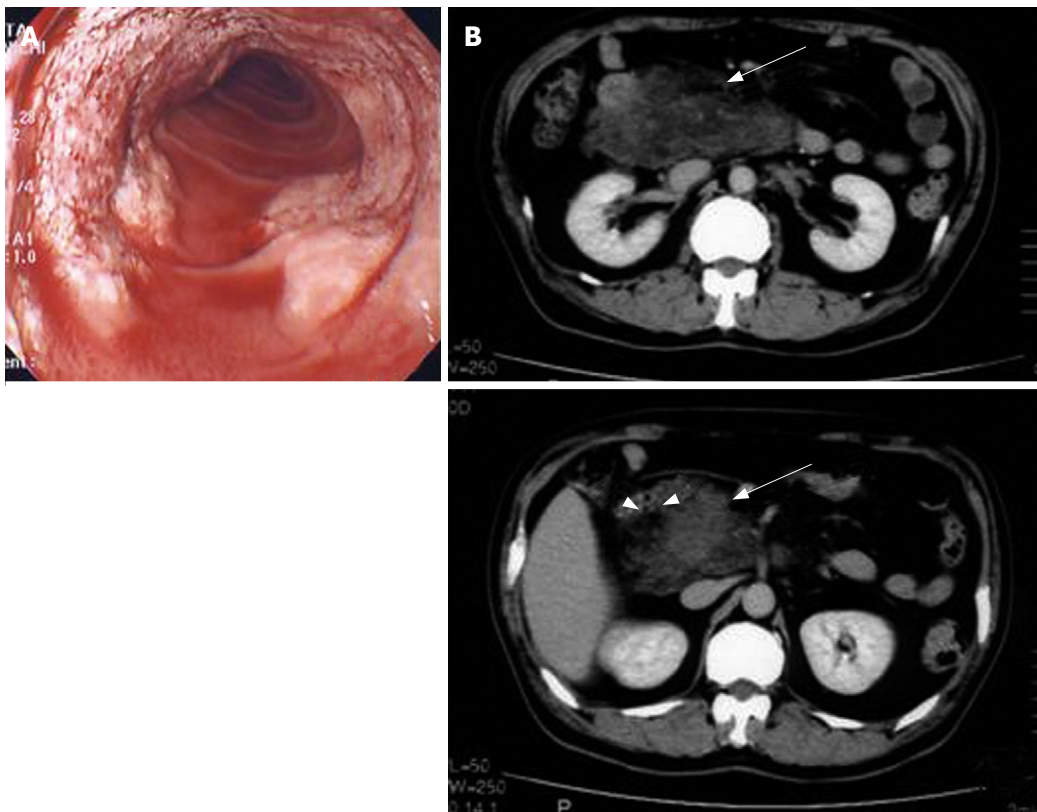
Hemolymphangioma of the pancreas is an uncommon disease and basically benign tumor. There were only five reports of this tumor of the pancreas until March 2008<sup>[1-5]</sup>. Vascular tumors of the pancreas are cystic tumors accounting for 0.1% of all pancreatic tumors<sup>[6]</sup>. The most frequent of these pancreatic tumors is lymphangioma.

Major symptoms in this hemolymphangioma are abdominal pain and distension associated with the enlarged tumor. This tumor is commonly a benign disease and has no invasion ability. Herein, we report a case of hemolymphangioma of the pancreas with gastrointestinal bleeding due to invasion to the duodenum.

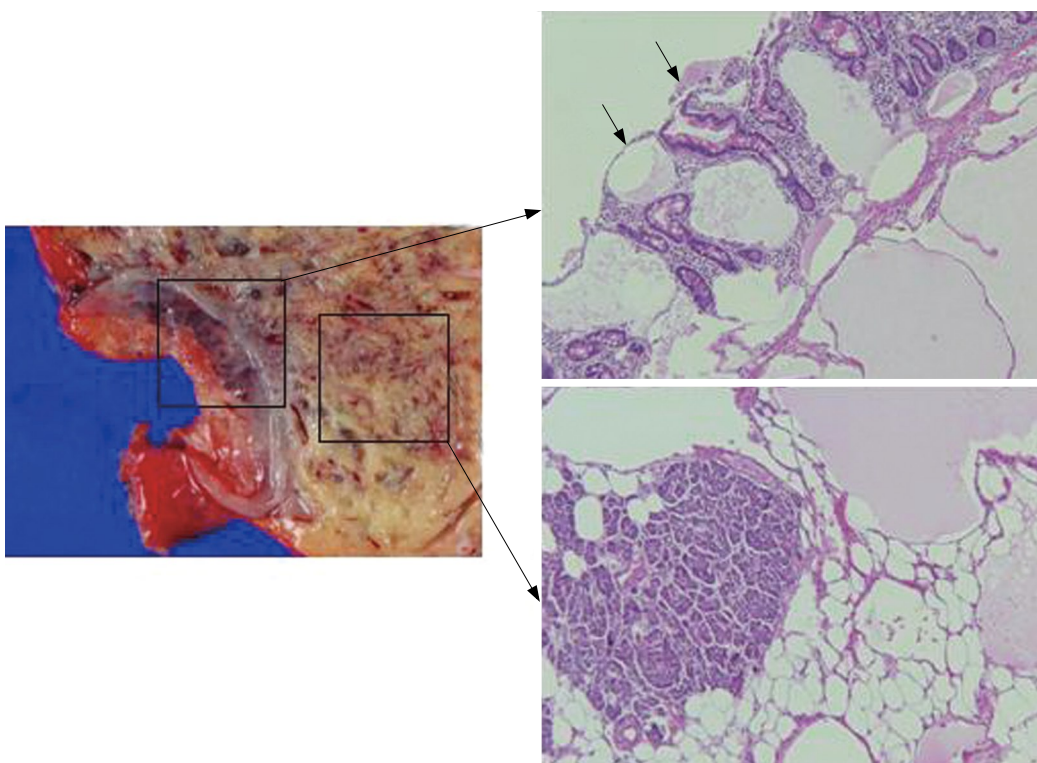
## CASE REPORT

A 53-year-old man was admitted to a referral hospital because of severe anemia due to gastrointestinal bleeding in December 2005. He was then transferred to our institute with a diagnosis of a tumor of the head of the pancreas with duodenal invasion in January 2006. No abnormalities were revealed except for anemia in laboratory data including tumor markers such as CEA and CA19-9. Gastrointestinal endoscopy revealed bleeding at the duodenum from a flat elevated lesion (Figure 1A). Computed tomography also demonstrated a heterogenous mass at the pancreatic head and suspected invasion to the duodenal wall (Figure 1B). Ultrasonography showed a huge mass at the pancreatic head with a mixture of high and low echoic areas. Endoscopic retrograde cholangio-pancreatography showed no abnormal findings in the bile duct and pancreatic duct. Pylorus-preserving pancreatoduodenectomy was





**Figure 1** A: Gastrointestinal endoscopy revealed bleeding at the duodenum; B: Computed tomography demonstrating a heterogenous mass (arrow) at the pancreatic head and suspected invasion to the duodenal wall (arrowhead).



**Figure 2** Histology consists of a benign soft tissue mass with lymphatic and blood vessels. Arrows show invasion to the duodenal wall.

performed. The pancreatic head tumor was soft and had invaded to the duodenum. Final pathological diagnosis of this tumor was a hemolymphangioma of the pancreas with invasion to the duodenum (Figure 2). His postoperative course was uneventful and he was discharged 26 d after surgery. He is currently enjoying normal life without signs of recurrence.

## DISCUSSION

Hemolymphangioma of the pancreas is an uncommon and benign tumor. In a literature review until March 2008 (PubMed), there were only five reports of this tumor occurring in the pancreas<sup>[1-5]</sup>. The first report by a French group, Couinaud *et al* reported a giant hemolymphangioma

of the pancreas in 1966<sup>[5]</sup>. Banchini *et al* considered this tumor a congenital malformation of the vascular system<sup>[2]</sup>. However, there are no reports that describe invasive features of this tumor.

Most frequent symptoms of this disease in these 5 case reports were abdominal pain and distension associated with enlarged tumor. Other symptoms such as vomiting and nausea are caused by occupied tumor<sup>[5]</sup>. This tumor is commonly a benign disease and has no invasion ability. In this case, the chief complaint was severe anemia caused by duodenal bleeding because of hemolymphangioma of the pancreas which invaded to the duodenum. This symptom is extremely rare. In generally, this disease is benign, but it is possible that this tumor invaded other organs like our case.

Despite its low frequency, this disease should be considered when gastrointestinal bleeding is seen. Surgery including local resection is a definitive modality. All cases in the literature had good postoperative courses as did our case. The risk of recurrence or metastasis seems very low, but careful follow-up is necessary. Herein, we reported a

case of hemolymphangioma of the pancreas head with gastrointestinal bleeding due to duodenal invasion.

## REFERENCES

- 1 **Balderramo DC**, Di Tada C, de Ditter AB, Mondino JC. Hemolymphangioma of the pancreas: case report and review of the literature. *Pancreas* 2003; **27**: 197-199
- 2 **Banchini E**, Bonati L, Villani LG. [A case of hemolymphangioma of the pancreas] *Minerva Chir* 1987; **42**: 807-813
- 3 **Montete P**, Marmuse JP, Claude R, Charleux H. [Hemolymphangioma of the pancreas] *J Chir (Paris)* 1985; **122**: 659-663
- 4 **Couinaud C**, Jouan, Prot, Chalut, Favre, Schneiter. [A rare tumor of the head of the pancreas. (Hemolymphangioma weighing 1,500 kg.)] *Presse Med* 1967; **75**: 1955-1956
- 5 **Couinaud, Jouan**, Prot, Chalut, Schneiter. [Hemolymphangioma of the head of the pancreas] *Mem Acad Chir (Paris)* 1966; **92**: 152-155
- 6 **Le Borgne J**, de Calan L, Partensky C. Cystadenomas and cystadenocarcinomas of the pancreas: a multiinstitutional retrospective study of 398 cases. French Surgical Association. *Ann Surg* 1999; **230**: 152-161

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# Hepatic angiosarcoma manifested as recurrent hemoperitoneum

Seung-Woo Lee, Chun-Young Song, Young-Hwa Gi, Sang-Beom Kang, Yon-Soo Kim, Soon-Woo Nam, Dong-Soo Lee, Jong-Ok Kim

Seung-Woo Lee, Chun-Young Song, Young-Hwa Gi, Sang-Beom Kang, Yon-Soo Kim, Soon-Woo Nam, Dong-Soo Lee, Division of Gastroenterology and Hepatology, Departments of Internal Medicine, The Catholic University of Korea, College of Medicine, Seoul 301-723, Korea

Jong-Ok Kim, Department of pathology, The Catholic University of Korea, College of Medicine, Seoul 301-723, Korea

**Author contributions:** Lee SW mainly wrote the manuscript; Lee DS revised the manuscript; Song CY, Gi YH, Kang SB, Kim YS and Nam SW were involved in the care of the patient; Kim JO is the pathologist who studied the specimen.

**Correspondence to:** Dr. Dong-Soo Lee, Division of Gastroenterology and Hepatology, Department of Internal Medicine, Dae Jeon St. Mary's Hospital, The Catholic University of Korea, 520-2 Dae Heung Dong, Joong Gu, Dae Jeon, Seoul 301-723, Korea. [cmcdj9502@catholic.ac.kr](mailto:cmcdj9502@catholic.ac.kr)

Telephone: +82-42-2209825 Fax: +82-42-2526807

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## Abstract

Angiosarcoma is a rare tumor that account for less than 1% of all sarcomas. Although hepatic angiosarcoma usually presents with unspecific symptoms, it rapidly progresses and has a high mortality. We report a rare case of primary hepatic angiosarcoma manifested as recurrent hemoperitoneum.

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**Key words:** Angiosarcoma; Hemoperitoneum; Primary hepatic tumor

**Peer reviewer:** Luis Rodrigo, Professor, Gastroenterology Service, Hospital Universitario Central de Asturias, c/Celestino Villamil, s.n., Oviedo 33.006, Spain

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## INTRODUCTION

Angiosarcoma is a rare tumor that account for less than 1% of all sarcomas<sup>[1]</sup>, and only 2% of all primary tumors of liver<sup>[2]</sup>. Although it is rarely observed, it is the third most common primary malignant liver tumor<sup>[3]</sup>. Clinical diagnosis

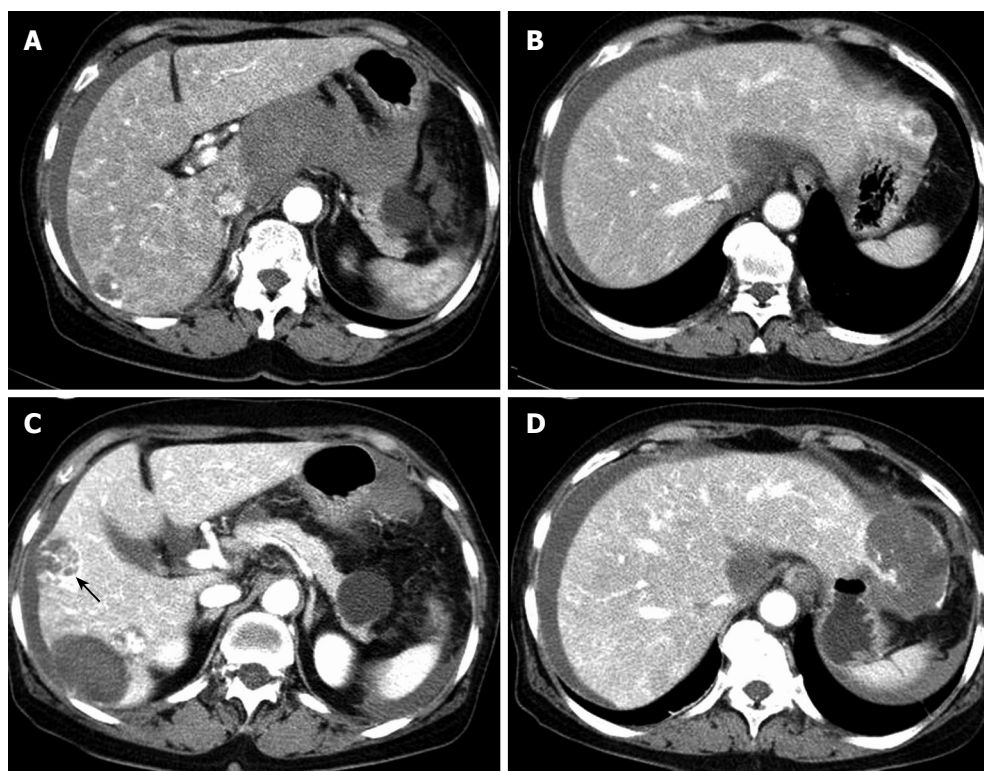
is usually difficult because the symptoms and signs are unspecific, and tumors are difficult to differentiate from other hepatic tumors radiologically<sup>[4]</sup>. Moreover, although tissue is required for diagnosis, the rate of diagnosis by needle biopsy is not high<sup>[5]</sup>. Angiosarcoma progresses rapidly and has a poor prognosis<sup>[6]</sup>. Treatment guidelines have not been issued as yet, however, we think it important to diagnose angiosarcoma as early as possible. Herein, we report a case of primary hepatic angiosarcoma, which was manifested as recurrent hemoperitoneum.

## CASE REPORT

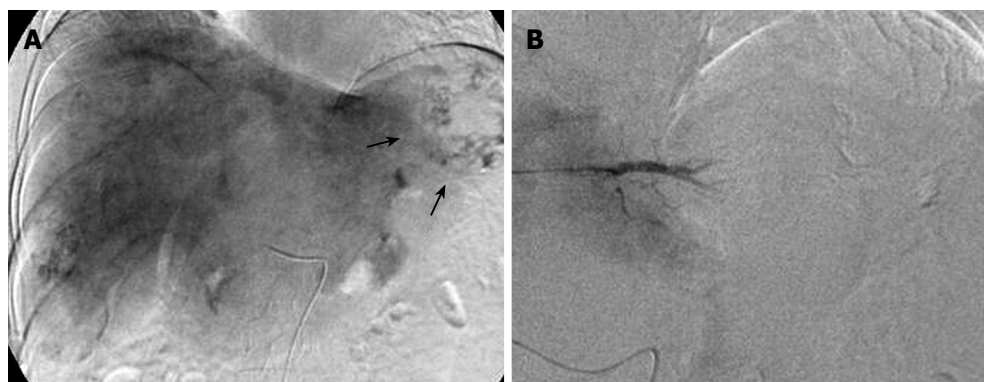
A 73-year-old woman presented with epigastric pain and right shoulder pain. She had suffered from hypertension for several years, but no other past medical histories. On physical examination, the patient appeared with acutely ill and had right upper quadrant pain and tenderness. A complete blood count and blood chemistry were: hemoglobin 11.3 g/dL, hematocrit 34.2%, white blood count 18400/mm<sup>3</sup> (segmented neutrophil 86.6%), platelet 284000/mm<sup>3</sup>, total protein/albumin 5.7/3.8 g/dL, total bilirubin 0.7 mg/dL, direct bilirubin 0.1 mg/dL, AST 24 IU/L, ALT 36 U/L, ALP 49 IU/L, G-GTP 59 IU/L, PT 13.2 s, and amylase 60. Tumor markers such as alpha fetoprotein, CEA, CA19-9 and CA125 were all within normal limit.

Computed tomography (CT) of the abdomen showed multiple hypervascular masses in the liver and a large amount of hematoma in lesser sac. We thought that a mass in the caudate lobe had ruptured into the abdominal cavity and had caused hemoperitoneum (Figure 1A and B). Transarterial embolization was performed. Her condition then stabilized and she was discharged. However, one month later she revisited the emergency room with identical symptoms. Abdominal CT revealed rupture of two hypervascular mass in the right hepatic lobe. After the 2nd transarterial embolization, she became destabilized. A sono-guided needle biopsy was performed but we failed to confirm a specific diagnosis. Five days after discharge, she visited the emergency room again with the same symptoms. Abdominal CT showed interval increased size of low attenuated lesions with peripheral nodular enhancement in the left lateral and right lobe. One of them shows septum-like and peripheral enhancement within the mass (Figure 1C and D). Transarterial embolization was performed repeatedly (Figure 2) but her vital signs were unstable and bleeding of the hepatic mass continued. On the 8th d after the 3rd embolization, we decided to resect





**Figure 1** Initial abdominal computed tomography images. **A** and **B**: A large amount of hematoma in lesser sac and a lowly attenuated nodular lesion with peripheral globular enhancement in the left lobe. Abdominal CT images after 1 mo; **C** and **D**: increased size of lowly attenuated lesions in the right lobe and the left lateral lobe. One showing septum-like appearance and peripheral enhancement within the mass (black arrow).



**Figure 2** Transarterial embolization images. **A**: A large tumor stain in the lateral segment of left lobe (arrows); **B**: Post-embolization angiogram showing no remained tumor stain.

the left hepatic lobe, which showed active bleeding on abdominal CT scan. In operation, there was much blood in the abdominal cavity. The rupture of hepatic mass at segment 2 and 3 was noted. So we decided to resect the left lateral segment which contained the bleeding mass. However, the intra-abdominal bleeding continued and she finally died of disseminated intravascular coagulation. The postoperative surgical specimen showed a great amount of atypical cells forming solid sheets filled with red blood cells (RBCs), which is suggestive of hepatic angiosarcoma. Immunohistochemical staining for CD31 and CD34 showed diffuse strong positive expression (Figure 3).

## DISCUSSION

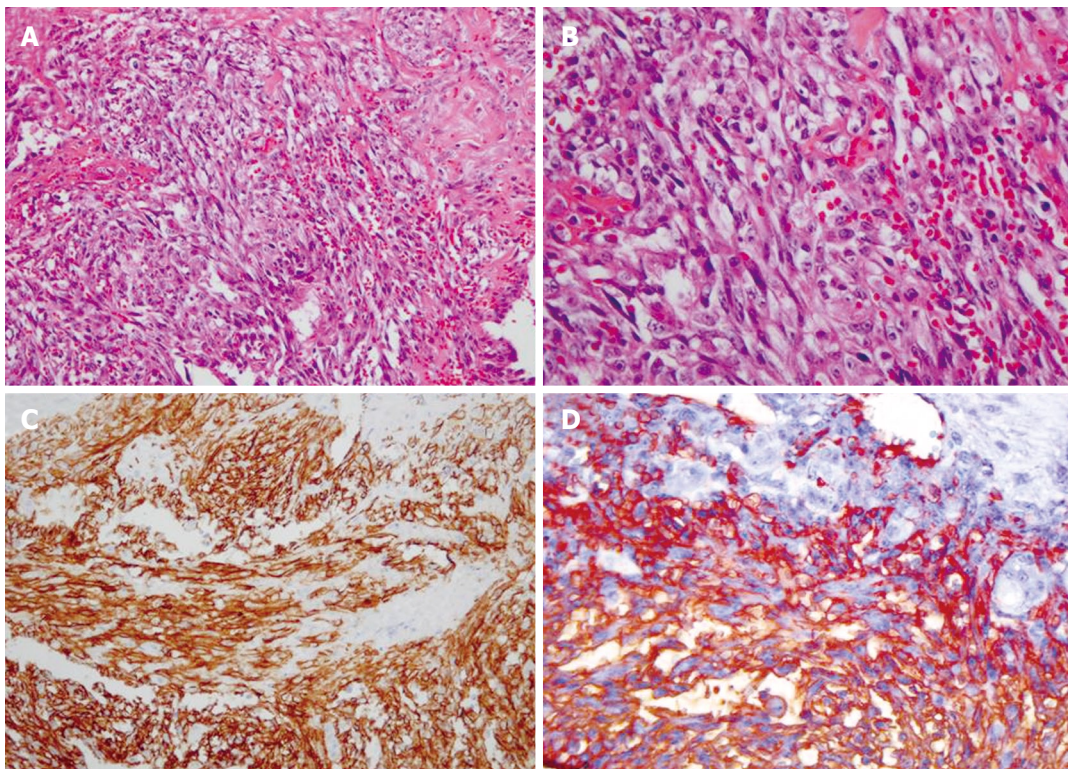
Angiosarcoma (AS) can originate from the endothelia of lymphatics or blood vessels and is a rarely encountered type of soft tissue sarcoma<sup>[7]</sup>. Angiosarcoma has a predilection for cutaneous sites in the head and neck region of elderly male patients<sup>[8]</sup>. Moreover, despite its

rarity, hepatic angiosarcoma is the third most common primary malignant tumor of the liver<sup>[3]</sup>. Environmental toxins are suggested to be the etiology of AS, for example, the industrial intermediates thorotrast and vinyl chloride and agricultural insecticides are associated with AS<sup>[9,10]</sup>, as do the anabolic steroids and synthetic estrogens<sup>[11,12]</sup>. However, the majority of hepatic angiosarcomas are not related to the above agents.

Angiosarcoma usually presents with nonspecific symptoms, such as abdominal pain, weight loss and general weakness<sup>[2]</sup>. Its physical findings are ascites, hepatomegaly and jaundice. In this case, the main symptoms were abdominal and right shoulder pain.

Primary hepatic angiosarcoma is difficult to differentiate from other vascular tumors of the liver using radiographic techniques. On ultrasound, single or multiple masses are demonstrated with different echotextures due to different levels of necrosis and hemorrhage<sup>[13]</sup>. On nonenhanced helical CT, tumors present as multiple hypodense masses or as a large solitary hypodense mass. In enhanced view,





**Figure 3** Pathologic findings. **A** and **B**: Atypical cells forming solid sheets filled with RBCs (HE stain,  $\times 200$ ,  $\times 400$ , respectively); **C** and **D**: Immunohistochemical staining for CD31 and CD34 showing diffuse strong positive expression ( $\times 200$ ,  $\times 400$ , respectively).

some of the masses are hyperattenuated while others remain hypoattenuated<sup>[14]</sup>. Within hyperattenuated mass areas, an area of hypoattenuation may be seen, which reflect areas of necrosis and/or old hemorrhage. The delayed scans of some lesions show progressive enhancement over minutes due to the vascular nature of the tumor<sup>[15]</sup>. In this case, multiple heterogeneous and lowly attenuated lesions with septum like structures were observed on CT scans. Arteriportal shunting is not seen in hemangiomas, and if it is present, it favors a diagnosis of angiosarcoma<sup>[16]</sup>. The magnetic resonance imaging findings of angiosarcomas and hemangiomas are also similar. Liver biopsies in angiosarcoma patients are accompanied by risks of morbidity and mortality, because the vascular nature of the tumor and its tendency to hemorrhage make the percutaneous biopsy dangerous<sup>[2]</sup>. Microscopic features include sinusoidal and solid patterns. The sinusoidal pattern is characterized by the proliferation of single or multilayered tumor cell along sinusoids which show sinusoidal dilation, atrophy of liver cell cords. The solid pattern is composed of spindle and polyhedral cells, which form a solid tumor nest without significant vascular spaces<sup>[17]</sup>. The immunohistochemical staining for CD31 and factor VIII related antigen can aid diagnosis<sup>[18]</sup>.

The treatment of hepatic angiosarcoma has not been defined because of its rarity and associated mortality. The median survival is 6 mo if not treated and only 3% of patients live longer than 2 years<sup>[2]</sup>. Hepatic resection is indicated when the tumor is localized to one lobe and the remainder of the liver is relatively normal<sup>[19]</sup>. However, patients meeting these criteria are extremely rare. Timaran *et al.*<sup>[20]</sup> reported a case of hepatic angiosarcoma with a long postoperative survival after complete surgical resection of the tumor.

Radiation therapy to the liver has been used to palliate liver metastases<sup>[21]</sup>, but soft tissue sarcoma is relatively radioresistant and the dosage required to treat sarcoma is higher than the tolerable dose<sup>[22]</sup>.

Chemotherapy plays a role in the treatment of soft tissue sarcomas. The drugs that show some activity are: alkylating agents, vincristine, actinomycin, adriamycin, and DTIC (Dimethyl triazeno imidazole carboxamide)<sup>[23]</sup>. Gershon *et al.*<sup>[2]</sup> reported that adriamycin produced two definite responses, as documented by angiography. Dannaer *et al.*<sup>[24]</sup> reported that chemotherapy produced an objective improvement in three of four patients and stabilized the disease in one patient. Transarterial chemoembolization is useful for preventing acute arterial bleeding from the liver. Catheter directed embolization allows planning and well structured surgery without the need of emergency liver surgery, which carries high risks of morbidity and lethality<sup>[25]</sup>. In this case, the patient underwent hepatic resection after transarterial chemoembolization due to acute arterial bleeding in the liver. Liver transplantation has been described in a patient with an unresectable primary hepatic tumor. However, no patient has been reported to survive for longer than 28 mo because of the high recurrence rate of hepatic angiosarcoma<sup>[26]</sup>.

When a ruptured mass causes the spillage of tumor cells into the peritoneal cavity and diffuse sarcomatosis in the peritoneum, the angiosarcomatous lesions subsequently formed induce diffuse and severe hemorrhage, thus appearing to be a leading cause of death<sup>[25]</sup>.

The prognosis of patients with hepatic angiosarcoma is dismal<sup>[6]</sup>. Although hepatic angiosarcoma is usually found to be unresectable at the time of diagnosis, early diagnosis and complete resection of the tumor may reduce the mortality associated with this disease. In conclusion,

we advise that the possibility of angiosarcoma should be considered when a bleeding hepatic mass is encountered.

## REFERENCES

- 1 **Bardwil JM**, Mocega EE, Butler JJ, Russin DJ. Angiosarcomas of the head and neck region. *Am J Surg* 1968; **116**: 548-553
- 2 **Locker GY**, Doroshow JH, Zwelling LA, Chabner BA. The clinical features of hepatic angiosarcoma: a report of four cases and a review of the English literature. *Medicine* (Baltimore) 1979; **58**: 48-64
- 3 **Wanebo HJ**, Falkson C, Order SE. Cancer of the hepatic biliary system. In: DeVita VT Jr, Hellman S, Rosenberg SA, editors. *Cancer: Principles and Practice of Oncology*, 3rd ed. Philadelphia: JB Lippincott, 1989: 836-868
- 4 **Molina E**, Hernandez A. Clinical manifestations of primary hepatic angiosarcoma. *Dig Dis Sci* 2003; **48**: 677-682
- 5 **Kew MC**, Dos Santos HA, Sherlock S. Diagnosis of primary cancer of the liver. *Br Med J* 1971; **4**: 408-411
- 6 **Falk H**, Herbert J, Crowley S, Ishak KG, Thomas LB, Popper H, Caldwell GG. Epidemiology of hepatic angiosarcoma in the United States: 1964-1974. *Environ Health Perspect* 1981; **41**: 107-113
- 7 **Naka N**, Ohsawa M, Tomita Y, Kanno H, Uchida A, Aozasa K. Angiosarcoma in Japan. A review of 99 cases. *Cancer* 1995; **75**: 989-996
- 8 **Pawlik TM**, Paulino AF, McGinn CJ, Baker LH, Cohen DS, Morris JS, Rees R, Sondak VK. Cutaneous angiosarcoma of the scalp: a multidisciplinary approach. *Cancer* 2003; **98**: 1716-1726
- 9 **Mazanet R**, Antman KH. Sarcomas of soft tissue and bone. *Cancer* 1991; **68**: 463-473
- 10 **Popper H**, Thomas LB, Telles NC, Falk H, Selikoff IJ. Development of hepatic angiosarcoma in man induced by vinyl chloride, thorotrast, and arsenic. Comparison with cases of unknown etiology. *Am J Pathol* 1978; **92**: 349-369
- 11 **Falk H**, Thomas LB, Popper H, Ishak KG. Hepatic angiosarcoma associated with androgenic-anabolic steroids. *Lancet* 1979; **2**: 1120-1123
- 12 **Hoch-Ligeti C**. Angiosarcoma of the liver associated with diethylstilbestrol. *JAMA* 1978; **240**: 1510-1511
- 13 **Buetow PC**, Buck JL, Ros PR, Goodman ZD. Malignant vascular tumors of the liver: radiologic-pathologic correlation. *Radiographics* 1994; **14**: 153-166; quiz 167-168
- 14 **Peterson MS**, Baron RL, Rankin SC. Hepatic angiosarcoma: findings on multiphasic contrast-enhanced helical CT do not mimic hepatic hemangioma. *AJR Am J Roentgenol* 2000; **175**: 165-170
- 15 **White PG**, Adams H, Smith PM. The computed tomographic appearances of angiosarcoma of the liver. *Clin Radiol* 1993; **48**: 321-325
- 16 **Ohmoto K**, Hirokawa M, Takesue M, Yamamoto S. Hepatic angiosarcoma with early central enhancement and arterioportal shunt on dynamic CT. *Hepatogastroenterology* 2000; **47**: 1717-1718
- 17 **Kojiro M**, Nakashima T, Ito Y, Ikezaki H, Mori T, Kido C. Thorium dioxide-related angiosarcoma of the liver. Pathomorphologic study of 29 autopsy cases. *Arch Pathol Lab Med* 1985; **109**: 853-857
- 18 **Saleh HA**, Tao LC. Hepatic angiosarcoma: aspiration biopsy cytology and immunocytochemical contribution. *Diagn Cytopathol* 1998; **18**: 208-211
- 19 **Adson MA**, Beart RW Jr. Elective hepatic resections. *Surg Clin North Am* 1977; **57**: 339-360
- 20 **Timaran CH**, Grandas OH, Bell JL. Hepatic angiosarcoma: long-term survival after complete surgical removal. *Am Surg* 2000; **66**: 1153-1157
- 21 **Turek-Maischeider M**, Kazem I. Palliative irradiation for liver metastases. *JAMA* 1975; **232**: 625-628
- 22 **McNeer GP**, Cantin J, Chu F, Nickson JJ. Effectiveness of radiation therapy in the management of sarcoma of the soft somatic tissues. *Cancer* 1968; **22**: 391-397
- 23 **Talley RW**. Chemotherapy of soft tissue sarcomas. *Proc Natl Cancer Conference* 1973; **7**: 889
- 24 **Dannaher CL**, Tamburro CH, Yam LT. Chemotherapy of vinyl chloride-associated hepatic angiosarcoma. *Cancer* 1981; **47**: 466-469
- 25 **Leowardi C**, Hormann Y, Hinz U, Wente MN, Hallscheidt P, Flechtenmacher C, Buchler MW, Friess H, Schwarzbach MH. Ruptured angiosarcoma of the liver treated by emergency catheter-directed embolization. *World J Gastroenterol* 2006; **12**: 804-808
- 26 **O'Grady JG**. Treatment options for other hepatic malignancies. *Liver Transpl* 2000; **6**: S23-S29

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# Chronic ulcerative gastroduodenitis as a first gastrointestinal manifestation of Hermansky-Pudlak syndrome in a 10-year-old child

Anselm Chi-Wai Lee, Kin-Hung Poon, Wing-Hong Lo, Lap-Gate Wong

Anselm Chi-Wai Lee, Kin-Hung Poon, Department of Pediatrics & Adolescent Medicine, Tuen Mun Hospital, New Territories, Hong Kong 999077, China

Wing-Hong Lo, Lap-Gate Wong, Department of Clinical Pathology, Tuen Mun Hospital, New Territories, Hong Kong 999077, China

Author contributions: Lee ACW conceptualized the report and wrote the paper; Poon KH, Lo WH and Wong LG prepared the illustrations and wrote the paper.

Correspondence to: Dr. Anselm Chi-Wai Lee, Children's Hematology & Cancer Centre, East Shore Medical Centre #05-01, 319 Joo Chiat Place, 427989, Singapore. [aclee@graduate.hku.hk](mailto:aclee@graduate.hku.hk)  
Telephone: +65-63408610 Fax: +65-63440117

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## INTRODUCTION

Hermansky-Pudlak syndrome (HPS) is a rare form of congenital platelet disorder, affecting 1 in 500 000-1 000 000 populations of different ethnicity<sup>[1]</sup>. A high prevalence is seen in northern Puerto Rico where 1 per 1800 people is affected. It is inherited as an autosomal recessive disorder of intracytoplasmic organelle-specific protein biosynthesis and trafficking<sup>[2]</sup>. Eight subtypes with known genetic mutations have been described<sup>[1,3-7]</sup>. The disease is characterized by oculocutaneous albinism and bleeding diathesis due to platelet storage pool deficiency. Colitis is an important bowel complication in patients with HPS<sup>[8]</sup>. Involvement of the upper gastrointestinal tract has not been reported and can be potentially confused with thrombocytopathic bleeding. Our successful diagnosis and management under such circumstances is reported below.

## CASE REPORT

The patient first presented at 5 years of age with a longstanding history of easy bruising and recurrent epistaxis. Both parents were of Chinese origin and unrelated. There was no history of bleeding into muscles, joints or the gastrointestinal tract. He had occasional mild eczema but no recurrent infections. Initial physical examination was remarkable only for multiple bruises of varying ages on his legs. The first laboratory investigations revealed mild thrombocytopenia (platelet counts, 118 and  $84 \times 10^9/L$  on two occasions) with normal platelet volume and morphology under light microscopy. A microcytic, hypochromic anemia (Hb, 10.4 g/dL) due to  $\beta$ -thalassaemia trait was also found. The coagulation screen was normal.

Four months later, he had a scalp laceration after a minor fall at home. The wound was sutured but he was re-admitted a few hours later because of progressive wound swelling. An excessive scalp hematoma was found but intracranial bleeding was excluded on computer tomography (CT) scan. The total blood counts were remarkable with Hb 9.3 g/dL and platelets  $91 \times 10^9/L$ . A prolonged bleeding time of 13 min was then documented. Platelet function studies showed a diminished response

## Abstract

A 10-year-old Chinese boy who had a history of congenital thrombocytopathy presented with severe iron deficiency anemia secondary to chronic gastric inflammation and duodenal ulcerations. Subtle oculocutaneous albinism led to the finding of diminished dense bodies in the platelets under electron microscopy, hence the diagnosis of Hermansky-Pudlak syndrome (HPS). Biopsies from the stomach and duodenum revealed a lymphocytic infiltration in the submucosa, but *H. pylori* infection was absent. The gastroduodenitis responded to the treatment with omeprazole while iron deficiency anemia was corrected by oral iron therapy. HPS is a rare cause of congenital bleeding disorder with multisystemic manifestations. Upper gastrointestinal involvement is rare and should be distinguished from a mere manifestation of the bleeding diathesis.

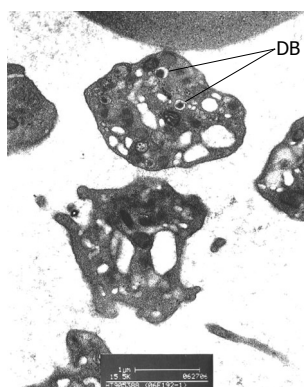
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**Key words:** Albinism; Duodenal ulceration; Hermansky-Pudlak syndrome; Inflammatory bowel disease; Thrombocytopathy

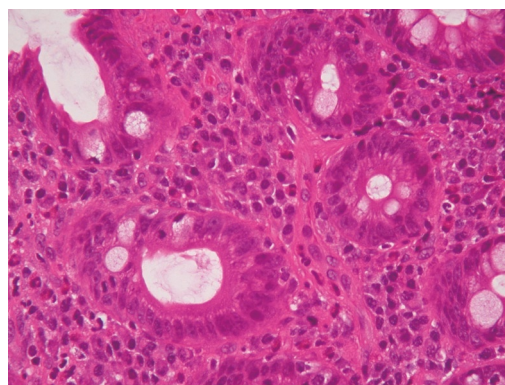
**Peer reviewer:** Limas Kupcinskas, Gastroenterology of Kaunas University of Medicine, Mickeviciaus 9, Kaunas LT 44307, Lithuania

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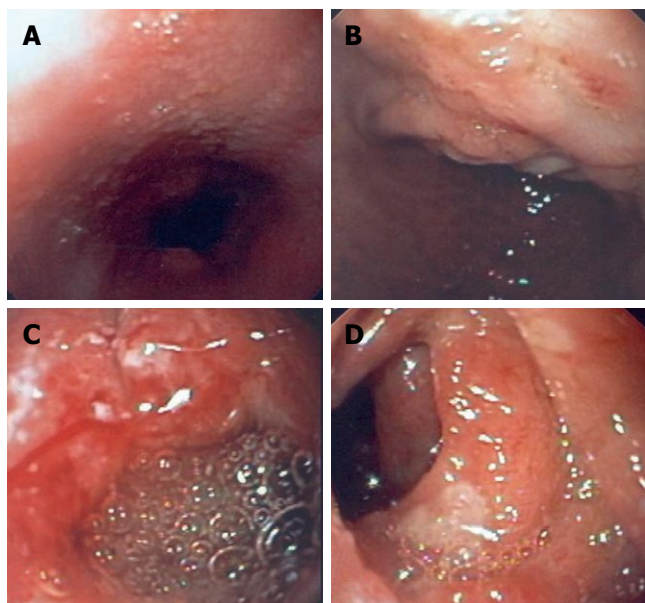




**Figure 1** Electron photomicrograph of the patient's platelets, showing a diminished number (normal, 4-8) of dense bodies (DB) in one, and absence of dense bodies in the adjacent platelets.



**Figure 3** Histologic section of duodenal mucosa showing intact villous architecture with a moderate inflammatory infiltrate of lymphocytes, plasma cells and eosinophils in lamina propria.



**Figure 2** Pictures obtained at esophagogastroduodenoscopy. **A:** Esophagus appeared normal except for mild nodularis near gastroesophageal junction; **B:** Stomach was covered with a coffee-ground substance and a patch of mucosal irregularity at proximal lesser curve was seen; **C:** A solitary ulcer of 2 cm in diameter with a rolled margin was found at anterior wall of the first part of duodenum; **D:** Multiple small, flat and oval aphthoid ulcers were found in the second part of duodenum.

to collagen and no response to arachidonic acid while response to adenosine diphosphate (ADP), adrenaline, ristocetin and calcium ionophore was normal. The findings were suggestive, but not typical, of storage pool deficiency.

The child has had only mild bleeding manifestations since then with cutaneous bruises and epistaxis. None of them required any hemostatic control other than local pressure. His skin color was paler compared with other children but the color of his hair and iris was not noticeably different. His mother recalled that he had once developed severe sunburn with skin blistering after having fun on the beach and he would never tan under the sun.

He presented at 10 years of age again because of a 3-mo history of recurrent severe epigastric pain, nausea and vomiting. There was no diarrhea, overt gastrointestinal bleeding or joint pain. He had lost 2.2 kg over the course of the illness. The blood counts showed Hb 5.9 g/dL and platelet  $237 \times 10^9/L$ . Serum biochemistries including hepatic transaminases and amylase were normal except

for diminished ferritin level (30 pmol/L, normal 67-896). Tests for C-reactive protein, erythrocyte sedimentation rate (ESR), anti-nuclear antibody, serum IgE, C3 and C4 were all normal. Bacterial, viral and parasitic studies of the stool were negative, while occult blood was found.  $C^{13}$ -urea breath test was negative. An ophthalmological consultation revealed diminished pigmentations in the iris and retina. Electron microscopy showed that dense bodies were diminished in some platelets and were absent in other platelets (Figure 1). The diagnosis of HPS was therefore made.

On esophagogastroduodenoscopy, scattered areas of inflammatory changes were noted in the esophagus and stomach. Mucosal ulcerations were noted in the first and second parts of the duodenum (Figure 2A-D). Biopsies taken from the stomach and the duodenum showed a mixed chronic infiltration with lymphocytes, plasma cells and some eosinophils in the lamina propria consistent with inactive chronic gastritis and non-specific duodenitis, respectively (Figure 3). *H. pylori*-like organism and intracellular ceroid-lipofuscin inclusions were not found.

The child was treated with omeprazole 20 mg (0.8 mg/kg) daily and iron (elemental iron 6 mg/kg per day) orally. He also received counseling on protection from solar damage with respect to his visual and dermatological vulnerabilities. Reassessment 5 wk later showed complete remission of epigastric pain, near total correction of anemia, complete ulcer healing and marked improvement in the gastric and duodenal inflammation on endoscopic and histologic examination. Omeprazole was given for 4 mo until his premorbid body weight and hemoglobin levels were restored. A normal fasting serum level of gastrin (16 pmol/L, normal < 55) obtained after cessation of omeprazole excluded hypergastrinemia.

However, two weeks after stopping omeprazole, the clinical symptoms and endoscopic signs of gastroduodenitis recurred. Although a full gastrointestinal workup was declined, the child responded again to omeprazole treatment, which would be given for long term acid suppression.

## DISCUSSION

Although the combination of oculocutaneous albinism



and platelet function disorder has only been described in HPS<sup>[9,10]</sup>, the definitive diagnosis was delayed in this patient. This was in part due to the subtleness of the hypopigmentation and the fact that the condition has not been previously reported in Hong Kong. Nevertheless, the emergence of an unusual complication during the course of the child's illness prompted to a re-evaluation, thus establishing the diagnosis of HPS. It is interesting that our case experienced marked upper gastrointestinal inflammation in association with his rare inherited disorder.

In addition to bleeding tendency, visual and cutaneous problems, patients with HPS may be complicated by diseases of the lungs, the gastrointestinal tract, the heart, and the kidneys<sup>[1,11]</sup>. Chronic neutropenia with recurrent infections have been reported in a few children with type 2 HPS<sup>[12]</sup>. Progressive pulmonary fibrosis, appearing during the fourth decade of life, is the most important cause of early mortality among patients with HPS<sup>[1,13]</sup>. It primarily affects individuals with types 1 and 4 HPS.

Gastrointestinal complications are an important manifestation in patients with HPS. Granulomatous colitis is the most frequently reported feature and may affect 10%-20% of patients with HPS<sup>[1,14]</sup>. The mean age at presentation is 15 years and about 15% of patients may present with severe inflammatory lesions. Granulomatous colitis may resemble Crohn's disease both clinically and histologically. The clinical features include signs and symptoms of inflammatory bowel disease like abdominal pain, bloody diarrhea, symptoms of bowel constriction, and perianal fistula<sup>[15]</sup>. Microscopically, the bowel shows areas of hyperemia and denuded epithelium with non-necrotizing granulomas. Ceroid deposits may be identified in the intestinal lesions but they may be absent even with severe disease<sup>[8]</sup>. Management of granulomatous colitis in HPS is also similar to Crohn's disease and consists of immunomodulatory therapy. Total colectomy has been required in a few patients with severe illness<sup>[1,8,14]</sup>.

Extra-colonic involvement of the gastrointestinal tract in HPS is unusual<sup>[8]</sup> and the clinical features and specific management have not been characterized. In the present case, the patient's clinical manifestations were similar to peptic ulceration except that *H pylori* infection was absent and rapid relapse followed cessation of therapy with proton-pump inhibitor. Treatment with a proton-pump inhibitor was found to be satisfactory, but the optimal duration of treatment was not known.

In summary, HPS is an unusual cause but an important differential diagnosis of upper gastrointestinal inflammatory disease in children. Endoscopic evaluation

is a safe and helpful adjunct in the diagnosis, but the optimal long-term management and outcomes of the gastrointestinal disease are unknown.

## REFERENCES

- 1 **Gahl WA.** Gene reviews: Hermansky-Pudlak syndrome. Last update on November 27, 2007. Available from: URL: <http://www.geneclinics.org/profiles/hps/details.html>
- 2 **Di Pietro SM, Dell'Angelica EC.** The cell biology of Hermansky-Pudlak syndrome: recent advances. *Traffic* 2005; **6**: 525-533
- 3 **Hermos CR, Huizing M, Kaiser-Kupfer MI, Gahl WA.** Hermansky-Pudlak syndrome type 1: gene organization, novel mutations, and clinical-molecular review of non-Puerto Rican cases. *Hum Mutat* 2002; **20**: 482
- 4 **Anderson PD, Huizing M, Claassen DA, White J, Gahl WA.** Hermansky-Pudlak syndrome type 4 (HPS-4): clinical and molecular characteristics. *Hum Genet* 2003; **113**: 10-17
- 5 **Huizing M, Hess R, Dorward H, Claassen DA, Helip-Wooley A, Kleta R, Kaiser-Kupfer MI, White JG, Gahl WA.** Cellular, molecular and clinical characterization of patients with Hermansky-Pudlak syndrome type 5. *Traffic* 2004; **5**: 711-722
- 6 **Li W, Zhang Q, Oiso N, Novak EK, Gautam R, O'Brien EP, Tinsley CL, Blake DJ, Spritz RA, Copeland NG, Jenkins NA, Amato D, Roe BA, Starcevic M, Dell'Angelica EC, Elliott RW, Mishra V, Kingsmore SF, Paylor RE, Swank RT.** Hermansky-Pudlak syndrome type 7 (HPS-7) results from mutant dysbindin, a member of the biogenesis of lysosome-related organelles complex 1 (BLOC-1). *Nat Genet* 2003; **35**: 84-89
- 7 **Morgan NV, Pasha S, Johnson CA, Ainsworth JR, Eady RA, Dawood B, McKeown C, Trembath RC, Wilde J, Watson SP, Maher ER.** A germline mutation in BLOC1S3/reduced pigmentation causes a novel variant of Hermansky-Pudlak syndrome (HPS8). *Am J Hum Genet* 2006; **78**: 160-166
- 8 **de Leusse A, Dupuy E, Huizing M, Danel C, Meyer G, Jian R, Marteau P.** Ileal Crohn's disease in a woman with Hermansky-Pudlak syndrome. *Gastroenterol Clin Biol* 2006; **30**: 621-624
- 9 **Hermansky F, Pudlak P.** Albinism associated with hemorrhagic diathesis and unusual pigmented reticular cells in the bone marrow: report of two cases with histochemical studies. *Blood* 1959; **14**: 162-169
- 10 **Scheinfeld NS.** Syndromic albinism: a review of genetics and phenotypes. *Dermatol Online J* 2003; **9**: 5
- 11 **Wei ML.** Hermansky-Pudlak syndrome: a disease of protein trafficking and organelle function. *Pigment Cell Res* 2006; **19**: 19-42
- 12 **Huizing M, Gahl WA.** Disorders of vesicles of lysosomal lineage: the Hermansky-Pudlak syndromes. *Curr Mol Med* 2002; **2**: 451-467
- 13 **Sandberg-Gertzen H, Eid R, Jarnerot G.** Hermansky-Pudlak syndrome with colitis and pulmonary fibrosis. *Scand J Gastroenterol* 1999; **34**: 1055-1056
- 14 **Schinella RA, Greco MA, Cobert BL, Denmark LW, Cox RP.** Hermansky-Pudlak syndrome with granulomatous colitis. *Ann Intern Med* 1980; **92**: 20-23
- 15 **Goswami GK, Sadler MA, Siegel S.** Small-bowel stricture in a woman with oculocutaneous albinism (Hermansky-Pudlak syndrome). *AJR Am J Roentgenol* 2000; **174**: 1163-1164

S- Editor Li DL L- Editor Ma JY E- Editor Yin DH



## CASE REPORT

# A case of primary malignant fibrous histiocytoma of the pancreas: CT and MRI findings

Ri-Sheng Yu, Jia-Wei Wang, Ying Chen, Wen-Hong Ding, Xiu-Fang Xu, Li-Rong Chen

Ri-Sheng Yu, Jia-Wei Wang, Ying Chen, Wen-Hong Ding, Xiu-Fang Xu, Department of Radiology, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, Zhejiang Province, China

Li-Rong Chen, Department of Pathology, Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, Zhejiang Province, China

**Author contributions:** Yu RS designed the research; Yu RS and Wang JW wrote the paper; Ding WH and Chen Y revised the paper; Chen Y, Ding WH and Xu XF performed the research; and Chen LR analyzed the pathological figures.

**Correspondence to:** Wen-Hong Ding, Department of Radiology, the Second Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou 310009, Zhejiang Province, China. [zheerfsk@163.com](mailto:zheerfsk@163.com)

Telephone: 86-571-87783860 Fax: 86-571-87784556

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## Abstract

Primary malignant fibrous histiocytoma (MFH) of the pancreas is rare and a distinct clinical entity. We report a case of recurrence of pancreatic MFH with computed tomography (CT) and magnetic resonance imaging (MRI) findings. A 67-year-old man presented with a history of decreased body weight over the past 6 mo. Abdominal CT revealed a large, multilocular cystic mass in the head of the pancreas with obvious atrophy in the body and tail of the pancreas. After 6 mo postoperatively, MRI demonstrated a recurrent large mass in the primary area of the head of the pancreas. The lesion was heterogeneous, hypointense to the liver on T1-weighted imaging, and heterogeneously hyperintense to the liver with a hypointense area in the central part of the tumor on fat-saturated T2-weighted imaging. Contrast-enhanced T1-weighted imaging demonstrated a large multilocular cystic mass with a cystic wall, fibrous septa and enhancement of solid components. To the best of our knowledge, this is the first report on recurrence of primary MFH of the pancreas, and the first with MRI findings.

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**Key words:** Pancreatic neoplasms; Malignant fibrous histiocytoma; Computed tomography; Magnetic resonance imaging; Tumor recurrence

**Peer reviewer:** Francesco Perri, MD, Department of Gastroenterology, CSS Hospital, IRCCS, Via Cappuccini, San Giovanni Rotondo 71013, Italy

Yu RS, Wang JW, Chen Y, Ding WH, Xu XF, Chen LR. A case of primary malignant fibrous histiocytoma of the pancreas: CT and MRI findings. *World J Gastroenterol* 2008; 14(18): 2942-2945 Available from: URL: <http://www.wjgnet.com/1007-9327/14/2942.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.2942>

## INTRODUCTION

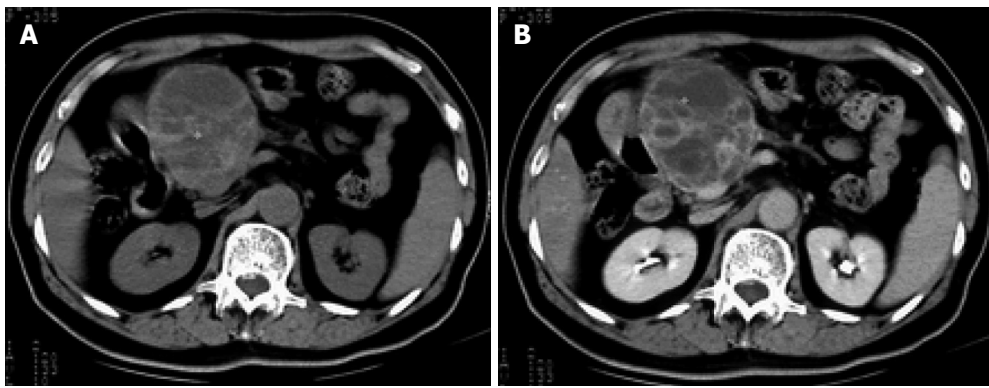
Malignant fibrous histiocytoma (MFH) is the most common type of soft tissue sarcoma of middle and late adulthood<sup>[1-4]</sup>, but it originates only infrequently in the pancreas<sup>[5-8]</sup>. Since the first MFH of the pancreas was described in 1976 by Margules *et al*<sup>[9]</sup>, only 20 cases have been documented in the world literature, including the present case<sup>[10-13]</sup>. Some of these cases have exhibited the characteristic radiological findings<sup>[10-12]</sup>, and no case with recurrence has been reported. Herein, we present a patient with primary MFH of the pancreas, which recurred 6 mo after surgery, and its computed tomography (CT) and magnetic resonance imaging (MRI) appearance. To the best of our knowledge, this is the first report on recurrence of primary MFH of the pancreas and the first with MRI findings.

## CASE REPORT

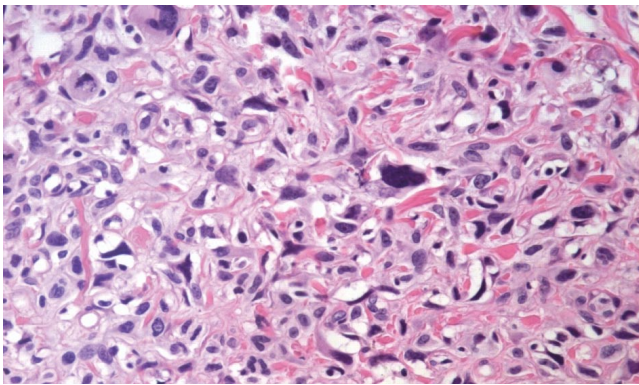
A 67-year-old man was referred to the hospital because of abnormal ultrasonographic findings in the pancreas, found by chance at medical checkup. He was asymptomatic apart from a 3-kg decrease in body weight over the past 6 mo. The patient had no history of alcohol consumption or smoking. Physical examination on admission was unremarkable except for a 9 cm × 10 cm hard mass located in the middle abdomen. No lymph nodes were detected at the body surface. Hepatitis B and C serology was negative, and blood cell counts, liver function tests and serum amylase were normal. Serum levels of carcinoembryonic antigen (CEA) and carbohydrate antigen 19-9 (CA19-9) were not elevated.

Abdominal ultrasound showed a huge soft-tissue mass with cystic and solid components in the head of the pancreas. Abdominal CT revealed a large (8.2 cm × 9.5 cm), non-homogeneous, dense mass in the head of the pancreas, with obvious atrophy in the body and tail of the pancreas on pre-contrast CT (Figure 1A). The lesion showed a well-defined, multilocular cystic





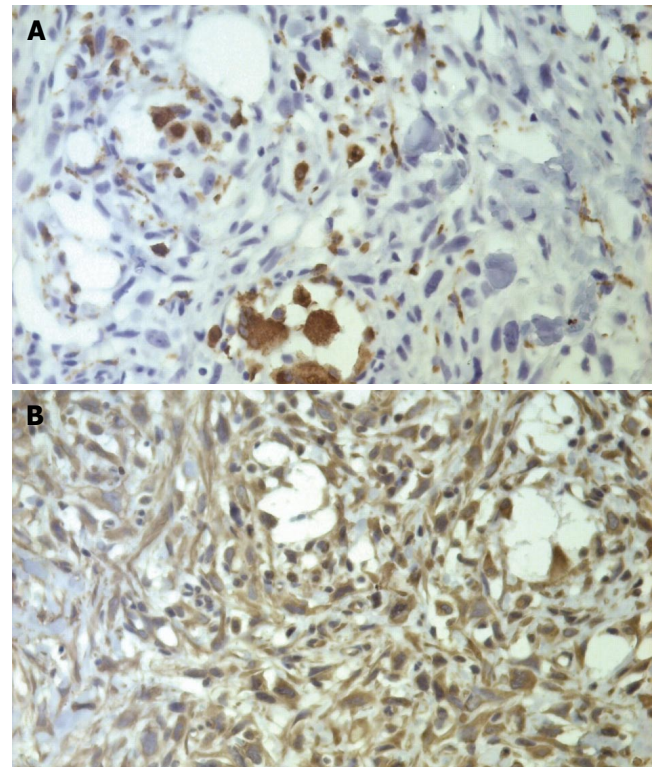
**Figure 1** A: Pre-contrast CT shows a large, non-homogeneous, density mass in the head of the pancreas, with obvious atrophy in the body and tail of the pancreas; B: Lesion illustrates a well-defined, multilocular cystic mass with a cystic wall, fibrous septa and enhancement of solid components on post-contrast CT.



**Figure 2** Pathology of the pancreatic mass shows spindle cells arranged in a storiform pattern, with varying numbers of polymorphic neoplastic and multinucleated cells (HE,  $\times 200$ ).

mass with a cystic wall, fibrous septa and enhancement of solid components on post-contrast CT (Figure 1B). None of the imaging examinations showed abnormal findings in the liver, spleen or retroperitoneum. There was no evidence of metastatic disease, and the patient underwent pancreaticoduodenectomy. Intraoperative findings demonstrated a large mass in the head of the pancreas and no adhesion to the liver or retroperitoneum. Macroscopically, the tumor specimen measured 10 cm  $\times$  11 cm  $\times$  8 cm in size, and was partially encapsulated by the normal pancreas. The cut surface of the tumor was gray-white in color, with a large area of hemorrhage and necrosis. Microscopically, the tumor was composed of spindle cells arranged in a storiform pattern with varying numbers of polymorphic neoplastic and multinucleated cells (Figure 2). To clarify the diagnosis of the tumor further, immunoperoxidase staining was performed on the tumor specimen. The tumor cells were negative for desmin, CEA and S-100; positive for CD68 (Figure 3A), vimentin (Figure 3B), lysozyme and  $\alpha$ -1-ACT; and no evidence of epithelial differentiation was found. In summary, the diagnosis of storiform-pleomorphic MFH of the pancreas was made.

The patient enjoyed a smooth recovery and was discharged on postoperative d 10. The patient was followed at 2-mo intervals by abdominal ultrasound. There was no evidence of recurrence or metastasis until 6 mo postoperatively. MRI demonstrated that a new mass had developed at the location of the MFH. T1-

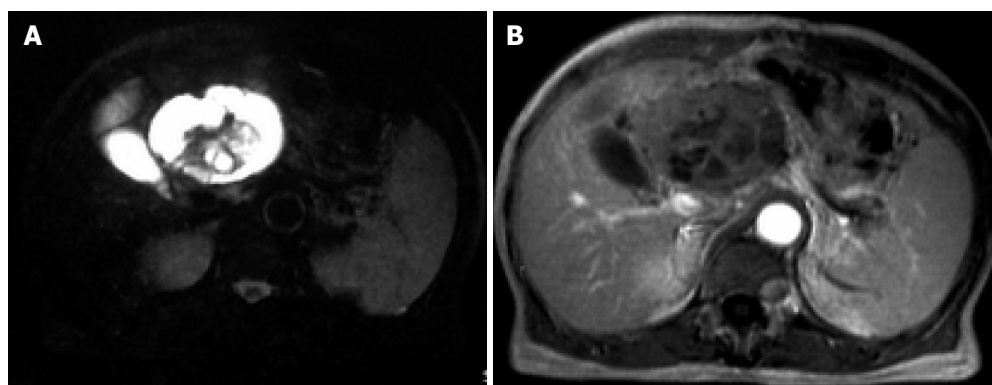


**Figure 3** Immunohistochemical staining shows that the tumor cells are positive for CD68 (A) and vimentin (B) ( $\times 250$ ).

weighted imaging showed a large (7 cm  $\times$  8.5 cm), non-heterogeneous, hypointense lesion of the liver. Fat-saturated T2-weighted imaging showed non-heterogeneous hyperintensity in the liver, with a hypointense area in the central part of the tumor (Figure 4A). Contrast-enhanced T1-weighted imaging demonstrated a large multilocular cystic mass with a cystic wall, fibrous septa and enhancement of solid components (Figure 4B). A diagnosis of recurrence was made. The patient died from the recurrence at 11 mo after operation.

## DISCUSSION

MFH accounts for > 30% of all soft tissue sarcomas<sup>[14]</sup>, and occurs most frequently in the extremities, trunk and retroperitoneum<sup>[15-18]</sup>. Men are affected twice as frequently as women<sup>[15,19]</sup>. Primary MFH of the pancreas is exceedingly



**Figure 4** A: MR fat-saturated T2-weighted image shows a large, non-heterogeneous, hyperintense lesion in the liver, with a hypointense area in the central part of the tumor; B: Contrast-enhanced T1-weighted image shows a large multilocular cystic mass with a cystic wall, fibrous septa and enhancement of solid components.

rare<sup>[5-8]</sup>. Akatsu *et al* have reviewed 16 cases and reported that the tumor has a male predominance with a median age at onset of 55 years. It is usually a large mass (mean tumor diameter is 13 cm, with a range of 4-35 cm)<sup>[11]</sup>. Of the documented cases, only three survived for > 48 mo<sup>[10-13]</sup>, which suggests a poor prognosis for long-term survival. Only one case had lymph node metastases<sup>[12]</sup>. Our case was the only one with recurrence.

MFH consists of histiocyte-like and fibroblast-like cells arranged in a storiform pattern, with other pleomorphic cells and multinucleated giant cells<sup>[20-23]</sup>. There are five subtypes of MFH according to histology<sup>[10,24]</sup>: storiform-pleomorphic, myxoid, giant cell, inflammatory, and angiomatoid. The storiform-pleomorphic pattern is the most common variant<sup>[25]</sup>. Three histological subtypes have been observed among the 20 cases previously reported in the literature: 13 (65%) storiform-pleomorphic, four (20%) giant cell, and three (15%) myxoid<sup>[2-5]</sup>. Our case belonged to the storiform-pleomorphic type.

Only a few radiological findings of primary MFH of the pancreas have been described<sup>[10-12]</sup>. The CT findings of the tumor include the following: a large, heterogeneous, low-attenuation density or multinodular mass, with intratumoral calcification on pre-contrast CT; non-homogeneously enhancing pancreatic mass with a large amount of necrosis<sup>[11,12]</sup>; and single enhancing peripheral pseudo-capsular mass on post-contrast CT<sup>[10]</sup>. To the best of our knowledge, we describe the first case of primary MFH of the pancreas, with CT showing a large multilocular cystic mass with a cystic wall, fibrous septa and enhancement of solid components. It is not clear whether or not the pathological features of pancreatic MFH correlate with the CT findings. Liu *et al* have described a predominant cystic MFH of the pancreas of the myxoid variety<sup>[10]</sup>, but other reports and our case suggest that not all cystic degeneration of the tumor belongs to the myxoid pattern<sup>[11,12]</sup>. Ko *et al* have considered that abdominal MFH, especially the storiform-pleomorphic subtype, exhibits metaplastic calcifications on CT, and they showed a heterogeneously enhanced mass with a large necrotic area in the pancreatic body and tail, and eccentrically located ring calcification<sup>[26]</sup>. Akatsu *et al* did not, however, support this sign and concluded that calcification is not always helpful in differentiating pancreatic MFH from other pancreatic tumors<sup>[11]</sup>. Therefore, we think that more cases need to be studied to prove the relationship between the CT findings

and the subtypes of primary MFH.

Mahajan and colleagues have retrospectively reviewed the MRI changes in 39 patients with MFH, however, none of these originated from the pancreas<sup>[27]</sup>. This study concluded that MRI sensitivity and specificity for detecting a neoplasm were 96% and 83%, respectively, but the signal changes were non-specific for MFH. When compared to CT in 14 patients, MRI better defined the extent of the MFH, its relationship to surrounding tissues and vessels, and best differentiated residual or recurrent disease from postoperative changes when examined at least 3 mo after surgery. There was no significant difference in signal intensity of 12 preoperative and 13 recurrent neoplasms<sup>[27]</sup>.

Tateishi *et al* have described the manner in which that primary MFH has a high signal intensity on T2-weighted MR images and non-homogeneous isosignal intensity compared with the surrounding muscle<sup>[28]</sup>. Miller *et al* have reported that a correlation between MRI appearance and histopathology may not be established, but concluded that MRI exhibits general features suggestive of malignant soft tissue neoplasm, namely, internal low signal septation, and heterogeneous high signal intensity on T2-weighted images<sup>[29]</sup>. Kitajima *et al*<sup>[30]</sup> have considered that MRI is helpful in the differential diagnosis between MFH of the renal capsule and other renal tumors, and that a hypointense area identified on T2-weighted images is an important characteristic of renal MFH. In our case, MRI showed a large multilocular cystic mass, which was similar to that on preoperative CT, and it could reveal the exact location and extent of the tumor recurrence. An obviously hypointense area on T2-weighted images that reflected the fibrous component was also seen in our pancreatic MFH.

In conclusion, primary MFH of the pancreas is rare and a distinct clinical entity. It may recur and the prognosis is poor. Although there are no unique findings of pancreatic MFH, a large, massive liquescent necrotic mass or multilocular cystic lesion, with calcification on CT or a hypointense area within the mass on T2-weighted imaging in an older male patient, should lead to consideration of a differential diagnosis of pancreatic MFH.

## REFERENCES

- Gutierrez JC, Perez EA, Franceschi D, Moffat FL Jr, Livingstone AS, Koniaris LG. Outcomes for soft-tissue sarcoma in 8249 cases from a large state cancer registry. *J Surg Res* 2007; **141**: 105-114



- 2 **Fu DL**, Yang F, Maskay A, Long J, Jin C, Yu XJ, Xu J, Zhou ZW, Ni QX. Primary intestinal malignant fibrous histiocytoma: two case reports. *World J Gastroenterol* 2007; **13**: 1299-1302
- 3 **Randall RL**, Albritton KH, Ferney BJ, Layfield L. Malignant fibrous histiocytoma of soft tissue: an abandoned diagnosis. *Am J Orthop* 2004; **33**: 602-608
- 4 **Issakov J**, Kollender Y, Soyfer V, Bickels J, Flusser G, Meller I, Merimsky O. A single-team experience of limb sparing approach in adults with high-grade malignant fibrous histiocytoma. *Oncol Rep* 2005; **14**: 1071-1076
- 5 **Basso L**, Pisanelli MC, Bovino A, Vietri F, D'Ermo G, De Toma G. Malignant fibrous histiocytoma of the mesentery: report of two cases and review of the literature. *G Chir* 2005; **26**: 43-46
- 6 **Mizukami H**, Yajima N, Wada R, Matsumoto K, Kojima M, Kloppel G, Yagihashi S. Pancreatic malignant fibrous histiocytoma, inflammatory myofibroblastic tumor, and inflammatory pseudotumor related to autoimmune pancreatitis: characterization and differential diagnosis. *Virchows Arch* 2006; **448**: 552-560
- 7 **Allen KB**, Skandalakis LJ, Brown BC, Gray SW, Skandalakis JE. Malignant fibrous histiocytoma of the pancreas. *Am Surg* 1990; **56**: 364-368
- 8 **Balén EM**, De Villa VH, Cienfuegos JA, Contreras F, Pardo F, Gonzalez J, Benito C. [Malignant fibrous histiocytoma of the pancreas] *Rev Esp Enferm Dig* 1993; **83**: 475-480
- 9 **Margules RM**, Allen RE, Dunphy JE. Pancreatic tumor of mesenchymal origin presenting as obstructive jaundice. *Am J Surg* 1976; **131**: 357-359
- 10 **Liu DM**, Jeffrey RB Jr, Mindelzun RE. Malignant fibrous histiocytoma presenting as cystic pancreatic mass. *Abdom Imaging* 1999; **24**: 299-300
- 11 **Akatsu T**, Tsugita M, Ro S, Kameyama K, Kitajima M. Primary malignant fibrous histiocytoma of the pancreas: a case with K-ras mutation and a review of the literature. *Dig Dis Sci* 2005; **50**: 2214-2217
- 12 **Bastian D**, Ramaswamy A, Barth PJ, Gerdes B, Ernst M, Bartsch D. Malignant fibrous histiocytoma of the pancreas: a case report with genetic analysis. *Cancer* 1999; **85**: 2352-2358
- 13 **Song HB**, Guo LN, Liu TH. Pancreatic multilocular myxoid cystadenoma cancerization incorporation with MFH of giant cell pattern: case report. *Chin J Pathol* 2001; **30**: 311-312
- 14 **Sternheim A**, Jin X, Shmookler B, Jelinek J, Malawer MM. 'Telangiectatic' transformation in soft tissue sarcomas. a clinicopathology analysis of an aggressive feature of high-grade sarcomas. *Ann Surg Oncol* 2008; **15**: 345-354
- 15 **Chen HC**, Chen CJ, Jeng CM, Yang CM. Malignant fibrous histiocytoma presenting as hemoperitoneum mimicking hepatocellular carcinoma rupture. *World J Gastroenterol* 2007; **13**: 6441-6443
- 16 **Weiss SW**, Enzinger FM. Malignant fibrous histiocytoma: an analysis of 200 cases. *Cancer* 1978; **41**: 2250-2266
- 17 **Nurdjanah S**, Bayupurnama P, Maduseno S, Ratnasari N. Abdominal Malignant Fibrous Histiocytoma Infiltrating Stomach with Chilaiditi's Sign Manifestation (A Rare Case Report). *Kobe J Med Sci* 2007; **53**: 119-124
- 18 **Peiper M**, Zurakowski D, Knoefel WT, Izbicki JR. Malignant fibrous histiocytoma of the extremities and trunk: an institutional review. *Surgery* 2004; **135**: 59-66
- 19 **Belal A**, Kandil A, Allam A, Khafaga Y, El-Husseiny G, El-Enbaby A, Memon M, Younge D, Moreau P, Gray A, Schultz H. Malignant fibrous histiocytoma: a retrospective study of 109 cases. *Am J Clin Oncol* 2002; **25**: 16-22
- 20 **Liu P**, Li J, Shen L. [A clinico-pathologic study of primary malignant fibrous histiocytoma of bone] *Zhonghua Zhongliu Zazhi* 1996; **18**: 146-149
- 21 **Tos AP**. Classification of pleomorphic sarcomas: where are we now? *Histopathology* 2006; **48**: 51-62
- 22 **Murphey MD**. World Health Organization classification of bone and soft tissue tumors: modifications and implications for radiologists. *Semin Musculoskelet Radiol* 2007; **11**: 201-214
- 23 **Scapolan M**, Perin T, Wassermann B, Canzonieri V, Colombatti A, Italia F, Spessotto P. Expression profiles in malignant fibrous histiocytomas: clues for differentiating 'spindle cell' and 'pleomorphic' subtypes. *Eur J Cancer* 2008; **44**: 298-309
- 24 **Munk PL**, Sallomi DF, Janzen DL, Lee MJ, Connell DG, O'Connell JX, Logan PM, Masri B. Malignant fibrous histiocytoma of soft tissue imaging with emphasis on MRI. *J Comput Assist Tomogr* 1998; **22**: 819-826
- 25 **Meister P**. Malignant fibrous histiocytoma. History, histology, histogenesis. *Pathol Res Pract* 1988; **183**: 1-7
- 26 **Ko SE**, Wan YL, Lee TY, Ng SH, Lin JW, Chen WJ. CT features of calcifications in abdominal malignant fibrous histiocytoma. *Clin Imaging* 1998; **22**: 408-413
- 27 **Mahajan H**, Kim EE, Wallace S, Abello R, Benjamin R, Evans HL. Magnetic resonance imaging of malignant fibrous histiocytoma. *Magn Reson Imaging* 1989; **7**: 283-288
- 28 **Tateishi U**, Kusumoto M, Hasegawa T, Yokoyama R, Moriyama N. Primary malignant fibrous histiocytoma of the chest wall: CT and MR appearance. *J Comput Assist Tomogr* 2002; **26**: 558-563
- 29 **Miller TT**, Hermann G, Abdelwahab IF, Klein MJ, Kenan S, Lewis MM. MRI of malignant fibrous histiocytoma of soft tissue: analysis of 13 cases with pathologic correlation. *Skeletal Radiol* 1994; **23**: 271-275
- 30 **Kitajima K**, Kaji Y, Morita M, Okuda Y, Sugimura K. Malignant fibrous histiocytoma arising from the renal capsule. *Magn Reson Med Sci* 2003; **2**: 199-202

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## LETTERS TO THE EDITOR

# Desperately seeking hepatitis C virus

Ricardo Moreno-Otero

Ricardo Moreno-Otero, Hepato-Gastroenterology Service and Ciberehd, Hospital Universitario de La Princesa, Autonomous University of Madrid, Madrid 28006, Spain

Author contributions: Moreno-Otero R wrote the paper.

Correspondence to: Ricardo Moreno-Otero, MD, Chief, Hepato-Gastroenterology Service and Ciberehd, Hospital Universitario de La Princesa, Autonomous University of Madrid, Madrid 28006, Spain. [rmoreno.hlpr@salud.madrid.org](mailto:rmoreno.hlpr@salud.madrid.org)  
Telephone: +34-91-3093911 Fax: +34-91-4022299

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## Abstract

Spanish investigators described recently the so-called occult hepatitis C virus (HCV) infection, emphasizing the detection of genomic and antigenomic HCV RNA strands in liver and peripheral blood mononuclear cells. Therefore, the persistence of viral replication in occult HCV infection should be considered as a putative source of infection among family members and patients undergoing invasive procedures, transfusion or transplantation. Additionally, the most worrisome finding is that an occult HCV infection may persist in patients with sustained virological response.

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**Key words:** Occult hepatitis C; Occult hepatitis C virus infection; Hepatitis C virus RNA; Peginterferon; Ribavirin

**Peer reviewers:** Giovanni Tarantino, MD, Professor, Department of Clinical and Experimental Medicine, Federico II University Medical School, VIA S. PANSINI, 5, Naples 80131, Italy; Peter L Lakatos, MD, PhD, Assistant Professor, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary; Hisato Nakajima, MD, Department of Gastroenterology and Hepatology, The Jikei University School of Medicine, 3-25-8, Nishi-Shinbashi, Minato-ku, Tokyo 105-8461, Japan

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response (SVR) in approximately 45% of patients with genotype 1 and in 80% of those with genotypes 2 or 3. SVR is associated with HCV eradication and liver damage reduction. Enormous scientific and economic efforts have dramatically changed the medical focus on HCV, and the prior condition of shipwrecked hepatologists has evolved into skilful navigators trained to cure chronic hepatitis C (CHC), adapting treatment to each patient instead of adapting the patient to an established therapy<sup>[1,2]</sup>.

Spanish investigators described recently the so-called occult hepatitis C virus (HCV) infection<sup>[3]</sup> summarizing its successive studies in a comprehensive review<sup>[4]</sup> that emphasizes the detection of genomic and antigenomic HCV RNA strands in liver and peripheral blood mononuclear cells (PBMCs). Therefore, the persistence of viral replication in occult HCV infection should be considered as a putative source of infection among family members and patients undergoing invasive procedures, transfusion or transplantation. Additionally, the most worrisome finding is that an occult HVC infection may persist in SVR patients, with HCV RNA presence in liver and PBMCs<sup>[4,5]</sup>. The hope of treated patients, who consider themselves having got rid of the virus, and of physicians, struggling for control and cure CHC, is most probably devastated.

These authors also found that HCV RNA is detected in the sera of patients with occult HCV infection after ultracentrifugation<sup>[6]</sup>. What is the significance of this new entity? Answers are a challenge for the scientific community in order to dissect the epidemiological aspects and pathological consequences of occult HCV infection, not to mention the design of therapeutic strategies. The social and medical costs can be astronomic, including work incapacity, absenteeism and intangible costs able to ruin the private life of some patients. Concerns and doubtful questions enunciated by frightened persons and medical doctors have been raised after an extensive report on occult hepatitis C was published, alluding the aforementioned study<sup>[6]</sup>, on a newspaper from Madrid (LA RAZÓN, July 10-2007, page 31). Whether all the information has any alarming relevance remains to be seen.

## TO THE EDITOR

Research into hepatitis C virus infection has progressed substantially over the past 15 years. Treatment with peginterferon plus ribavirin yields a sustained virological

## REFERENCES

- 1 Hoofnagle JH, Seeff LB. Peginterferon and ribavirin for chronic hepatitis C. *N Engl J Med* 2006; **355**: 2444-2451
- 2 Moreno-Otero R. Therapeutic modalities in hepatitis C: challenges and development. *J Viral Hepat* 2005; **12**: 10-19

- 3 **Castillo I**, Pardo M, Bartolomé J, Ortiz-Movilla N, Rodríguez-Iñigo E, de Lucas S, Salas C, Jiménez-Heffernan JA, Pérez-Mota A, Graus J, López-Alcorocho JM, Carreño V. Occult hepatitis C virus infection in patients in whom the etiology of persistently abnormal results of liver-function tests is unknown. *J Infect Dis* 2004; **189**: 7-14
- 4 **Carreño V**. Occult hepatitis C virus infection: a new form of hepatitis C. *World J Gastroenterol* 2006; **12**: 6922-6925
- 5 **Castillo I**, Rodríguez-Iñigo E, López-Alcorocho JM, Pardo M, Bartolomé J, Carreño V. Hepatitis C virus replicates in the liver of patients who have a sustained response to antiviral treatment. *Clin Infect Dis* 2006; **43**: 1277-1283
- 6 **Bartolomé J**, López-Alcorocho JM, Castillo I, Rodríguez-Iñigo E, Quiroga JA, Palacios R, Carreño V. Ultracentrifugation of serum samples allows detection of hepatitis C virus RNA in patients with occult hepatitis C. *J Virol* 2007; **81**: 7710-7715

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Third Department of Internal Medicine, Ehime University School of Medicine, Shigenobu-Cho, Ehime 791-0295, Japan

**Claudio Bassi, MD, Professor,**

Department of Surgery and Gastroenterology, Hospital GB Rossi, University of Verona, Piazza LA Scuro 37134 Verona, Italy

**Reinhard Buettner, Professor,**

Institute of Pathology, University Hospital Bonn, Sigmund-Freud-Str. 25, D-53127 Bonn, Germany

**David L Carr-Locke, MD,**

Director of Endoscopy, Brigham and Women's Hospital, Endoscopy Center, Brigham and Women's Hospital, 75 Francis St, Boston MA 02115, United States

**Massimo Colombo, Professor,**

1st Division of Gastroenterology, Fondazione IRCCS Maggiore Hospital, Policlinico, Mangiagalli e Regina Elena, University of Milan, Via F. Sforza 35, 20122 Milan, Italy

**David Cronin II, MD, PhD, FACS, Associate Professor,**

Department of Surgery, Yale University School of Medicine, 330 Cedar Street, FMB 116, P. O. Box 208062, New Haven, Connecticut 06520-8062, United States

**Arno J Dormann, PD, MED, Habil,**

Medizinische Klinik, Krankenhaus Holweide, Kliniken der Stadt Köln gGmbH, Neufelder St. 32, 51067 Köln, Germany

**James E East, BSc, MBChB, MRCP,**

St. Mary's Hospital, Endoscopy Unit, Clarence Wing, 3rd Floor, Praed Street, London, W2 1NY, United Kingdom

**Francesco Feo, Professor,**

Dipartimento di Scienze Biomediche, Sezione di Patologia Sperimentale e Oncologia, Università di Sassari, Via P. Manzella 4, 07100 Sassari, Italy

**Mitsuhiro Fujishiro, Dr,**

Department of Gastroenterology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

**Toru Ishikawa, MD,**

Department of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan

**Syed MW Jafri, Professor,**

Medicine/Gastroenterology, Aga Khan University, POB 3500, Karachi 74800, Pakistan

**Tom H Karlsen, MD,**

Institute of Immunology, Rikshospitalet University Hospital, N-0027 Oslo, Norway

**Ioannis E Koutroubakis, MD, PhD,**

Assistant Professor of Medicine, University Hospital Heraklion, Department of Gastroenterology, PO Box 1352, 71110 Heraklion, Crete, Greece

**Shiu-Ming Kuo, MD,**

University at Buffalo, 15 Farber Hall, 3435 Main Street, Buffalo 14214, United States

**Ezio Laconi, MD, PhD,**

Professor of General Pathology, Department of Sciences and Biomedical Technologies, Unit of Experimental Pathology, University of Cagliari, Via Porcell, 4 - IV Piano, 09125 - Cagliari, Italy

**James YW Lau,**

Department of Surgery, Prince of Wales Hospital, the Chinese University of Hong Kong, Hong Kong, China

**Laura Lladó, PhD,**

Department of Surgery, Liver Transplant Unit, Hospital Universitari de Bellvitge, IDIBELL, 08907 Barcelona, Spain

**Mercedes Susan Mandell, MD, PhD,**

Department of Anesthesiology, University of Colorado Health Sciences Ctr., 12401 E. 17th Ave, B113 Aurora, CO 80045, United States

**Robert D Odze, MD, FRCPc, Chief,**

Gastrointestinal Pathology Service, Associate Professor of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston MA, United States

**Natalia A Osna,**

Liver Study Unit, Research Service (151), VA Medical Center, 4101 Woolworth Avenue, Omaha NE 68105, United States

**Kostas Pantopoulos, Associate Professor,**

Department of Medicine, McGill University, Lady Davis Institute for Medical Research, 3755 Cote Ste-Catherine Road, Montreal, Quebec, H3T 1E2, Canada

**George Papatheodoridis, MD,**

Assistant Professor in Medicine & Gastroenterology, 2nd Department of Internal Medicine, Athens University Medical School, Hippokraton General Hospital of Athens, 114 Vas. Sophias Ave., 115 27 Athens, Greece

**Gustav Paumgartner, Professor,**

University of Munich, Klinikum Grosshadern, Marchioninstr. 15, Munich, D-81377, Germany

**Jose L del Pozo, MD, PhD,**

Infectious Diseases Division, Mayo Clinic College of Medicine, 200 1st St SW, Rochester, MN 55905, United States

**Paul E Sijens, PhD, Associate Professor,**

Radiology, UMCG, Hanzeplein 1, 9713GZ Groningen, The Netherlands

**Akihito Tsubota, Assistant Professor,**

Institute of Clinical Medicine and Research, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan

**Marie-Catherine Vozenin-brotons, PhD,**

UPRES EA 27-10, IRSN/IGR, 39 rue C. Desmoulins, Villejuif Cedex 94305, France

**Eddie Wisse, Professor,**

Irisweg 16, Keerbergen 3140, Belgium

**Harry HX Xia, PhD, MD,**

Novartis Pharmaceuticals Corporation, One Health Plaza, East Hanover, NJ 07936-1080, United States

**Yoshio Yamaoka, MD, PhD, Associate Professor,**

Department of Medicine/Gastroenterology, Baylor College of Medicine and VA Medical Center (111D), 2002 Holcombe Blvd, Houston, Texas 77030, United States

**Ta-Sen Yeh, MD, PhD,**

Department of Surgery, Chang Gung Memorial Hospital, 5 Fu-Hsing Street, Taoyuan, Taiwan, China

**Yuan Yuan, Professor,**

Cancer Institute of China Medical University, 155 North Nanjing Street, Heping District, Shenyang 110001, Liaoning Province, China



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January 24-25, Frankfurt, Germany  
 Falk Workshop: Perspectives in Liver Transplantation

#### International Gastroenterological Congresses 2008

February 14-16, Paris, France  
 EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
 8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
 3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

March 10-13, Birmingham, UK  
 British Society of Gastroenterology Annual Meeting  
 E-mail: [BSG@mailbox.ulcc.ac.uk](mailto:BSG@mailbox.ulcc.ac.uk)

March 14-15, HangZhou, China  
 Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
 Asian Pacific Association for the Study of the Liver  
 18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
 Shanghai - Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
 OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation - Management of Adeno- carcinomas  
 Email: [robert.giuli@oeso.org](mailto:robert.giuli@oeso.org)

April 18-22, Buenos Aires, Argentina  
 9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
 Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
 43<sup>rd</sup> Annual Meeting of the European

Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary  
 Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA  
 Digestive Disease Week 2008  
 June 4-7, Helsinki, Finland

The 39<sup>th</sup> Nordic Meeting of Gastroenterology  
[www.congex.com/ngc2008](http://www.congex.com/ngc2008)  
 June 6-8, Prague, Czech Republic  
 3<sup>rd</sup> Annual European Meeting: Perspectives in Inflammatory Bowel Diseases  
 Email: [meetings@imedex.com](mailto:meetings@imedex.com)

June 13-14, Amsterdam, Netherlands  
 Falk Symposium 165: XX International Bile Acid Meeting. Bile Acid Biology and Therapeutic Actions

June 25-28, Barcelona, Spain  
 10<sup>th</sup> World Congress on Gastrointestinal Cancer  
 Imedex and ESMO  
 Email: [meetings@imedex.com](mailto:meetings@imedex.com)

June 25-28, Lodz, Poland  
 Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatology (IAP)  
 E-mail: [office@epc-iap2008.org](mailto:office@epc-iap2008.org)  
[www.e-p-c.org](http://www.e-p-c.org)  
[www.pancreatology.org](http://www.pancreatology.org)

June 26-28, Bratislava, Slovakia  
 5<sup>th</sup> Central European Gastroenterology Meeting  
[www.ceurgem2008.cz](http://www.ceurgem2008.cz)  
 September 10-13, Budapest, Hungary

11<sup>th</sup> World Congress of the International Society for Diseases of the Esophagus  
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September 13-16, New Delhi, India  
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III FALK GASTRO-CONFERENCE  
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 Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany  
 Falk Symposium 166:  
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September 18-20, Prague, Czech Republic  
 Prague Hepatology Meeting 2008  
[www.czech-hepatology.cz/phm2008](http://www.czech-hepatology.cz/phm2008)

September 20-21, Mainz, Germany  
 Falk Symposium 167:  
 Liver Under Constant Attack - From

Fat to Viruses  
 September 24-27, Nantes, France  
 Third Annual Meeting  
 European Society of Coloproctology  
[www.escp.eu.com](http://www.escp.eu.com)



October 8-11, Istanbul, Turkey  
 18<sup>th</sup> World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists  
 E-mail: [orkun.sahin@serenas.com.tr](mailto:orkun.sahin@serenas.com.tr)

October 18-22, Vienna, Austria  
 16<sup>th</sup> United European Gastroenterology Week  
[www.negf.org](http://www.negf.org)  
[www.acv.at](http://www.acv.at)

October 22-25, Brisbane, Australia  
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 Email: [gesa@gesa.org.au](mailto:gesa@gesa.org.au)

October 31-November 4, Moscone West Convention Center, San Francisco, CA  
 59<sup>th</sup> AASLD Annual Meeting and Postgraduate Course  
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November 6-9, Lucerne, Switzerland  
 Neurogastroenterology & Motility Joint International Meeting 2008  
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[www.ngm2008.com](http://www.ngm2008.com)

November 12, Santiago de Chile, Chile  
 Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

December 7-9, Seoul, Korea  
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For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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#### Abstract

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- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

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- 6 21st century heart solution may have a sting in the tail. *BMJ*



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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

### Books

#### Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

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- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

#### Author(s) and editor(s)

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Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

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## Chronic intestinal pseudo-obstruction

Alexandra Antonucci, Lucia Fronzoni, Laura Cogliandro, Rosanna F Cogliandro, Carla Caputo, Roberto De Giorgio, Francesca Pallotti, Giovanni Barbara, Roberto Corinaldesi, Vincenzo Stanghellini

Alexandra Antonucci, Lucia Fronzoni, Laura Cogliandro, Rosanna F Cogliandro, Carla Caputo, Roberto De Giorgio, Francesca Pallotti, Giovanni Barbara, Roberto Corinaldesi, Vincenzo Stanghellini, Department of Internal Medicine and Gastroenterology, University of Bologna, Bologna I-40138, Italy  
**Author contributions:** Antonucci A, Fronzoni L performed PubMed and Medline searches pertinent to the objective of the present article and reviewed the literature; Cogliandro L contributed analyzing the text, reviewing literature; Caputo C and Pallotti F performed PubMed and Medline searches particularly focusing on previously published articles on pathophysiology of chronic intestinal pseudo-obstruction; Cogliandro RF, De Giorgio R, Barbara G, Corinaldesi R and Stanghellini V contributed to the writing manuscript, section coordination and English editing.

**Correspondence to:** Vincenzo Stanghellini, MD, Department of Internal Medicine & Gastroenterology, St. Orsola-Malpighi Hospital, Via Massarenti 9, Bologna I-40138, Italy. [v.stanghellini@unibo.it](mailto:v.stanghellini@unibo.it)

Telephone: +39-51-6364101 Fax: +39-51-345864

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### Abstract

Chronic intestinal pseudo-obstruction (CIPO) is a severe digestive syndrome characterized by derangement of gut propulsive motility which resembles mechanical obstruction, in the absence of any obstructive process. Although uncommon in clinical practice, this syndrome represents one of the main causes of intestinal failure and is characterized by high morbidity and mortality. It may be idiopathic or secondary to a variety of diseases. Most cases are sporadic, even though familial forms with either dominant or recessive autosomal inheritance have been described. Based on histological features intestinal pseudo-obstruction can be classified into three main categories: neuropathies, mesenchymopathies, and myopathies, according on the predominant involvement of enteric neurones, interstitial cells of Cajal or smooth muscle cells, respectively. Treatment of intestinal pseudo-obstruction involves nutritional, pharmacological and surgical therapies, but it is often unsatisfactory and the long-term outcome is generally poor in the majority of cases.

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**Key words:** Chronic intestinal pseudo-obstruction; Small bowel manometry; Immunohistochemistry; Prokinetics; Intestinal transplantation

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Research Unit, University Hospital Vall d'Hebron, Paseo Vall d'Hebron, 119-129, Barcelona 08035, Spain

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### INTRODUCTION

Chronic intestinal pseudo-obstruction (CIPO) is a rare, severe disease characterized by the failure of the intestinal tract to propel its contents which results in a clinical picture mimicking mechanical obstruction in the absence of any lesion occluding the gut. CIPO is one of the most important causes of chronic intestinal failure both in pediatric (15%) and adult cases (20%)<sup>[1-5]</sup>, since affected individuals are often unable to maintain normal body weight and/or normal oral nutrition. The severity of clinical picture, generally characterized by disabling digestive symptoms even between sub-occlusive episodes, contributes to deterioration of quality of life of the patients. Furthermore, CIPO often passes unrecognized for long time, so that patients almost invariably undergo repeated, useless and potentially dangerous surgical procedures.

This article is aimed at reviewing the current knowledge on pathophysiology, clinical features and management of patients affected by CIPO.

### ETIOLOGY AND PATHOPHYSIOLOGY

CIPO is idiopathic in the majority of cases. In our experience organic, systemic or metabolic causes of the disease were identified in only 4 patients of 77 CIPO patients consecutively referred in our laboratory (5%)<sup>[2]</sup>. Nevertheless, it is mandatory to investigate affected individuals by traditional diagnostic procedures (radiology, endoscopy, lab tests, etc) in order to exclude every possible cause of secondary CIPO. The main secondary causes of CIPO are specified in Table 1.

In fact, every disease that affects one of the control mechanisms of intestinal functioning, including intrinsic and extrinsic neural supplies as well as muscle cells, can be responsible for secondary and potentially curable forms of CIPO. The extrinsic autonomic nervous system can be affected both centrally (i.e. Parkinson syndrome, Shy-Drager syndrome, stroke, encephalitis, neoplasm and any



Table 1 Main causes of secondary chronic idiopathic pseudo-obstruction and relative gut tissue that is predominantly involved

Underlying disease	Main causes
Diseases of central autonomic and enteric nervous systems	Stroke, encephalitis, calcification of basal ganglia, orthostatic hypotension, Von Recklinghausen, Hirschsprung
Immune-mediated and collagen diseases	Paraneoplastic (CNS neoplasms, lung microstoma, bronchial carcinoid, leiomyosarcomas), scleroderma, dermatomyositis, amyloidosis, Ehlers-Danlos, LES
Endocrine and metabolic diseases	Diabetes, hypothyroidism, hypoparathyroidism, pheochromocytoma
Other	Iatrogenic (radiation enteritis, clonidine, phenothiazines, antidepressants, antiparkinsonians, antineoplastics, bronchodilators, anthraquinones) jejunal diverticulosis, chagas

other disease that could affect the encephalic autonomous centres), and peripherally (i.e. diabetic neuropathy, or other neuropathies potentially involving the enteric nervous system including Hirschsprung, Chagas, Von Recklinghausen, as well as non-specific diseases, like paraneoplastic syndromes, autoimmune diseases, viral infections). Enteric smooth muscle cells can be markedly damaged in patients affected by myotonic dystrophy or progressive systemic sclerosis. Collagenosis, Ehlers-Danlos syndrome, jejunal diverticulosis and radiation enteritis can be responsible for both a neuronal and myogenic impairment.

Nonetheless, diseases like hypothyroidism, hypoparathyroidism, and celiac disease have been described to be responsible for some cases of secondary CIPO, even if the underlying mechanism remains undetermined<sup>[1,4]</sup>.

CIPO is generally sporadic, but familial forms have also been described both with autosomal dominant, autosomal recessive and X-linked transmission<sup>[1,6,7]</sup>. Some genes and loci have been identified in syndromic forms of CIPO, including the transcription factor *SOX10* on chromosome 22 (22p12), the DNA polymerase gamma gene (*POLG*) on chromosome 21 (21q17) and a locus on chromosome 8<sup>[7-9]</sup>. In terms of X-linked transmission, recently Gargiulo *et al* have identified a 2-base pair deletion in exon 2 of the *filamin A* gene (encoding for a large cytoskeletal protein involved in the modulation of the cellular response to chemical and mechanical environmental factors) that is present at the heterozygous state in the carrier females of a family with syndromic CIPO<sup>[10]</sup>. Familial cases are more frequent in mitochondrial neurogastrointestinal encephalomyopathy (MNGIE), which is characterized by subocclusive episodes and lactic acidosis, skeletal muscle abnormalities (i.e. “ragged red fiber”) and specific mitochondrial changes at the ultrastructural level<sup>[11,12]</sup>. Mutations of the gene encoding the thymidine phosphorylase gene (*TP* or endothelial cell growth factor-1, *ECGF1*), mapped to locus 22q13.32qter have a pathogenic role and are responsible for MNGIE<sup>[11-15]</sup>. The biochemical dysfunctions underlying MNGIE consists of decreased TP activity leading to accumulation of thymidine (dThd) and deoxyuridine (dUrd) in blood and tissues<sup>[16,17]</sup>.

Toxic levels of dThd and dUrd induce nucleotide pool imbalance that, in turn, leads to mitochondrial DNA abnormalities including point mutations, multiple deletions and depletion<sup>[16,18]</sup>.

### Histopathology and putative pathogenic mechanisms

Examination of full-thickness biopsies of the intestinal

wall may help in establishing a correct diagnosis, revealing pathological abnormalities underlying the neuromuscular impairment. Histopathologic features of CIPO include neuropathic, mesenchymopathic and myopathic forms based on abnormalities affecting the integrity of nerve pathways supplying the gut (either intrinsic or extrinsic), interstitial cells of Cajal (ICC) and smooth muscle cells, respectively. Neuropathic, mesenchymopathic and myopathic changes may contribute to gut dysmotility either individually or in combination (e.g. neuro-myopathies or neuro-ICC alterations) (Table 2)<sup>[1,6,7]</sup>.

### Enteric neuropathies and enteroglia cell abnormalities

Enteric neurodegenerative abnormalities and immune-mediated changes may occur in gut specimens of patients with neuropathic CIPO. Inflammatory neuropathies are characterized by a dense inflammatory infiltrate characterized by CD3 positive (composed of both CD4 and CD8) lymphocytes almost invariably confined to the myenteric plexus (hence the term of *lymphocytic myenteric ganglionitis*)<sup>[7-9,19]</sup>. The close apposition of CD3 lymphocytes to myenteric neurons provides the basis to neuro-immune interactions targeting and affecting ganglion cell structure and survival<sup>[20,21]</sup>. Indeed, experimental evidence indicates that inflammation/immune activation in the gastrointestinal tract can profoundly affect both morphology and function of the enteric nervous system (ENS).

The evidence that patients with inflammatory neuropathy have circulating anti-neuronal auto-antibodies (e.g. anti-Hu anti-neuronal antibodies) also suggests the role of the immune system in neuronal dysfunction<sup>[19]</sup>. Previous results indicated that these autoantibodies alter ascending reflex pathway of peristalsis in *in vitro* preparations<sup>[22]</sup> and elicit neuronal hyperexcitability as demonstrated by Ca<sup>2+</sup>-imaging technique<sup>[23]</sup>. In addition, anti-HuD neuronal antibodies evoked activation of caspase-3 and apaf-1 along with apoptosis when incubated with primary culture of myenteric neurons<sup>[24]</sup>. Taken together, these experimental data suggest that anti-Hu antibodies may exert either a direct pathogenic role or contribute in association with the lymphocytic infiltrate in ENS dysfunction in patients with CIPO related to an inflammatory neuropathy. Although the etiology of inflammatory neuropathies remains undetermined, the demonstration showing herpes virus DNA in the myenteric plexus of patients with CIPO<sup>[25]</sup> raises the exciting possibility that infectious agents can be involved in the pathogenic cascade leading to inflammatory damage of the ENS.

Table 2 Immunohistochemical markers to analyze full thickness biopsy of patients with CIPO

Markers	Cell targets and sites	Description
PGP9.5, NSE, MAP-2, NFs, tubulins, Hu C/D	Neurons: Membrane/Cytoplasmic	Identification of the general structure of the ENS
$\beta$ -S-100, GFAP	Glial cells: Cytoplasmic	Detection of enteroglia cells
Kit	Interstitial cells of Cajal: Membrane/Cytoplasmic	Different ICC networks
SP, VIP, PACAP, CGRP, NPY, Galanin, 5-HT, NOS, ChAT, somatostatin, Calbindin, NeuN, NK1, NK2 and NK3	Subclasses of enteric neurons; interstitial cells of Cajal: Membrane/Cytoplasmic	Characterization of neurochemical coding and enteric neuron subclasses; subsets of interstitial cells of Cajal
Bcl-2, TUNEL, Caspase-3, Caspase-8, Apaf-1	Apoptosis and related mechanisms: Nuclear/Cytoplasmic	Assessment of apoptosis and related pathways
Actin, myosin, desmin, smoothelin	Smooth muscle cells: Cytoplasmic	Assessment of smooth muscle integrity
CD3, CD4, CD8, CD79 $\alpha$ , CD68; MIP-1 $\alpha$ , TNF- $\alpha$ , IFN- $\gamma$	Immune cells, chemokines and cytokines: Membrane/Cytoplasmic	Evaluation of B (CD79 $\alpha$ ) and T-lymphocytes (CD3), T-helper (CD4), T-suppressor (CD8), macrophages (CD68) in enteric ganglionitis; MIP-1 $\alpha$ is a chemokine; TNF- $\alpha$ and IFN- $\gamma$ are inflammatory cytokines

Bcl-2: B cell lymphoma-2 protein; ChAT: Choline acetyltransferase; CGRP: Calcitonin gene-related peptide; ENS: Enteric nervous system; GFAP: Glial fibrillary acidic protein; Hu C/D: Hu C/D molecular antigen; IFN- $\gamma$ : Interferon  $\gamma$ ; MAP-2: Microtubule associated protein-2; MIP-1 $\alpha$ : Macrophage inflammatory protein-1 $\alpha$ ; NeuN: Neuronal-specific nuclear protein; NFs: Neurofilaments; NK1, NK2, NK3: Neurokinin1, neurokinin2, neurokinin3; NOS: Nitric oxide synthase; NPY: Neuropeptide Y; NSE: Neuron-specific enolase; PACAP: Pituitary adenylate cyclase activating polypeptide; PGP9.5: Protein gene product 9.5; 5-HT: 5-hydroxytryptamine (serotonin); SP: Substance P; TNF- $\alpha$ : Tumor necrosis factor  $\alpha$ ; Tubulins: Cytoskeletal proteins; TUNEL: Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling; VIP: Vasoactive intestinal polypeptide.

Further to lymphocytic ganglionitis, Schappi *et al* have reported on eosinophilic ganglionitis characterized by eosinophils infiltrating the myenteric plexus of pediatric patients with CIPO<sup>[26]</sup>. In contrast to lymphocytic, the eosinophilic ganglionitis does not appear to evoke neuronal degeneration and loss and, therefore, gut dysmotility may be interpreted as a functional impairment of the ENS due to the infiltrate *per se* or humoral messengers released by eosinophils. Recently, mast cell predominant ganglionitis has been described in patients with severe gut dysmotility (including CIPO)<sup>[27]</sup>. The mast cells detected within myenteric ganglia in these patients were associated with markedly reduced neuronal nitric oxide synthase expression identified at molecular and immunohistochemical level. These findings suggest an impaired enteric inhibitory innervation in these peculiar subsets of CIPO.

Degenerative (or non inflammatory) neuropathies may be regarded as the end result of several putative pathogenic mechanisms, such as altered calcium signaling, mitochondrial dysfunction and production of free radicals, leading to degeneration and loss of the intrinsic neurons of the gut<sup>[28]</sup>. Degenerative neuropathies can be familial (related to a genetic background-see above) or sporadic and classified into primary (idiopathic) or secondary forms to a variety of causes, such as radiations, vinka alkaloids, myxedema, diabetes mellitus, muscular dystrophy and amyloidosis. Typical neuropathological findings reported in neurodegenerative CIPO include various qualitative (neuronal swelling, intranuclear inclusions, axonal degeneration and other lesions) and quantitative (especially hypoganglionosis) abnormalities of the ENS. Sporadic cases of visceral neuropathies are associated with two major patterns of alterations: (1) A marked reduction of intramural (especially myenteric) neural cells mainly associated with swollen neural cell bodies and processes, fragmentation and loss of axons and proliferation of glial cells; (2) A loss of the normal staining in subsets of enteric neurons, in the absence of

dendritic swelling or glial proliferation<sup>[6,20,21]</sup>. Since no reliable models of degenerative neuropathies exist, the mechanisms through which exogenous noxae or other triggering factors initiate degenerative processes in enteric neurons remain obscure. Enteric neurons of patients with severe forms of idiopathic intrinsic neuropathy display a decreased expression of the protein encoded by *Bcl-2*, a gene related to one of the intracellular pathways leading to programmed cell death<sup>[4,29,30]</sup>. Indeed, this finding has been associated with an increased number of neurons displaying TUNEL, a marker of apoptosis<sup>[31]</sup>.

Abnormalities of enteric glia may also contribute to intrinsic neuropathy either attracting immune cells to the ENS or resulting in insufficient support/trophism to enteric neurons and thus eliciting neurodegenerative events in the absence of inflammation<sup>[32]</sup>.

### Enteric mesenchymopathies

Abnormalities to ICCs have been detected in gut tissues of patients with CIPO. These include decreased ICC density, loss of processes and damaged intracellular cytoskeleton and organelles as revealed by immunohistochemical analysis and electron microscopy<sup>[33-37]</sup>. As a result, it has been proposed that the impairment of the major functional subclasses of ICC (i.e. those involved in pacemaker activity and neurotransmission to smooth muscle) may contribute to enteric motility abnormalities detectable in patients with CIPO.

### Enteric myopathies

Histopathological analysis of the enteric muscle layer may reveal the existence of muscular abnormalities (i.e. smooth muscle fibrosis and vacuolization) of the circular and longitudinal layers in patients with primary visceral myopathy<sup>[38,39]</sup>. A controlled multinational study conducted by Knowles *et al* has proposed that a selective decrease or even absence of  $\alpha$ -actin in the circular muscle of the small bowel wall can be regarded as biological markers of

CIPO<sup>[40]</sup>. Although exciting, the possibility that a defective expression/localization of  $\alpha$ -actin may be a biomarker of a heterogeneous disease such as CIPO awaits solid confirmatory evidence.

The histopathological details concerning other segments of the gut as well as extra-digestive systems (i.e. urinary tract, gall-bladder) is poorly characterized and further studies are awaited to elucidate this important aspect.

## CLINICAL FEATURES

Subocclusive episodes can strike in apparently healthy people, but the onset of CIPO is generally insidious, with gastrointestinal symptoms which precede the first acute episode.

The typical clinical manifestation of CIPO is characterized by recurrent episodes of abdominal pain, abdominal distension and inability to defecate (flatus may not be completely suppressed), with or without vomiting, mimicking a mechanical sub-occlusion. During acute episodes radiological evidence of distended bowel loops and air-fluid levels in the upright position is an important diagnostic marker of this pathological condition. Acute episodes can last only a few hours, but in the most severe cases intestinal loops are chronically distended and air-fluid levels are invariably detected. Due to this misleading clinical manifestation, a history of multiple, useless surgeries are typical of the syndrome. Thus, many patients have abdominal adhesions and the concomitant presence of functional and mechanical (secondary to adhesions) obstruction is often impossible to rule out despite extensive investigations.

Between subocclusive episodes patients are very rarely asymptomatic, and almost invariably complain of severe digestive symptoms<sup>[1,2]</sup> suggestive of delayed transit in the proximal and/or distal portions of the alimentary canal. Nausea, vomiting and weight loss are predominant symptoms when the functional derangement primarily affects the upper gastrointestinal tract, while diffuse abdominal pain, abdominal distension and constipation are suggestive of a more distal involvement of the gut. Dysphagia is present in a low proportion of CIPO patients although it is relatively frequent in forms secondary to progressive systemic sclerosis.

Diarrhea and steatorrhea often occur as a consequence to small bowel bacterial overgrowth.

This pathologically accelerated transit is often well accepted by patients since it is associated with partial relief of other digestive symptoms, but it contributes to determine intestinal malabsorption and deteriorate nutritional conditions. Indeed, many patients are afflicted by inability to maintain a normal body weight, despite dietary manipulations, both because of the deranged digestive functions and because food ingestion often exacerbates digestive symptoms and consequently patients tend to avoid a normal oral nutrition.

Urinary symptoms, generally associated with evidence of urinary tract distension, are also frequent.

Depression or other psychological disturbances are often secondary to the disabling digestive problems and the disappointing quality of healthcare received.

## DIAGNOSTIC PROCEDURES

The diagnosis of CIPO is mainly clinical, supported by radiographic documentation of dilated bowel with air-fluid level, after exclusion of organic lesions occluding the gut lumen, as detected by radiologic and/or endoscopic investigations. Thus, diagnostic tests in patients with suspected CIPO are necessary to exclude mechanical occlusion, identify possible causes of secondary forms, explore underlying pathophysiological mechanisms and disclose possible complications.

### Radiology

Radiology is one of the most important examinations in the diagnosis of CIPO. Plain abdominal films identify typical signs of intestinal occlusion such as distended bowel loops with air-fluid levels, the latter obtained with the patients in the upright position (as specified above). Contrast studies are necessary to exclude the presence of organic lesions responsible for the occlusion. Entero-CT scan allows simultaneous internal and external views of the gut wall, abdominal CT and MR scans are important in investigating possible causes of gut compression, while MR angiography may non-invasively identify congenital or acquired vascular abnormalities. Excretory urograms should be performed in patients with urinary symptoms.

Symptoms suggestive of a subocclusive state in the absence of dilated bowel with air fluid levels at radiology have been defined by some authors as a "mild forms of CIPO"<sup>[41]</sup>. Nonetheless, this definition has been criticized<sup>[4]</sup>. In fact, preliminary studies suggest that patients with extremely severe digestive symptoms and malnutrition, but no radiological evidence of intestinal occlusion, have a significantly reduced probability of undergoing abdominal surgery and present less severe motility disorders<sup>[42]</sup>.

### Endoscopy

The main indication of upper gastrointestinal endoscopy is exclusion of mechanical occlusions in the gastro-jejunal and ileo-colonic regions. It allows to exclusion of false positive radiologic diagnoses of mechanical occlusion in the duodenum and proximal small bowel, as in many cases of the so-called "aorto-mesenteric compression syndrome"<sup>[43]</sup>. Mucosal biopsies of the small bowel should be taken to rule out celiac disease. Colonoscopy also has a therapeutic potential, since it can be used to try to decompress the large bowel<sup>[44]</sup>.

### Laboratory tests

Laboratory tests are useful to identify the presence of potentially curable diseases responsible for secondary forms, but also to monitor hydro-electrolyte balance and circulating levels of essential elements in patients on parenteral nutrition or, in general, with a severe malnutrition.

### Manometry

Small bowel manometry is invariably abnormal in CIPO patients<sup>[2,3,45]</sup>; however, the test is not of diagnostic value due to its low specificity. At best, it can play a supportive role in defining the diagnosis, since it can contribute to differentiate mechanical from functional obstruction and



to recognize the underlying pathophysiological mechanism<sup>[2,3,45]</sup>.

Describing in detail small bowel manometric abnormalities of CIPO goes beyond the scope of the present review. They can be summarized as follows: uncoordinated bursts of powerful contractions with variable duration are suggestive of an underlying intrinsic neuropathy<sup>[1-4,45-48]</sup> conversely, normally coordinated motor patterns with low amplitude have been reported in patients with a myogenic disorder<sup>[1-4,45-48]</sup>. Nonetheless, low amplitude contractions may merely reflect the inability of the manometric technique to record non-occlusive contractions, such as in the case of dilated bowel loops<sup>[1-4,45-48]</sup>.

Unlike what is observed in pseudo-obstruction, the manometric pattern of mechanical occlusion is characterized by giant contractions (prolonged contractions lasting at least 10 s and can be either propagated or non propagated) or clustered contractions (3-10 regular contractions, occurring 1 per 5 s preceded and followed by  $\geq 1$  min of absent motor activity lasting at least 20 min and can be either propagated or non propagated)<sup>[1-4,45-49]</sup>.

Esophageal manometry generally adds very little to the diagnostic work-up of CIPO, but it plays an important diagnostic and prognostic role if the disease is secondary to scleroderma. Ano-rectal manometry is important to rule out Hirschsprung's disease, particularly in patients with intractable constipation and a marked distension of the large intestine.

### **Biopsy and pathologic examination**

Full thickness biopsies should be obtained from dilated and nor dilated tracts of the alimentary canal in all patients with suspected CIPO who undergo surgery for unexplained occlusive episodes. Biopsies should be processed for in depth pathological evaluation by both traditional staining and immunohistochemistry techniques in dedicated laboratories with a specific interest in this area, as specified above.

## **NATURAL HISTORY**

Even if clinical experience shows that CIPO is a progressive disease that often leads to death, only few studies have precisely described the natural history of this pathology and its symptoms prognostic values, especially in the adult age. Children generally present the first manifestations of CIPO at birth or during the first years of age<sup>[50-53]</sup>. The pediatric expression of the disease is often characterized by a particularly severe course, with mortality rates extremely high within the first year of age, mainly due to surgical and parenteral nutrition complications<sup>[52,53]</sup>. Several predictors of poor outcome have been identified in children including myopathic forms, malrotation, short bowel syndrome, and urinary tract involvement<sup>[50,51]</sup>.

In the adult population, the first sub-occlusive episode is often preceded by a long history of non-specific, progressively more severe digestive symptoms. An acute onset of the disease occurs in only one-fourth of the cases<sup>[2]</sup>.

After diagnosis is established the frequency of sub-

occlusive episodes and, consequently, also of surgical procedures tend to decrease. Nevertheless, the clinical course of CIPO is almost invariably severe<sup>[1,2,52-54]</sup> with progressive deterioration of bowel function and digestive symptoms. In order to control both the body weight and the abdominal pain most patients progressively limit oral nutrition and end up on long-term parenteral nutrition.

The main causes of death are TPN-related complications, surgery-related complications, and post-transplantation complication, together with septic shock of GI origin. A variety of clinical, histological and manometric parameters have been found to be predictive of a poor clinical outcome in adult patients, including myopathy and decreased contractile activity<sup>[2,50,52,53,55-60]</sup>. MNGIE has a particularly poor prognosis with slowly progressive evolution and death around 40 years of age<sup>[11]</sup>.

## **THERAPY**

The treatment of CIPO is difficult and often provides unsatisfactory results. Of course, treatment of the underlying disease is mandatory in secondary forms whenever available<sup>[57]</sup>.

### **Treatment of the acute phase**

During acute phases patients should be treated as those with acute mechanical obstruction. Fluid and electrolytes balance should be maintained *via IV* infusions; abdominal decompression should be attempted by positioning of nasogastric and rectal tubes. The former generally prevents vomiting and *ab ingestis* while the latter is generally ineffective and colonic decompression can be attempted by colonoscopy or cecostomy (see below). In case of prolonged subocclusive episodes systemic or poorly absorbable antibiotics are necessary to prevent bacterial overgrowth. Appropriate caloric support must be provided by *IV* infusion. Erythromycin, somatostatin and neostigmine can be used to promote transit and decrease the duration of acute episodes<sup>[61-63]</sup>.

### **Nutritional support**

The nutritional status of patients with CIPO is generally poor. Frequent small meals with liquid or homogenized foods, with or without oral nutritional supplements, may help patients with sufficient residual digestive functions. Enteral nutrition is an option for patients whose motility disorder is mainly localized in the stomach and duodenum. It presents fewer complications than parenteral nutrition, but clinical experience suggests that enteral feeding is rarely tolerated by patients. In the most severe cases, when small bowel function is diffusely affected, parenteral nutrition is necessary to satisfy nutritional requirements. The main limitations of this nutritional support include liver insufficiency, pancreatitis, glomerulonephritis and catheter-related complications (i.e. thrombosis and septicemia)<sup>[58,59]</sup>.

### **Pharmacological therapy**

The pharmacological treatment of CIPO is aimed at controlling symptoms and avoiding complications. Co-



prescription of antiemetics, antisecretory, antispasmodics, laxatives or antidiarrheal and analgesic drugs is often necessary. Prokinetics are often prescribed, with the intention to improve gastrointestinal motility and to control visceral sensitivity<sup>[2,54]</sup>.

Some prokinetics seem to be more effective than others: metoclopramide, domperidone, bethanechol or neostigmine are often used, but with only limited success, while cisapride, that is currently available only in some parts of the world, has been reported to exert positive effects<sup>[60,64-67]</sup>. Two controlled trials including CIPO patients described positive effects of cisapride in accelerating gastric emptying<sup>[60]</sup> and improving symptoms<sup>[66]</sup>. Erythromycin is a macrolide antibiotic with a specific agonist action on the motilin receptors of the proximal gastrointestinal tract. It increases antral contraction and promotes gastric emptying, while its effects on colonic motility are controversial: at low doses it stimulates intestinal contractions, but doses normally used to enhance gastric emptying decrease motility of the small intestine<sup>[61]</sup>. Octreotide is a long-acting somatostatin analog which increases intestinal motor activity and decreases bacterial overgrowth<sup>[62]</sup>. Co-prescription of erythromycin and octreotide can be useful to control both the gastric emptying and the intestinal motility<sup>[68]</sup>. Anticholinesterase drugs have been described as effective in autoimmune gastrointestinal motor disorders<sup>[69]</sup>. Tegaserod, a more recent 5-HT<sub>4</sub> agonist, was also recommended for the treatment of subocclusive episodes in CIPO, but the drug has been withdrawn from the market<sup>[70]</sup>. A preliminary open study describes encouraging results exerted gastric electrical stimulation on nausea and vomiting in a small number of CIPO patients<sup>[71]</sup>.

Opioids are required in patients with intractable pain, but their constipating effect can further deteriorate digestive functions<sup>[72,73]</sup>.

Antibiotics are often useful to contrast bacterial overgrowth. Poorly absorbable antibiotics such as paramomycin and rifaximine should be preferred, but alternating cycles with metronidazole and tetracycline are necessary to limit resistances<sup>[74]</sup>.

Steroids or other immunosuppressive treatments are recommended when CIPO is related to an underlying inflammatory neuropathy. These cases have to be selected through tissue analysis or at least suspected by the identification of circulating anti-neuronal antibodies<sup>[6]</sup>. Treatment of MNGIE is largely supportive, being based on parenteral nutrition and/or supplementation with coenzyme Q, riboflavin and other vitamins (vitamin C, vitamin K3, carnitine). Prompt treatment of fever and infections and avoidance of extremes in temperature, over exercise, drugs known to interfere with mitochondrial functions (phenytoin, chloramphenicol, tetracycline, macrolides, and aminoglycosides), are also recommended. Infusion platelets to reduce thymidine level have been reported to exert some positive effect in preliminary study in MNGIE patients<sup>[75]</sup>.

### **Surgical therapy**

Even if CIPO patients often undergo surgical procedures, this kind of approach has only a limited role in the

management of the disease and has to be considered only in some carefully selected patients. Specifically, since CIPO generally involves the whole alimentary canal, only rare cases can benefit from surgical resections. Indeed surgery can precipitate deterioration of the clinical conditions and should be performed only if strictly necessary. Full thickness biopsies should be obtained whenever possible for pathological examination as stated above. In particular, surgery can be considered in patients having what appears to be localized involvement of the gastrointestinal tract, but CIPO is often a progressive disease and the benefit is likely temporary<sup>[41,76]</sup>.

Gastrostomies and enterostomies can effectively decrease retching, vomiting and abdominal distension and represent a possible option in patients who can be fed by enteral nutrition. Furthermore, decompression of distended bowel loops can exert a positive effect on the transport capacities of the alimentary canal which, in turn, results in a decreased frequency of further hospital admissions and surgeries.

Small bowel or, when needed, multivisceral transplantation is available only in a few highly specialized centers. The general outcome of this surgical procedure has markedly improved with the use of the immunosuppressive agent tacrolimus associated with steroid and together a number of induction agents such as alemtuzumab, antithymocyte globulins and daclizumab<sup>[77]</sup>. However, the need for long-term parenteral nutrition, re-laparotomies, organ rejection and, especially, bacterial infections are frequent complications and the procedure still have mortality rates approaching 50% at 5 years. Predictors of post-transplant complications are: concomitant neuromuscular disorders of the urinary tract, chronic use of opioids and technical problems determined by previous multiple laparotomies and/or the need of gastrectomy for gastroparesis. Nonetheless, transplantation should be considered when all other therapeutic options have failed according to the following indications: chronic intestinal failure with a high risk of mortality, life-threatening complications of parenteral nutrition, lack of venous access, disease-related poor quality of life despite optimal parenteral nutrition<sup>[58]</sup>.

## **CONCLUSION**

CIPO is a rare and often misdiagnosed pathological condition. Even if the acute phases can be hardly differentiated by mechanical occlusions and the inter-crisis digestive symptoms can mimic other severe functional digestive syndromes, the syndrome should be recognized based on the typical combination of clinical features, natural course and radiological signs. The diagnostic suspicion should be then confirmed by more accurate examinations, in order to identify possible causes of secondary forms and underlying pathophysiological mechanisms.

Management of CIPO remains extremely challenging and often disappointing.

A greater awareness of the clinical features of CIPO would help to limit surgical procedures to a minimum and, even more importantly, to collect full-thickness biopsies

for analysis of the gut neuromuscular layer at an early and potentially curable stage of the disease.

## REFERENCES

- 1 **Stanghellini V**, Camilleri M, Malagelada JR. Chronic idiopathic intestinal pseudo-obstruction: clinical and intestinal manometric findings. *Gut* 1987; **28**: 5-12
- 2 **Stanghellini V**, Cogliandro RF, De Giorgio R, Barbara G, Morselli-Labate AM, Cogliandro L, Corinaldesi R. Natural history of chronic idiopathic intestinal pseudo-obstruction in adults: a single center study. *Clin Gastroenterol Hepatol* 2005; **3**: 449-458
- 3 **Cogliandro RF**, De Giorgio R, Barbara G, Cogliandro L, Concordia A, Corinaldesi R, Stanghellini V. Chronic intestinal pseudo-obstruction. *Best Pract Res Clin Gastroenterol* 2007; **21**: 657-669
- 4 **Stanghellini V**, Cogliandro RF, de Giorgio R, Barbara G, Salvioli B, Corinaldesi R. Chronic intestinal pseudo-obstruction: manifestations, natural history and management. *Neurogastroenterol Motil* 2007; **19**: 440-452
- 5 **Di Lorenzo C**. Pseudo-obstruction: current approaches. *Gastroenterology* 1999; **116**: 980-987
- 6 **De Giorgio R**, Camilleri M. Human enteric neuropathies: morphology and molecular pathology. *Neurogastroenterol Motil* 2004; **16**: 515-531
- 7 **De Giorgio R**, Sarnelli G, Corinaldesi R, Stanghellini V. Advances in our understanding of the pathology of chronic intestinal pseudo-obstruction. *Gut* 2004; **53**: 1549-1552
- 8 **De Giorgio R**, Seri M, Cogliandro R, Cusano R, Fava M, Caroli F, Panetta D, Forabosco P, Barbara G, Ravazzolo R, Ceccherini I, Corinaldesi R, Stanghellini V. Analysis of candidate genes for intrinsic neuropathy in a family with chronic idiopathic intestinal pseudo-obstruction. *Clin Genet* 2001; **59**: 131-133
- 9 **Degincerti A**, De Giorgio R, Cefle K, Devoto M, Pippucci T, Castegnaro G, Panza E, Barbara G, Cogliandro RF, Mungan Z, Palanduz S, Corinaldesi R, Romeo G, Seri M, Stanghellini V. A novel locus for syndromic chronic idiopathic intestinal pseudo-obstruction maps to chromosome 8q23-q24. *Eur J Hum Genet* 2007; **15**: 889-897
- 10 **Gargiulo A**, Auricchio R, Barone MV, Cotugno G, Reardon W, Milla PJ, Ballabio A, Ciccodicola A, Auricchio A. Filamin A is mutated in X-linked chronic idiopathic intestinal pseudo-obstruction with central nervous system involvement. *Am J Hum Genet* 2007; **80**: 751-758
- 11 **Finsterer J**. Mitochondriopathies. *Eur J Neurol* 2004; **11**: 163-186
- 12 **Hirano M**, Silvestri G, Blake DM, Lombes A, Minetti C, Bonilla E, Hays AP, Lovelace RE, Butler I, Bertorini TE. Mitochondrial neurogastrointestinal encephalomyopathy (MNGIE): clinical, biochemical, and genetic features of an autosomal recessive mitochondrial disorder. *Neurology* 1994; **44**: 721-727
- 13 **Nishino I**, Spinazzola A, Papadimitriou A, Hammans S, Steiner I, Hahn CD, Connolly AM, Verloes A, Guimaraes J, Maillard I, Hamano H, Donati MA, Semrad CE, Russell JA, Andreu AL, Hadjigeorgiou GM, Vu TH, Tadesse S, Nygaard TG, Nonaka I, Hirano I, Bonilla E, Rowland LP, DiMauro S, Hirano M. Mitochondrial neurogastrointestinal encephalomyopathy: an autosomal recessive disorder due to thymidine phosphorylase mutations. *Ann Neurol* 2000; **47**: 792-800
- 14 **Gillis L**, Kaye E. Diagnosis and management of mitochondrial diseases. *Pediatr Clin North Am* 2002; **49**: 203-219
- 15 **Marti R**, Spinazzola A, Tadesse S, Nishino I, Nishigaki Y, Hirano M. Definitive diagnosis of mitochondrial neurogastrointestinal encephalomyopathy by biochemical assays. *Clin Chem* 2004; **50**: 120-124
- 16 **Spinazzola A**, Marti R, Nishino I, Andreu AL, Naini A, Tadesse S, Pela I, Zammarchi E, Donati MA, Oliver JA, Hirano M. Altered thymidine metabolism due to defects of thymidine phosphorylase. *J Biol Chem* 2002; **277**: 4128-4133
- 17 **Valentino ML**, Marti R, Tadesse S, Lopez LC, Manes JL, Lyzak J, Hahn A, Carelli V, Hirano M. Thymidine and deoxyuridine accumulate in tissues of patients with mitochondrial neurogastrointestinal encephalomyopathy (MNGIE). *FEBS Lett* 2007; **581**: 3410-3414
- 18 **Nishigaki Y**, Marti R, Hirano M. ND5 is a hot-spot for multiple atypical mitochondrial DNA deletions in mitochondrial neurogastrointestinal encephalomyopathy. *Hum Mol Genet* 2004; **13**: 91-101
- 19 **King PH**, Redden D, Palmgren JS, Nabors LB, Lennon VA. Hu antigen specificities of ANNA-I autoantibodies in paraneoplastic neurological disease. *J Autoimmun* 1999; **13**: 435-443
- 20 **Krishnamurthy S**, Schuffler MD. Pathology of neuromuscular disorders of the small intestine and colon. *Gastroenterology* 1987; **93**: 610-639
- 21 **De Giorgio R**, Guerrini S, Barbara G, Cremon C, Stanghellini V, Corinaldesi R. New insights into human enteric neuropathies. *Neurogastroenterol Motil* 2004; **16** Suppl 1: 143-147
- 22 **Caras SD**, McCallum HR, Brashear HR, Smith TK. The effect of human antineuronal antibodies on the ascending excitatory reflex and peristalsis in isolated guinea pig ileum: "Is the paraneoplastic syndrome a motor neuron disorder?". *Gastroenterology* 1996; **110**: A643
- 23 **Talamonti L**, Li Q, Beyak M, Trevisani M, Michel K, Campi B, Barbara G, Stanghellini V, Corinaldesi R, Geppetti P, Grundy D, Schemann M, De Giorgio R. Sensory. motor abnormalities in severe gut dysmotility: role of anti-HuD neuronal antibodies. *Neurogastroenterol Motil* 2006; **18**: 669
- 24 **De Giorgio R**, Bovara M, Barbara G, Canossa M, Sarnelli G, De Ponti F, Stanghellini V, Tonini M, Cappello S, Pagnotta E, Nobile-Orazio E, Corinaldesi R. Anti-HuD-induced neuronal apoptosis underlying paraneoplastic gut dysmotility. *Gastroenterology* 2003; **125**: 70-79
- 25 **Debinski HS**, Kamm MA, Talbot IC, Khan G, Kangro HO, Jeffries DJ. DNA viruses in the pathogenesis of sporadic chronic idiopathic intestinal pseudo-obstruction. *Gut* 1997; **41**: 100-106
- 26 **Schappi MG**, Smith VV, Milla PJ, Lindley KJ. Eosinophilic myenteric ganglionitis is associated with functional intestinal obstruction. *Gut* 2003; **52**: 752-755
- 27 **Accarino A**, Colucci R, Barbara G, Malagelada C, Gori A, Vera G, Cogliandro RF, Ghisu N, Bernardini N, Blandizzi C, Stanghellini V, Corinaldesi R, Azpiroz F, Del Tacca M, Malagelada JR, De Giorgio R. Mast cell neuromuscular involvement in patients with severe gastrointestinal motility disorders. *Gut* 2007; **56**: A18
- 28 **Hall KE**, Wiley JW. Neural injury, repair and adaptation in the GI tract. I. New insights into neuronal injury: a cautionary tale. *Am J Physiol* 1998; **274**: G978-G983
- 29 **Hockenbery D**, Nunez G, Millman C, Schreiber RD, Korsmeyer SJ. Bcl-2 is an inner mitochondrial membrane protein that blocks programmed cell death. *Nature* 1990; **348**: 334-336
- 30 **De Giorgio R**, Barbara G, Stanghellini V, De Ponti F, Guerrini S, Cogliandro L, Ceccarelli C, Salvioli B, Adamo C, Cogliandro R, Tonini M, Corinaldesi R. Reduced bcl-2 expression in the enteric nervous system (ENS) as a marker for neural degeneration in patients with gastrointestinal motor disorders (GIMD). *Gastroenterology* 2000; **118**: A867
- 31 **Sarnelli G**, Stanghellini V, Barbara G, Pasquinelli G, Di Nardo G, Cremon C, Cogliandro RF, Salvioli B, Gori A, Cuomo R, Corinaldesi R, De Giorgio R. Reduced Bcl-2 expression and increased myenteric neuron apoptosis in patients with idiopathic enteric neuropathy. *Gastroenterology* 2005; **128**: A23
- 32 **Ruhl A**. Glial cells in the gut. *Neurogastroenterol Motil* 2005; **17**: 777-790
- 33 **Boeckxstaens GE**, Rumessen JJ, de Wit L, Tytgat GN, Vanderwinden JM. Abnormal distribution of the interstitial cells of cajal in an adult patient with pseudo-obstruction and megaduodenum. *Am J Gastroenterol* 2002; **97**: 2120-2126
- 34 **Isozaki K**, Hirota S, Miyagawa J, Taniguchi M, Shinomura Y, Matsuzawa Y. Deficiency of c-kit+ cells in patients with

- a myopathic form of chronic idiopathic intestinal pseudo-obstruction. *Am J Gastroenterol* 1997; **92**: 332-334
- 35 **Huizinga JD**, Thuneberg L, Vanderwinden JM, Rumessen JJ. Interstitial cells of Cajal as targets for pharmacological intervention in gastrointestinal motor disorders. *Trends Pharmacol Sci* 1997; **18**: 393-403
  - 36 **Feldstein AE**, Miller SM, El-Youssef M, Rodeberg D, Lindor NM, Burgart LJ, Szurszewski JH, Farrugia G. Chronic intestinal pseudoobstruction associated with altered interstitial cells of cajal networks. *J Pediatr Gastroenterol Nutr* 2003; **36**: 492-497
  - 37 **Sanders KM**, Ordog T, Ward SM. Physiology and pathophysiology of the interstitial cells of Cajal: from bench to bedside. IV. Genetic and animal models of GI motility disorders caused by loss of interstitial cells of Cajal. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G747-G756
  - 38 **De Giorgio R**, Guerrini S, Barbara G, Stanghellini V, De Ponti F, Corinaldesi R, Moses PL, Sharkey KA, Mawe GM. Inflammatory neuropathies of the enteric nervous system. *Gastroenterology* 2004; **126**: 1872-1883
  - 39 **Smith VV**, Gregson N, Foggensteiner L, Neale G, Milla PJ. Acquired intestinal aganglionosis and circulating autoantibodies without neoplasia or other neural involvement. *Gastroenterology* 1997; **112**: 1366-1371
  - 40 **Knowles CH**, Silk DB, Darzi A, Veress B, Feakins R, Raimundo AH, Crompton T, Browning EC, Lindberg G, Martin JE. Deranged smooth muscle alpha-actin as a biomarker of intestinal pseudo-obstruction: a controlled multinational case series. *Gut* 2004; **53**: 1583-1589
  - 41 **Murr MM**, Sarr MG, Camilleri M. The surgeon's role in the treatment of chronic intestinal pseudoobstruction. *Am J Gastroenterol* 1995; **90**: 2147-2151
  - 42 **Cogliandro R**, Stanghellini V, Cogliandro L, Guidi M, Bini L, Barbara G, De Giorgio R, Morselli Labate AM, Corinaldesi R. Small Bowel manometric findings in different forms of severe digestive syndromes. *Neurogastroenterol Motil* 2004; **16**: A838
  - 43 **Malagelada JR**, Stanghellini V. Manometric evaluation of functional upper gut symptoms. *Gastroenterology* 1985; **88**: 1223-1231
  - 44 **Attar A**, Kuoch V, Ducreux M, Benamouzig R, Malka D. Simultaneous decompression colonoscopy and radiologic G-tube insertion in a patient with megacolon because of chronic colonic pseudo-obstruction. *Gastrointest Endosc* 2005; **62**: 975-976; discussion 976
  - 45 **Kellow JE**. Small intestine: normal function and clinical disorders. Manometry. In: Schuster MM, Crowell MD, Koch KL, editors. Schuster atlas of gastrointestinal motility in health and disease. Hamilton-London: BC Decker, 2002: 219-236
  - 46 **Hyman PE**, McDiarmid SV, Napolitano J, Abrams CE, Tomomasa T. Antroduodenal motility in children with chronic intestinal pseudo-obstruction. *J Pediatr* 1988; **112**: 899-905
  - 47 **Boige N**, Faure C, Cargill G, Mashako LM, Cordeiro-Ferreira G, Viarme F, Cezard JP, Navarro J. Manometrical evaluation in visceral neuropathies in children. *J Pediatr Gastroenterol Nutr* 1994; **19**: 71-77
  - 48 **Cucchiara S**, Annese V, Minella R, Franco MT, Iervolino C, Emiliano M, Auricchio S. Antroduodenal manometry in the diagnosis of chronic idiopathic intestinal pseudoobstruction in children. *J Pediatr Gastroenterol Nutr* 1994; **18**: 294-305
  - 49 **Camilleri M**. Jejunal manometry in distal subacute mechanical obstruction: significance of prolonged simultaneous contractions. *Gut* 1989; **30**: 468-475
  - 50 **Fell JM**, Smith VV, Milla PJ. Infantile chronic idiopathic intestinal pseudo-obstruction: the role of small intestinal manometry as a diagnostic tool and prognostic indicator. *Gut* 1996; **39**: 306-311
  - 51 **Heneyke S**, Smith VV, Spitz L, Milla PJ. Chronic intestinal pseudo-obstruction: treatment and long term follow up of 44 patients. *Arch Dis Child* 1999; **81**: 21-27
  - 52 **Mousa H**, Hyman PE, Cocjin J, Flores AF, Di Lorenzo C. Long-term outcome of congenital intestinal pseudoobstruction. *Dig Dis Sci* 2002; **47**: 2298-2305
  - 53 **Faure C**, Goulet O, Ategbro S, Breton A, Tounian P, Ginies JL, Roquelaure B, Despres C, Scaillon M, Maurage C, Paquot I, Hermier M, De Napoli S, Dabadie A, Huet F, Baudon JJ, Larchet M. Chronic intestinal pseudoobstruction syndrome: clinical analysis, outcome, and prognosis in 105 children. French-Speaking Group of Pediatric Gastroenterology. *Dig Dis Sci* 1999; **44**: 953-959
  - 54 **Mann SD**, Debinski HS, Kamm MA. Clinical characteristics of chronic idiopathic intestinal pseudo-obstruction in adults. *Gut* 1997; **41**: 675-681
  - 55 **Hyman PE**, Di Lorenzo C, McAdams L, Flores AF, Tomomasa T, Garvey TQ 3rd. Predicting the clinical response to cisapride in children with chronic intestinal pseudo-obstruction. *Am J Gastroenterol* 1993; **88**: 832-836
  - 56 **Di Lorenzo C**, Flores AF, Buie T, Hyman PE. Intestinal motility and jejunal feeding in children with chronic intestinal pseudo-obstruction. *Gastroenterology* 1995; **108**: 1379-1385
  - 57 **Stanghellini V**, Corinaldesi R, Ghidini C, Ricci Maccarini M, De Giorgio R, Biasco G, Brillanti S, Paparo GF, Barbara L. Reversibility of gastrointestinal motor abnormalities in chronic intestinal pseudo-obstruction. *Hepatogastroenterology* 1992; **39**: 34-38
  - 58 **Pironi L**, Spinucci G, Paganelli F, Merli C, Masetti M, Miglioli M, Pinna AD. Italian guidelines for intestinal transplantation: potential candidates among the adult patients managed by a medical referral center for chronic intestinal failure. *Transplant Proc* 2004; **36**: 659-661
  - 59 **Guglielmi FW**, Boggio-Bertinet D, Federico A, Forte GB, Guglielmi A, Loguercio C, Mazzuoli S, Merli M, Palmo A, Panella C, Pironi L, Francavilla A. Total parenteral nutrition-related gastroenterological complications. *Dig Liver Dis* 2006; **38**: 623-642
  - 60 **Camilleri M**, Malagelada JR, Abell TL, Brown ML, Hench V, Zinsmeister AR. Effect of six weeks of treatment with cisapride in gastroparesis and intestinal pseudoobstruction. *Gastroenterology* 1989; **96**: 704-712
  - 61 **Emmanuel AV**, Shand AG, Kamm MA. Erythromycin for the treatment of chronic intestinal pseudo-obstruction: description of six cases with a positive response. *Aliment Pharmacol Ther* 2004; **19**: 687-694
  - 62 **Soudah HC**, Hasler WL, Owyang C. Effect of octreotide on intestinal motility and bacterial overgrowth in scleroderma. *N Engl J Med* 1991; **325**: 1461-1467
  - 63 **De Giorgio R**, Barbara G, Stanghellini V, Tonini M, Vasina V, Cola B, Corinaldesi R, Biagi G, De Ponti F. Review article: the pharmacological treatment of acute colonic pseudo-obstruction. *Aliment Pharmacol Ther* 2001; **15**: 1717-1727
  - 64 **Di Lorenzo C**, Reddy SN, Villanueva-Meyer J, Mena I, Martin S, Hyman PE. Cisapride in children with chronic intestinal pseudoobstruction. An acute, double-blind, crossover, placebo-controlled trial. *Gastroenterology* 1991; **101**: 1564-1570
  - 65 **Camilleri M**, Balm RK, Zinsmeister AR. Determinants of response to a prokinetic agent in neuropathic chronic intestinal motility disorder. *Gastroenterology* 1994; **106**: 916-923
  - 66 **Camilleri M**, Balm RK, Zinsmeister AR. Symptomatic improvement with one-year cisapride treatment in neuropathic chronic intestinal dysmotility. *Aliment Pharmacol Ther* 1996; **10**: 403-409
  - 67 **Cogliandro R**, Stanghellini V, Cogliandro L, Tosetti C, Salvioli B, Zamboni PF, Barbara G, De Giorgio R, Corinaldesi R. Symptomatic response to short-term treatment with cisapride but not small bowel manometry predicts a positive outcome in adult patients with chronic idiopathic intestinal pseudo-obstruction (CIIP). *Gastroenterology* 1999; **116**: A1087
  - 68 **Verne GN**, Eaker EY, Hardy E, Sninsky CA. Effect of octreotide and erythromycin on idiopathic and scleroderma-associated intestinal pseudoobstruction. *Dig Dis Sci* 1995; **40**: 1892-1901
  - 69 **Pasha SF**, Lunsford TN, Lennon VA. Autoimmune gastrointestinal dysmotility treated successfully with pyridostigmine. *Gastroenterology* 2006; **131**: 1592-1596
  - 70 **Lyford G**, Foxx-Orenstein A. Chronic Intestinal Pseudo-

- bstruction. *Curr Treat Options Gastroenterol* 2004; **7**: 317-325
- 71 **Andersson S**, Lonroth H, Simren M, Ringstrom G, Elfvin A, Abrahamsson H. Gastric electrical stimulation for intractable vomiting in patients with chronic intestinal pseudoobstruction. *Neurogastroenterol Motil* 2006; **18**: 823-830
- 72 **Zimmerman DM**, Gidda JS, Cantrell BE, Schoepp DD, Johnson BG, Leander JD. Discovery of a potent, peripherally selective trans-3,4-dimethyl-4-(3-hydroxyphenyl)piperidine opioid antagonist for the treatment of gastrointestinal motility disorders. *J Med Chem* 1994; **37**: 2262-2265
- 73 **Wolff BG**, Michelassi F, Gerkin TM, Techner L, Gabriel K, Du W, Wallin BA. Alvimopan, a novel, peripherally acting mu opioid antagonist: results of a multicenter, randomized, double-blind, placebo-controlled, phase III trial of major abdominal surgery and postoperative ileus. *Ann Surg* 2004; **240**: 728-734; discussion 734-735
- 74 **Barbara G**, Stanghellini V, Brandi G, Cremon C, Di Nardo G, De Giorgio R, Corinaldesi R. Interactions between commensal bacteria and gut sensorimotor function in health and disease. *Am J Gastroenterol* 2005; **100**: 2560-2568
- 75 **Lara MC**, Weiss B, Illa I, Madoz P, Massuet L, Andreu AL, Valentino ML, Anikster Y, Hirano M, Marti R. Infusion of platelets transiently reduces nucleoside overload in MNGIE. *Neurology* 2006; **67**: 1461-1463
- 76 **Kim HY**, Kim JH, Jung SE, Lee SC, Park KW, Kim WK. Surgical treatment and prognosis of chronic intestinal pseudo-obstruction in children. *J Pediatr Surg* 2005; **40**: 1753-1759
- 77 **Masetti M**, Di Benedetto F, Cautero N, Stanghellini V, De Giorgio R, Lauro A, Begliomini B, Siniscalchi A, Pironi L, Cogliandro R, Pinna AD. Intestinal transplantation for chronic intestinal pseudo-obstruction in adult patients. *Am J Transplant* 2004; **4**: 826-829

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EDITORIAL

## Endoscopic submucosal dissection for gastrointestinal neoplasms

Naomi Kakushima, Mitsuhiro Fujishiro

Naomi Kakushima, Mitsuhiro Fujishiro, Department of Gastroenterology, The University of Tokyo, Graduate School of Medicine, Tokyo 113-8655, Japan

**Author contributions:** Kakushima N and Fujishiro M contributed equally to this work; Kakushima N and Fujishiro M performed research, and wrote the paper.

**Correspondence to:** Naomi Kakushima, MD, PhD, Department of Gastroenterology, The University of Tokyo, Graduate School of Medicine, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan. [kakushin-ky@umin.ac.jp](mailto:kakushin-ky@umin.ac.jp)

Telephone: +81-3-38155411 Fax: +81-3-58008806

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### Abstract

Endoscopic submucosal dissection (ESD) is an advanced technique of therapeutic endoscopy for superficial gastrointestinal neoplasms. Three steps characterize it: injecting fluid into the submucosa to elevate the lesion, cutting the surrounding mucosa of the lesion, and dissecting the submucosa beneath the lesion. The ESD technique has rapidly permeated in Japan for treatment of early gastric cancer, due to its excellent results of en-bloc resection compared to endoscopic mucosal resection (EMR). Although there is still room for improvement to lessen its technical difficulty, ESD has recently been applied to esophageal and colorectal neoplasms. Favorable short-term results have been reported, but the application of ESD should be well considered by three aspects: (1) the possibility of nodal metastases of the lesion, (2) technical difficulty such as location, ulceration and operator's skill, and (3) organ characteristics.

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**Key words:** Endoscopic submucosal dissection; Gastric cancer; Esophageal cancer; Colorectal cancer; Endoscopic mucosal resection; Therapeutic endoscopy

**Peer reviewers:** Zvi Fireman, Professor, Department of Gastroenterology, Hillel- yaffe Medical Center, Hadera 38100, Israel; Chee Lim, Dr, Department of Gastroenterology, Good Hope Hospital, Heart of England Foundation NHS Trust, W Midlands B75 7RR, United Kingdom

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### INTRODUCTION

Application of endoscopic resection (ER) to gastrointestinal (GI) neoplasms is limited to lesions with no risk of nodal metastasis. Either polypectomy or endoscopic mucosal resection (EMR) is beneficial for patients because of its low level of invasiveness. However, to ensure the curative potential of these treatment modalities, accurate histopathologic assessment of the resected specimens is essential because the depth of invasion and lymphovascular infiltration of the tumor is associated with considerable risk for lymph node metastasis. For accurate assessment of the appropriateness of the therapy, en bloc resection is more desirable than piecemeal resection. For a reliable en bloc resection of GI neoplasms, a new method of ER called endoscopic submucosal dissection (ESD) has been developed. In this article, an outline of the current status of ESD will be discussed.

### DEVELOPMENT OF ESD

The ESD technique has developed from one of the EMR techniques, namely endoscopic resection after local injection of a solution of hypertonic saline-epinephrine (ERHSE)<sup>[1]</sup>. Initially, the ESD technique was called by various names such as cutting EMR, exfoliating EMR, EMR with circumferential incision *etc.* However, a new name was proposed to this technique in 2003, as a treatment positioned between EMR and laparoscopic surgery, since this technique is innovative and enables complete resection of neoplasms that were impossible to resect en bloc by EMR.

At present, numerous electrosurgical knives such as insulation-tipped diathermic knife (IT-knife)<sup>[2-6]</sup>, needle knife<sup>[7]</sup>, hook knife<sup>[8]</sup>, flex knife<sup>[9-11]</sup>, triangle-tipped knife<sup>[12]</sup>, flush knife<sup>[13]</sup>, mucosectomy<sup>[14]</sup>, splash needle<sup>[15]</sup> and a special device called a small-caliber tip transparent (ST) hood<sup>[7]</sup> are available for this technique. One or two of these electrosurgical knives are used in combination with a high frequency electrosurgical current (HFEC) generator with an automatically controlled system (Endocut mode, Erbotom ICC200, ICC350, VIO300D, ERBE, Tubingen, Germany) (PSD-60, Olympus, Tokyo, Japan). New types of endoscopes are available for ESD, such as an endoscope with a water jet system (EG-2931, Pentax, Tokyo, Japan, GIF-Q260J, Olympus, Tokyo, Japan), an endoscope with a multi-bending system (M-scope: XGIF-Q240M, R-scope: XGIF-2TQ240R, Olympus, Tokyo, Japan) to facilitate the ESD procedure<sup>[16-19]</sup>. As another approach to successful

ESD, investigations of submucosal injection solutions have been actively done. It was reported that a hyaluronic acid solution makes a better long-lasting submucosal cushion without tissue damage than other available solutions<sup>[7,20-23]</sup>. As a further improvement of hyaluronic acid solution, usefulness of a mixture of high-molecular-weight hyaluronic acid, glycerin, and sugar has also been reported<sup>[24,25]</sup>.

ESD is characterized by three steps: injecting fluid into the submucosa to elevate the lesion from the muscle layer, circumferential cutting of the surrounding mucosa of the lesion, and subsequent dissection of the connective tissue of the submucosa beneath the lesion. Major advantages of this technique in comparison with polypectomy or EMR are as follows. The resected size and shape can be controlled, en bloc resection is possible even in a large neoplasm, and neoplasms with submucosal fibrosis are also resectable. So this technique can be applied to the resection of complex neoplasms such as large neoplasms, ulcerative non-lifting neoplasms, and recurrent neoplasms. The disadvantages of this technique are the requirement of two or more assistants, it is time-consuming, there is a higher risk of bleeding and perforation than EMR. In Japan, ESD is now gaining acceptance as the standard endoscopic resection technique for stomach neoplasms in an early stage, especially for large or ulcerative neoplasms. Recently, the ESD technique is applied to esophageal or colorectal neoplasms in some institutions, although it is still controversial considering the technical difficulty, associated risks, and favorable outcomes by EMR.

## INDICATION FOR ENDOSCOPIC RESECTION

### *Gastric cancer*

Early gastric cancer (EGC) is defined to a mucosal or submucosal invasive cancer (T1 cancer) irrespective of the presence of lymph node metastasis. Lesions indicated for ER should be EGC with no risk of nodal metastasis and that can be resected in a single fragment. Using a large database of more than 5000 EGC patients who underwent gastrectomy with D2 lymph node dissection, a criteria of node negative cancer has been defined<sup>[26]</sup>. At present, lesions with preoperative endoscopic diagnosis of differentiated type intramucosal cancer without ulcer findings, differentiated type intramucosal cancer no larger than 3 cm in diameter with ulcer findings, differentiated type minute invasive submucosal (less than 500 micrometers below muscularis mucosa) cancer no larger than 3 cm in diameter are considered as expanding indication for ER<sup>[27]</sup>. Undifferentiated type cancer lesions, and preoperative diagnosis of ulcerative findings is difficult, so that ER for these lesions should be carefully considered.

### *Esophageal cancer*

Early esophageal cancer (EEC) involving the epithelium (m1: carcinoma in situ) or the lamina propria (m2) are candidates for ER because no lymph node metastasis have been reported in cancers limited to these two layers<sup>[28]</sup>. For EEC invading the muscularis mucosa (m3), the lymph

node metastasis rate is reported as 9%, and for cancer with minute submucosal invasion (< 200 micrometers below the muscularis mucosa; sm1) the rate is 19%<sup>[29]</sup>. The lymph node metastasis rate of m3 or sm1 cancer without lymphovascular infiltration of the tumor is reported as 4.7%<sup>[29]</sup>. Therefore, for patients unwilling for esophagectomy or patients with comorbid diseases not suited for surgery, ER may be a relative indication for m3 or sm1 cancer. Also, for lesions spreading more than three-quarter of circumference of the esophagus are considered as relative indication for ER because post-operative stricture occurs in a high rate.

### *Colorectal cancer*

Early colorectal cancer (ECC) limited to the mucosa or with slight submucosal invasion (< 1000 micrometers below the muscularis mucosa; sm1) are candidates for ER<sup>[30]</sup>. However, even for lesions that meet the criteria above, laparoscopic or open surgery may be selected in some institutions considering the location and size of the lesion. In institutions actively performing ESD for colorectal lesions, depressed lesions and laterally spreading tumors of non-granular type (LST-NG) are considered as good candidates for ESD because these lesions have a high possibility of submucosal invasion which may be difficult to diagnose preoperatively, and a thorough histopathological assessment of the resected specimen is essential.

### *Preoperative evaluation for candidates of ER*

Endoscopy with chromoendoscopy is essential to define the lesion. To evaluate the depth of the lesion, size, redness, presence or absence of ulceration, superficial structure of the lesion, and deformity of the wall of the organ in compliance with air-flow rate are carefully observed by endoscopy and chromoendoscopy. Magnification endoscopy with narrow band imaging technique (NBI) has been reported as a promising new modality to evaluate the depth of ECC. Magnification endoscopy with NBI is also useful to distinguish the border of EGC in case of lack of utility of chromoendoscopy with indigocarmine. Magnification endoscopy with crystal violet staining or NBI is useful in estimating the depth of colorectal lesions. Endoscopic ultrasonography is often performed to evaluate the depth of invasion, and computed tomography may be performed to detect lymph node metastasis if any, if the diagnosis of node negative cancer is difficult to judge even with multiple diagnostic modalities.

### *Pathological evaluation of the removed specimen*

Whether a lesion may be included into the criteria of node-negative neoplasms is considered before treatment. However, at present, it is impossible to make a definite diagnosis of a neoplasm regarding depth, histological type and lymphatic vessel invasion before treatment. It is often experienced that although a biopsy specimen shows adenoma/dysplasia of a lesion, a diagnosis of cancer is made after total resection of the lesion. Therefore, a precise pathological evaluation of the resected specimen is essential, and an en bloc resection of the lesion is desirable in this respect.

After removal, the specimen should be oriented immediately before it is immersed in formalin. Orientation of the specimen is accomplished by fixing the periphery with thin needles on a plate of rubber or wood. The submucosal side of the specimen is faced to the plate. After fixation, the specimen is sectioned serially at 2 mm intervals parallel to a line that includes the closest part between the margin of the specimen and of the neoplasm, so that both lateral and vertical margins are assessed. The depth of invasion is then evaluated microscopically along with the degree of differentiation and lymphovascular infiltration, if any.

In result of thorough pathological assessment, if the lesion is resected en bloc with negative margins of neoplasm and fulfills the criteria of node-negative neoplasms with no lymphovascular infiltration, the treatment is judged as curative resection. For lesions with piecemeal resection but being judged as node-negative neoplasms, or lesions with histologically non-evaluable areas due to artifact or tissue burning, a periodical endoscopic follow-up should be performed to detect residual neoplasm or local recurrence. On the other hand, for lesions that do not fulfill the criteria of node-negative neoplasms, additional surgery with nodal dissection should be strongly recommended.

## OUTCOMES OF ESD

### En bloc resection rate

Recent results of en bloc resection rate and local recurrence of ESD for neoplasms in the stomach, esophagus and colorectum are described in Table 1. For gastric neoplasms larger than 20 mm, en bloc resection rate is extremely low among conventional EMR methods, and local recurrence rates are around 10%<sup>[44]</sup>. Although ESD was considered as a difficult and complicated technique when it was first described in the stomach, after maturity of the techniques of ESD, en bloc resection rates became greater than 90%, regardless of size, and local recurrence rates became almost zero. Technical feasibility and favorable results of ESD have also been reported in recurrent neoplasms<sup>[45-47]</sup>, neoplasms of the esophago-gastric junction<sup>[48]</sup>, and duodenal neoplasms although the number of cases is small. Few reports of ESD for resection of subepithelial tumors have also been published<sup>[49]</sup>.

### Complication

Complications of ESD include pain, bleeding, perforation, and stricture. Pain after ESD is often mild and lasts one or two days after the procedure although the frequency is low. Patients of esophageal ESD are more likely to develop pain than gastric or colorectal ESD.

Complications of post-operative bleeding and perforation among various ESD methods in the stomach, esophagus and colorectum are described in Table 2. Bleeding is more frequent in the stomach cases, whereas perforation is more frequent in the colorectal cases. To prevent post-procedural bleeding, hemostasis of appearing vessels on the artificial ulcer after removing the specimen is essential. Hemostasis is performed by hemostatic forceps (HDB2422/HDB2418, Pentax), coagrasper (FD-410LR,

Table 1 Recent outcomes of various endoscopic submucosal dissection methods for stomach, esophagus and colorectum

Site	Author	Yr	Method	En bloc resection rate (%)	Local recurrence rate (%)
Stomach	Yamamoto <sup>[33]</sup>	2002	EMRSH	76 (53/70)	3 (2/67)
	Ishigooka <sup>[34]</sup>	2004	s-ERHSE	79 (27/34)	0 (0/34)
	Oda <sup>[35]</sup>	2005	ESD-IT knife	93 <sup>1</sup> (957/1033)	-
	Kakushima <sup>[32]</sup>	2006	ESD-Flex knife	91 <sup>1</sup> (347/383)	-
	Imagawa <sup>[36]</sup>	2006	ESD-Flex knife	84 <sup>1</sup> (181/195)	0 (0/164)
	Oyama <sup>[37]</sup>	2006	ESD-Hook knife	94 (104/111)	0 (0/111)
	Onozato <sup>[38]</sup>	2006	ESD-Flex knife	94 <sup>1</sup> (161/171)	0 (0/99)
	Hirasaki <sup>[39]</sup>	2007	ESD-IT knife	96	-
Esophagus	Oyama <sup>[8]</sup>	2005	ESD-Hook knife	95 (95/102)	0 (0/102)
	Fujishiro <sup>[11]</sup>	2006	ESD-Flex knife	100 (58/58)	2.5 (1/40)
	Fujishiro <sup>[31]</sup>	2007	ESD-Flex knife	91.5 (183/200)	1.8 (2/111)
Colorectum	Saito <sup>[40]</sup>	2007	ESD several knives	84 (168/200)	0.5 (1/180)
	Tanaka <sup>[41]</sup>	2007	ESD several knives	80 (56/70)	0 (0/62)
	Tamegai <sup>[42]</sup>	2007	ESD-Hook knife	98.6 (33/42)	11 (4/36)
	Onozato <sup>[43]</sup>	2007	ESD-Flex knife	77 (27/35)	0 (0/23)

<sup>1</sup>En bloc resection + R0 resection rate.

Olympus), hot biopsy forceps, argon plasma coagulation or endoclips. According to perforation, recent case series suggest that small perforation immediately recognized can be successfully sealed with endoclips and treated conservatively by nasogastric suction, fasting and antibiotics without emergency laparotomy<sup>[51,52]</sup>. However, there are rare cases of delayed perforation, which requires surgical rescue. Delayed perforation may occur in the esophagus, stomach, duodenum and colorectum<sup>[31,53-56]</sup>, mostly at two or more days after a successful ESD. The reason for delayed perforation is unknown, however patients with uncontrolled diabetes mellitus, patients on permanent hemodialysis, lesions located on surgical anastomosis, and too much coagulation are considered as possible risk factors.

Stricture after ESD may occur in esophageal ESD when the ESD ulcer is larger than two-third of circumference of the esophageal lumen, or in gastric ESD when the ESD ulcer involves more than three quarter of the pylorus or pre-pylorus area. In these cases, early intervention to avoid passage obstruction is required. Dilation using bougie or balloon are often applied one week after ESD and repeated several times until healing of the ESD ulcer<sup>[8,11,57]</sup>.

## MANAGEMENTS AFTER ESD

In Japan, ESD is performed on hospitalized patients. After ESD, eating is usually started on the next or 2 d after ESD if there is no complication, and the patient

**Table 2** Bleeding and perforation rate of various endoscopic submucosal dissection methods for stomach, esophagus and colorectum

Site	Author	Year	Method	Total cases	Bleeding (%)	Perforation (%)
Stomach	Yamamoto <sup>[33]</sup>	2002	EMRSH	70	4	0
	Ishigooka <sup>[34]</sup>	2004	s-ERHSE	34	0	12
	Oda <sup>[35]</sup>	2005	ESD-IT knife	1033	6	4
	Kakushima <sup>[32]</sup>	2006	ESD-Flex knife	383	3.4	3.9
	Imagawa <sup>[36]</sup>	2006	ESD-Flex knife	159	0	6.1
	Oyama <sup>[37]</sup>	2006	ESD-Hook knife	111	-	1
	Onozato <sup>[38]</sup>	2006	ESD-Flex knife	171	7.6	3.5
	Hirasaki <sup>[39]</sup>	2007	ESD-IT knife	112	4	1
	Oyama <sup>[8]</sup>	2005	ESD-Hook knife	102	-	0
Esophagus	Fujishiro <sup>[11]</sup>	2006	ESD-Flex knife	58	0	6.9
Colorectum	Fujishiro <sup>[31]</sup>	2007	ESD-Flex knife	200	1	6
	Saito <sup>[40]</sup>	2007	ESD-several knives	200	2	5
	Tanaka <sup>[41]</sup>	2007	ESD-several knives	70	1.4	10
	Tamegai <sup>[42]</sup>	2007	ESD-Hook knife	74	-	1.4
	Hurlstone <sup>[50]</sup>	2007	ESD-Flex knife	42	12	2.4
	Onozato <sup>[43]</sup>	2007	ESD-Flex knife	35	0	2.9

may be discharged within a few days. Antacids are usually administered to gastric and esophageal ESD patients to relieve pain, prevent postoperative bleeding and promote ulcer healing. A recent study showed that proton pump inhibitors more effectively prevented bleeding from the gastric ulcer created after ESD than did H<sub>2</sub>-receptor antagonists<sup>[58]</sup>. Ulcers after ESD are reported to heal within 6 to 8 wk in the esophagus, stomach and colorectum<sup>[59-63]</sup>.

Endoscopic surveillance should be carried out in patients after ESD not only to detect local recurrence but also metachronous cancer especially in the esophagus and stomach. A recent study showed that the average time to detect a first metachronous gastric cancer (MGC) was 3.1  $\pm$  1.7 years after EMR/ESD, and the cumulative 3-year incidence was 5.9%<sup>[64]</sup>. In order to detect MGC at an early stage to perform a successful ER, annual endoscopic surveillance program may be practical for post-ER patients.

## LONG-TERM OUTCOMES AFTER ESD

Long-term outcomes after ESD for gastric cancers within the expanded indication are currently under investigation. Survival data is still lacking in the literature, however in the 2007 annual meeting of Japanese gastroenterological endoscopy society (JGES), a symposium was held upon long-term outcomes after gastric and esophageal ESD. For gastric ESD, 3-year disease free survival rate was reported as 90%-92%, local recurrence rate was reported as 0.8%-12%. For lesions within the criteria of node negative cancers, there were no reports of distant metastasis. Metachronous gastric cancer detection rate during follow-up was reported as 3.4%-10.2%. In comparison, long-term outcomes after EMR for small differentiated mucosal EGC less than 20 mm in diameter have been reported as comparable to those after gastrectomy. The disease-specific 5- and 10-year survival rates were 99% and 99%<sup>[65]</sup>. For esophageal ESD, in the 2007 JGES meeting, 3-year survival rate for m1-2 cancer and m3-sm1 cancer were 95.1% and 86.7%, respectively. According to colorectal ESD, there is still no long-term data at present.

## FUTURE PERSPECTIVES

With the development of ESD, more than half of GI cancers in the early stage are removed by ER in advanced institutions in Japan. En bloc retrieval of lesions is essential for detailed histopathologic studies, which form the basis for stratification of treatment outcomes and patient's prognosis. ESD theoretically offers greater histopathological accuracy than conventional EMR methods or piecemeal resection. However, ESD requires highly skilled endoscopists, and a suitable training program is demanded for permeation of this technique. For trainees starting ESD, skills of routine endoscopy and colonoscopy, target biopsy, endoscopic hemostasis techniques and simple EMR techniques should be required. A trainee would gain early proficiency of ESD after 30 cases under supervision of a mentor<sup>[32,66]</sup>. On the other hand, serious complications such as delayed perforation have been reported, and a thorough patient care before and after ESD is essential. At present, selection of a lesion within the criteria for ER, selection of the patient with adequate general function should be well considered. It is important to share the information and experience among endoscopists to skill up and avoid serious complications. The ESD technique is still not a treatment at ease, and further refinements of the technique is required to popularize ESD as a safe and reliable, less invasive treatment for patients with GI neoplasms.

## REFERENCES

- 1 **Hirao M**, Masuda K, Asanuma T, Naka H, Noda K, Matsuura K, Yamaguchi O, Ueda N. Endoscopic resection of early gastric cancer and other tumors with local injection of hypertonic saline-epinephrine. *Gastrointest Endosc* 1988; **34**: 264-269
- 2 **Ono H**, Kondo H, Gotoda T, Shirao K, Yamaguchi H, Saito D, Hosokawa K, Shimoda T, Yoshida S. Endoscopic mucosal resection for treatment of early gastric cancer. *Gut* 2001; **48**: 225-229
- 3 **Ohkuwa M**, Hosokawa K, Boku N, Ohtu A, Tajiri H, Yoshida S. New endoscopic treatment for intramucosal gastric tumors using an insulated-tip diathermic knife. *Endoscopy* 2001; **33**: 221-226
- 4 **Miyamoto S**, Muto M, Hamamoto Y, Boku N, Ohtsu A, Baba S, Yoshida M, Ohkuwa M, Hosokawa K, Tajiri H, Yoshida S.



- A new technique for endoscopic mucosal resection with an insulated-tip electrosurgical knife improves the completeness of resection of intramucosal gastric neoplasms. *Gastrointest Endosc* 2002; **55**: 576-581
- 5 Rosch T, Sarbia M, Schumacher B, Deinert K, Frimberger E, Toerner T, Stolte M, Neuhaus H. Attempted endoscopic en bloc resection of mucosal and submucosal tumors using insulated-tip knives: a pilot series. *Endoscopy* 2004; **36**: 788-801
  - 6 Gotoda T. A large endoscopic resection by endoscopic submucosal dissection procedure for early gastric cancer. *Clin Gastroenterol Hepatol* 2005; **3**: S71-S73
  - 7 Yamamoto H, Kawata H, Sunada K, Sasaki A, Nakazawa K, Miyata T, Sekine Y, Yano T, Satoh K, Ido K, Sugano K. Successful en-bloc resection of large superficial tumors in the stomach and colon using sodium hyaluronate and small-caliber-tip transparent hood. *Endoscopy* 2003; **35**: 690-694
  - 8 Oyama T, Tomori A, Hotta K, Morita S, Kominato K, Tanaka M, Miyata Y. Endoscopic submucosal dissection of early esophageal cancer. *Clin Gastroenterol Hepatol* 2005; **3**: S67-S70
  - 9 Yahagi N, Fujishiro M, Kakushima N, Kobayashi K, Hashimoto T, Oka M, Iguchi M, Enomoto S, Ichinose M, Niwa H, Omata M. Endoscopic submucosal dissection for early gastric cancer using the tip of an electrosurgical snare (thin type). *Dig Endosc* 2004; **16**: 34-38
  - 10 Fujishiro M, Yahagi N, Nakamura M, Kakushima N, Kodashima S, Ono S, Kobayashi K, Hashimoto T, Yamamichi N, Tateishi A, Shimizu Y, Oka M, Ogura K, Kawabe T, Ichinose M, Omata M. Endoscopic submucosal dissection for rectal epithelial neoplasia. *Endoscopy* 2006; **38**: 493-497
  - 11 Fujishiro M, Yahagi N, Kakushima N, Kodashima S, Muraki Y, Ono S, Yamamichi N, Tateishi A, Shimizu Y, Oka M, Ogura K, Kawabe T, Ichinose M, Omata M. Endoscopic submucosal dissection of esophageal squamous cell neoplasms. *Clin Gastroenterol Hepatol* 2006; **4**: 688-694
  - 12 Inoue H, Kudo S. A novel procedure of en bloc EMR using triangle-tipped knife (abstract). *Gastrointest Endosc* 2003; **57**: AB86
  - 13 Toyonaga T, Nishino E, Hirooka T, Dozaiku T, Sujiyama T, Iwata Y, Ono W, Ueda C, Tomita M, Hirooka T, Makimoto S, Hayashibe A, Sonomura T. Use of short needle knife for esophageal endoscopic submucosal dissection. *Dig Endosc* 2005; **17**: 246-252
  - 14 Kawahara Y, Takenaka R, Okada H. Risk management to prevent perforation during endoscopic submucosal dissection. *Dig Endosc* 2007; **19**: S9-S13
  - 15 Fujishiro M, Kodashima S, Goto O, Ono S, Muraki Y, Kakushima N, Omata M. Successful en bloc resection of superficial esophageal cancer treated by endoscopic submucosal dissection with a splash-needle (with video). *Endoscopy* 2007 Available from: URL: <http://www.thieme-connect.de/ejournals/html/endoscopy/doi/10.1055/s-2007-995538>
  - 16 Yahagi N, Fujishiro M, Imagawa A, Kakushima N, Iguchi M, Omata M. Endoscopic submucosal dissection for the reliable en bloc resection of colorectal mucosal tumors. *Dig Endosc* 2004; **16**: S89-S92
  - 17 Yahagi N, Fujishiro M, Kakushima N, Kodashima S, Nakamura M, Omata M. Clinical evaluation of the multi-bending scope in various endoscopic procedures of the upper GI tract. *Dig Endosc* 2005; **17**: S94-S96
  - 18 Yonezawa J, Kaise M, Sumiyama K, Goda K, Arakawa H, Tajiri H. A novel double-channel therapeutic endoscope ("R-scope") facilitates endoscopic submucosal dissection of superficial gastric neoplasms. *Endoscopy* 2006; **38**: 1011-1015
  - 19 Neuhaus H, Costamagna G, Deviere J, Fockens P, Ponchon T, Rosch T. Endoscopic submucosal dissection (ESD) of early neoplastic gastric lesions using a new double-channel endoscope (the "R-scope"). *Endoscopy* 2006; **38**: 1016-1023
  - 20 Yamamoto H, Yube T, Isoda N, Sato Y, Sekine Y, Higashizawa T, Ido K, Kimura K, Kanai N. A novel method of endoscopic mucosal resection using sodium hyaluronate. *Gastrointest Endosc* 1999; **50**: 251-256
  - 21 Conio M, Rajan E, Sorbi D, Norton I, Herman L, Filiberti R, Gostout CJ. Comparative performance in the porcine esophagus of different solutions used for submucosal injection. *Gastrointest Endosc* 2002; **56**: 513-516
  - 22 Fujishiro M, Yahagi N, Kashimura K, Mizushima Y, Oka M, Enomoto S, Kakushima N, Kobayashi K, Hashimoto T, Iguchi M, Shimizu Y, Ichinose M, Omata M. Comparison of various submucosal injection solutions for maintaining mucosal elevation during endoscopic mucosal resection. *Endoscopy* 2004; **36**: 579-583
  - 23 Fujishiro M, Yahagi N, Kashimura K, Matsuura T, Nakamura M, Kakushima N, Kodashima S, Ono S, Kobayashi K, Hashimoto T, Yamamichi N, Tateishi A, Shimizu Y, Oka M, Ichinose M, Omata M. Tissue damage of different submucosal injection solutions for EMR. *Gastrointest Endosc* 2005; **62**: 933-942
  - 24 Fujishiro M, Yahagi N, Kashimura K, Mizushima Y, Oka M, Matsuura T, Enomoto S, Kakushima N, Imagawa A, Kobayashi K, Hashimoto T, Iguchi M, Shimizu Y, Ichinose M, Omata M. Different mixtures of sodium hyaluronate and their ability to create submucosal fluid cushions for endoscopic mucosal resection. *Endoscopy* 2004; **36**: 584-589
  - 25 Fujishiro M, Yahagi N, Nakamura M, Kakushima N, Kodashima S, Ono S, Kobayashi K, Hashimoto T, Yamamichi N, Tateishi A, Shimizu Y, Oka M, Ogura K, Kawabe T, Ichinose M, Omata M. Successful outcomes of a novel endoscopic treatment for GI tumors: endoscopic submucosal dissection with a mixture of high-molecular-weight hyaluronic acid, glycerin, and sugar. *Gastrointest Endosc* 2006; **63**: 243-249
  - 26 Gotoda T, Yanagisawa A, Sasako M, Ono H, Nakanishi Y, Shimoda T, Kato Y. Incidence of lymph node metastasis from early gastric cancer: estimation with a large number of cases at two large centers. *Gastric Cancer* 2000; **3**: 219-225
  - 27 Soetikno R, Kaltenbach T, Yeh R, Gotoda T. Endoscopic mucosal resection for early cancers of the upper gastrointestinal tract. *J Clin Oncol* 2005; **23**: 4490-4498
  - 28 The Japan Esophageal Society. Guidelines for the clinical and pathologic studies on carcinoma of the esophagus [in Japanese]. 10th ed. Tokyo: Kanehara Shuppan, 2007
  - 29 Oyama T, Miyata Y, Shimaya S. Lymph nodal metastasis of m3, sm1 esophageal cancer [in Japanese]. *Stomach Intestine* 2002; **37**: 71-74
  - 30 Yokoyama J, Ajioka Y, Watanabe H, Asakura H. Lymph node metastasis and micrometastasis of submucosal invasive colorectal carcinoma: an indicator of the curative potential of endoscopic treatment. *Acta Medica Biologica* 2002; **50**: 1-8
  - 31 Fujishiro M, Yahagi N, Kakushima N, Kodashima S, Muraki Y, Ono S, Yamamichi N, Tateishi A, Oka M, Ogura K, Kawabe T, Ichinose M, Omata M. Outcomes of endoscopic submucosal dissection for colorectal epithelial neoplasms in 200 consecutive cases. *Clin Gastroenterol Hepatol* 2007; **5**: 678-683; quiz 645
  - 32 Kakushima N, Fujishiro M, Kodashima S, Muraki Y, Tateishi A, Omata M. A learning curve for endoscopic submucosal dissection of gastric epithelial neoplasms. *Endoscopy* 2006; **38**: 991-995
  - 33 Yamamoto H, Kawata H, Sunada K, Satoh K, Kaneko Y, Ido K, Sugano K. Success rate of curative endoscopic mucosal resection with circumferential mucosal incision assisted by submucosal injection of sodium hyaluronate. *Gastrointest Endosc* 2002; **56**: 507-512
  - 34 Ishigooka M, Uchisawa M, Kusama K, Furuyama J, Morizono R, Takahashi B. Endoscopic resection for early gastric cancer by direct incision of the submucosa, with local injection of HSE solution (in Japanese with English abstract). *Stomach Intest* 2002; **37**: 1163-1168
  - 35 Oda I, Gotoda T, Hamanaka H, Eguchi T, Saito Y, Matsuda T, Bhandari P, Emura F, Saito D, Ono H. Endoscopic submucosal dissection for early gastric cancer: technical feasibility, operation time and complications from a large consecutive series. *Dig Endosc* 2005; **17**: 54-58
  - 36 Imagawa A, Okada H, Kawahara Y, Takenaka R, Kato J, Kawamoto H, Fujiki S, Takata R, Yoshino T, Shiratori Y. Endoscopic submucosal dissection for early gastric cancer:

- results and degrees of technical difficulty as well as success. *Endoscopy* 2006; **38**: 987-990
- 37 **Oyama T**, Tanaka M, Tomori A, Hotta K, Morita S, Furutachi S, Takahashi A, Miyata Y. Prognosis of endoscopic submucosal dissection for early gastric cancer, results of 3 years or more after treatment. (in Japanese with English abstract) *Stomach Intest* 2006; **41**: 87-90
  - 38 **Onozato Y**, Ishihara H, Iizuka H, Sohara N, Kakizaki S, Okamura S, Mori M. Endoscopic submucosal dissection for early gastric cancers and large flat adenomas. *Endoscopy* 2006; **38**: 980-986
  - 39 **Hirasaki S**, Kanzaki H, Matsubara M, Fujita K, Ikeda F, Taniguchi H, Yumoto E, Suzuki S. Treatment of over 20 mm gastric cancer by endoscopic submucosal dissection using an insulation-tipped diathermic knife. *World J Gastroenterol* 2007; **13**: 3981-3984
  - 40 **Saito Y**, Uraoka T, Matsuda T, Emura F, Ikehara H, Mashimo Y, Kikuchi T, Fu KI, Sano Y, Saito D. Endoscopic treatment of large superficial colorectal tumors: a case series of 200 endoscopic submucosal dissections (with video). *Gastrointest Endosc* 2007; **66**: 966-973
  - 41 **Tanaka S**, Oka S, Kaneko I, Hirata M, Mouri R, Kanao H, Yoshida S, Chayama K. Endoscopic submucosal dissection for colorectal neoplasia: possibility of standardization. *Gastrointest Endosc* 2007; **66**: 100-107
  - 42 **Tamegai Y**, Saito Y, Masaki N, Hinohara C, Oshima T, Kogure E, Liu Y, Uemura N, Saito K. Endoscopic submucosal dissection: a safe technique for colorectal tumors. *Endoscopy* 2007; **39**: 418-422
  - 43 **Onozato Y**, Kakizaki S, Ishihara H, Iizuka H, Sohara N, Okamura S, Mori M, Itoh H. Endoscopic submucosal dissection for rectal tumors. *Endoscopy* 2007; **39**: 423-427
  - 44 **Fujishiro M**. Endoscopic resection for early gastric cancer. In: Kaminishi M, Takubo K, Mafune K (Eds). The diversity of gastric carcinoma; Pathogenesis, diagnosis, and therapy. Springer-Verlag Tokyo 2005: 243-252
  - 45 **Yokoi C**, Gotoda T, Hamanaka H, Oda I. Endoscopic submucosal dissection allows curative resection of locally recurrent early gastric cancer after prior endoscopic mucosal resection. *Gastrointest Endosc* 2006; **64**: 212-218
  - 46 **Oka S**, Tanaka S, Kaneko I, Mouri R, Hirata M, Kanao H, Kawamura T, Yoshida S, Yoshihara M, Chayama K. Endoscopic submucosal dissection for residual/local recurrence of early gastric cancer after endoscopic mucosal resection. *Endoscopy* 2006; **38**: 996-1000
  - 47 **Fujishiro M**, Goto O, Kakushima N, Kodashima S, Muraki Y, Omata M. Endoscopic submucosal dissection of stomach neoplasms after unsuccessful endoscopic resection. *Dig Liver Dis* 2007; **39**: 566-571
  - 48 **Kakushima N**, Yahagi N, Fujishiro M, Kodashima S, Nakamura M, Omata M. Efficacy and safety of endoscopic submucosal dissection for tumors of the esophagogastric junction. *Endoscopy* 2006; **38**: 170-174
  - 49 **Lee IL**, Lin PY, Tung SY, Shen CH, Wei KL, Wu CS. Endoscopic submucosal dissection for the treatment of intraluminal gastric subepithelial tumors originating from the muscularis propria layer. *Endoscopy* 2006; **38**: 1024-1028
  - 50 **Hurlstone DP**, Atkinson R, Sanders DS, Thomson M, Cross SS, Brown S. Achieving R0 resection in the colorectum using endoscopic submucosal dissection. *Br J Surg* 2007; **94**: 1536-1542
  - 51 **Minami S**, Gotoda T, Ono H, Oda I, Hamanaka H. Complete endoscopic closure of gastric perforation induced by endoscopic resection of early gastric cancer using endoclips can prevent surgery (with video). *Gastrointest Endosc* 2006; **63**: 596-601
  - 52 **Fujishiro M**, Yahagi N, Kakushima N, Kodashima S, Muraki Y, Ono S, Kobayashi K, Hashimoto T, Yamamichi N, Tateishi A, Shimizu Y, Oka M, Ogura K, Kawabe T, Ichinose M, Omata M. Successful nonsurgical management of perforation complicating endoscopic submucosal dissection of gastrointestinal epithelial neoplasms. *Endoscopy* 2006; **38**: 1001-1006
  - 53 **Toyonaga T**. Complications of endoscopic submucosal dissection and their practical management. (in Japanese with an English abstract) *Shokakinaishikyo* 2005; **17**: 639-649
  - 54 **Doyama H**, Oomori T, Narumi K, Takemura K, Shimazaki H, Hiranuma C, Koizumi H. Experience of delayed perforation after ESD of an adenoma in the duodenal 2nd portion. (in Japanese, abstract) *Endoscopic forum for digestive disease* 2006; **22**: 175
  - 55 **Onozato Y**, Iizuka H, Sagawa T, Yoshimura S, Sakamoto I, Arai H, Ishihara H, Tomizawa N, Ogawa T, Takayama H, Abe T, Motegi A, Ito H. A case report of delayed perforation due to endoscopic submucosal dissection (ESD) for early gastric cancer. (in Japanese) *Progress of Digestive Endosc* 2006; **68**: 114-115
  - 56 **Tanaka M**, Oyama T, Miyata Y, Tomori A, Hotta K, Morita S, Kominato K, Takeuchi M, Hisa T, Furutake M, Takamatsu M. A case of delayed perforation 6 days after esophageal ESD successfully recovered by conservative treatment. (in Japanese, abstract) *Endoscopic forum for digestive disease* 2005; **21**: 98
  - 57 **Fujishiro M**, Yahagi N, Kakushima N, Kodashima S, Ichinose M, Omata M. En bloc resection of a large semicircular esophageal cancer by endoscopic submucosal dissection. *Surg Laparosc Endosc Percutan Tech* 2006; **16**: 237-241
  - 58 **Uedo N**, Takeuchi Y, Yamada T, Ishihara R, Ogiyama H, Yamamoto S, Kato M, Tatsumi K, Masuda E, Tamai C, Higashino K, Iishi H, Tatsuta M. Effect of a proton pump inhibitor or an H2-receptor antagonist on prevention of bleeding from ulcer after endoscopic submucosal dissection of early gastric cancer: a prospective randomized controlled trial. *Am J Gastroenterol* 2007; **102**: 1610-1616
  - 59 **Kakushima N**, Yahagi N, Fujishiro M, Iguchi M, Oka M, Kobayashi K, Hashimoto T, Omata M. The healing process of gastric artificial ulcers after endoscopic submucosal dissection. *Dig Endosc* 2004; **16**: 327-331
  - 60 **Kakushima N**, Fujishiro M, Kodashima S, Kobayashi K, Tateishi A, Iguchi M, Imagawa A, Motoi T, Yahagi N, Omata M. Histopathologic characteristics of gastric ulcers created by endoscopic submucosal dissection. *Endoscopy* 2006; **38**: 412-415
  - 61 **Kakushima N**, Fujishiro M, Yahagi N, Kodashima S, Nakamura M, Omata M. Helicobacter pylori status and the extent of gastric atrophy do not affect ulcer healing after endoscopic submucosal dissection. *J Gastroenterol Hepatol* 2006; **21**: 1586-1589
  - 62 **Iguchi M**, Yahagi N, Fujishiro M, Kakushima N, Oka M, Enomoto S, Yanaoka K, Arii K, Shimizu Y, Kitauchi S, Omata M, Ichinose M. The healing process of large artificial ulcers in the colorectum after endoscopic mucosal resection. [abstract]. *Gastrointest Endosc* 2003; **57**: AB226
  - 63 **Fujishiro M**, Yahagi N, Kakushima N, Kodashima S, Ichinose M, Omata M. Successful endoscopic en bloc resection of a large laterally spreading tumor in the rectosigmoid junction by endoscopic submucosal dissection. *Gastrointest Endosc* 2006; **63**: 178-183
  - 64 **Nakajima T**, Oda I, Gotoda T, Hamanaka H, Eguchi T, Yokoi C, Saito D. Metachronous gastric cancers after endoscopic resection: how effective is annual endoscopic surveillance? *Gastric Cancer* 2006; **9**: 93-98
  - 65 **Uedo N**, Iishi H, Tatsuta M, Ishihara R, Higashino K, Takeuchi Y, Imanaka K, Yamada T, Yamamoto S, Yamamoto S, Tsukuma H, Ishiguro S. Longterm outcomes after endoscopic mucosal resection for early gastric cancer. *Gastric Cancer* 2006; **9**: 88-92
  - 66 **Gotoda T**, Friedland S, Hamanaka H, Soetikno R. A learning curve for advanced endoscopic resection. *Gastrointest Endosc* 2005; **62**: 866-867



## CLINICAL PRACTICE GUIDELINES

# Pharmacological approach to acute pancreatitis

Ulrich Christian Bang, Synne Semb, Camilla Nøjgaard, Flemming Bendtsen

Ulrich Christian Bang, Synne Semb, Camilla Nøjgaard, Flemming Bendtsen, Department of Gastroenterology, Hvidovre Hospital, Hvidovre DK-2650, Denmark

Flemming Bendtsen, Faculty of Health Sciences, University of Copenhagen, Blegdamsvej 3B, København N DK-2200, Denmark

**Author contributions:** Bang UC and Bendtsen F wrote the manuscript; Semb S and Nøjgaard C revised the manuscript.

**Correspondence to:** Ulrich Bang, Department of Gastroenterology, Hvidovre Hospital, Kettegaard Allé 30, Hvidovre DK-2650, Denmark. [ulrich\\_bang@dadlnet.dk](mailto:ulrich_bang@dadlnet.dk)

Telephone: +45-26748942 Fax: +45-36473311

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**Peer reviewer:** Minoti V Apte, Associate Professor, Pancreatic Research Group, South Western Sydney Clinical School, The University of New South Wales, Liverpool, NSW 2170, Australia

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## Abstract

The aim of the present review is to summarize the current knowledge regarding pharmacological prevention and treatment of acute pancreatitis (AP) based on experimental animal models and clinical trials. Somatostatin (SS) and octreotide inhibit the exocrine production of pancreatic enzymes and may be useful as prophylaxis against Post Endoscopic retrograde cholangiopancreatography Pancreatitis (PEP). The protease inhibitor Gabexate mesilate (GM) is used routinely as treatment to AP in some countries, but randomized clinical trials and a meta-analysis do not support this practice. Nitroglycerin (NGL) is a nitrogen oxide (NO) donor, which relaxes the sphincter of Oddi. Studies show conflicting results when applied prior to ERCP and a large multicenter randomized study is warranted. Steroids administered as prophylaxis against PEP has been validated without effect in several randomized trials. The non-steroidal anti-inflammatory drugs (NSAID) indomethacin and diclofenac have in randomized studies showed potential as prophylaxis against PEP. Interleukin 10 (IL-10) is a cytokine with anti-inflammatory properties but two trials testing IL-10 as prophylaxis to PEP have returned conflicting results. Antibodies against tumor necrosis factor-alpha (TNF- $\alpha$ ) have a potential as rescue therapy but no clinical trials are currently being conducted. The antibiotics beta-lactams and quinolones reduce mortality when necrosis is present in pancreas and may also reduce incidence of infected necrosis. Evidence based pharmacological treatment of AP is limited and studies on the effect of potent anti-inflammatory drugs are warranted.

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**Key words:** Acute pancreatitis; Diclofenac; Gabexate; Indomethacin; Interleukin-10; Necrotizing pancreatitis; Nitrogen oxides; Octreotide; Protease inhibitors; Somatostatin

## INTRODUCTION

Acute pancreatitis (AP) is a localized inflammatory condition, which may extent to other organs. The etiology is usually excessive consumption of alcohol or gallstone disease, but is in some cases iatrogenic following medication or endoscopic retrograde cholangiopancreatography (ERCP).

Only a few reviews summarizing the available pharmacological options for treating AP have been published despite various experimental and clinical testing of potential drugs<sup>[1]</sup>. The aim of the present review is to validate the current literature covering the pharmacological treatment of AP. The main focus is on human clinical trials but some animal experimental models have been included as well (Table 1). AP after ERCP [post-ERCP pancreatitis (PEP)] can be regarded as a clinical "experimental" model of AP and hence is subject to preventive measures. The studies of potentially prophylactic drugs to PEP are therefore included (Table 2).

## PATHOPHYSIOLOGY

The pathobiological processes have primarily been investigated in experimental animal models and it is widely accepted that the acinar cells play a central role in the development of AP. The secretory acinar cells (SAC) contain zymogen precursors and the intra-acinar activation of digestive enzymes is a key event in the pathogenesis of AP. The molecular mechanism by which zymogen in AP fails to leave the SAC is unknown. Studies suggest a loss of the terminal actin web or a displacement of one of the SNARE membrane proteins, which regulate exocytosis<sup>[2]</sup>.

The inflammatory response is partly caused by the release of chemokines from SAC, which is followed by recruitment of helper T lymphocytes and macrophages leading to pancreatic edema and accumulation of neutrophils. The systemic release of cytokines including major pro-inflammatory cytokines causes a systemic

**Table 1** Pharmacological treatment of acute pancreatitis: Overview of drugs tested in animal experimental models and clinical trials

Name	Mechanism	Effect in animal models	Result in human trials
Somatostatin	Inhibition of pancreatic secretion	No reduced mortality	No reduced mortality
Octreotid	Inhibition of pancreatic secretion	No effect (divergent results)	No reduced mortality
Gabexate mesilate	Protease inhibitor	Reduced histology score	Maybe reduced mortality
N-acetyl-cysteine	Reduction of oxidative stress	Reduced severity	No reduced mortality
Nitrogen oxide	Improvement of micro-circulation	Reduced edema	No published trials
Steroids	Non-specific anti-inflammatory	Reduced mortality	No published trials
Interleukin-10	Anti-inflammatory	Reduced mortality	No published trials
TNF-alpha antibody	Specific anti-inflammatory	Reduced mortality	No published trials
PAF inhibitor	Specific anti-inflammatory	No reduced mortality	No reduced mortality
Antibiotics	Antibacterial	-	Reduced mortality
Probiotics	Prevention of colonization of the gut	-	No reduced mortality

**Table 2** Pharmacological prevention of PEP: Overview of drugs tested in clinical trials

Name	Mechanism	Incidence of PEP
Somatostatin	Inhibition of pancreatic secretion	No effect
Octreotid	Inhibition of pancreatic secretion	Probably reduced
Gabexate mesilate	Protease inhibitor	No effect
N-acetylcysteine	Reduction of oxidative stress	No effect
Nitrogen oxide	Improvement of micro-circulation	No effect
Steroids	Non-specific anti-inflammatory	No effect
Interleukin-10	Anti-inflammatory	Probably no effect
TNF-alpha	Specific anti-inflammatory	No published trials
Antibiotics	Antibacterial	Reduced

response, which may include remote organs<sup>[3]</sup>. The inflammatory process is followed by interstitial edema and the disease will in 10%-15% of the cases progress to necrosis in parts of the pancreas and possible bacterial infection. During an attack of AP the microvascular circulation is affected, which compromises oxygenation of the tissue<sup>[2]</sup>.

The clinical manifestations of AP include upper abdominal pain and symptoms related to the systemic inflammatory response. In complicated cases with involvement of remote organ systems mortality is increased to 5% with ranges from 0% to 47% depending on the severity of the disease. The current treatment of AP is mainly supportive care using analgesics and relevant measures when other organs are involved. Enteral nutrition must be initiated as soon as possible and whenever patients are unable to eat, feeding tubes should be used. Supplementary treatment often involves antibiotics when an infection is suspected and surgery or endoscopic ultrasonic (EUS)-guided drainage in case of infected necrotizing pancreatitis<sup>[4]</sup>.

AP after ERCP is a common complication and reported incidences vary from 5% to 15% in larger series. The majority of the cases are mild self-limiting conditions but up to 1% may develop a severe and potentially fatal pancreatitis. Although the pathophysiological mechanism remains to be elucidated several risk factors are identified: known sphincter Oddi dysfunction, sphincterotomy, injection of contrast more than one time and experience of the endoscopists<sup>[5-7]</sup>.

## METHODS

Published trials were identified on PubMed using the MeSH term "AP" in combination with the following MeSH terms: steroids, cortisone, hydrocortisone, corticosteroids, nitroglycerin (NGL), non-steroidal anti-inflammatory drugs (NSAID), celecoxib, COX- II, diclofenac, indomethacin, interleukin 10 (IL-10), tumor necrosis factor-alpha (TNF- $\alpha$ ), infliximab, Remicade<sup>®</sup>, etanercept, adalimumab, Humira<sup>®</sup>, platelet activating-factor (PAF), lexipafant, antibiotics, somatostatin (SS), octreotide, Sandostatin<sup>®</sup>, probiotics, gabexate, nutrition. Only articles in English were included. Earlier published reviews were manually examined for other relevant studies.

### SS/octreotide

SS is a peptide hormone mainly produced in the gastrointestinal tract, where it has an inhibitory effect on gastric emptying, intestinal motility and intestinal blood flow. Furthermore, SS strongly inhibits the production of pancreatic enzymes, which has been the basis for using SS or its analogue octreotide as treatment for AP<sup>[8]</sup>.

Both experimental and clinical trials have been conducted with SS and octreotide, but no effect on the course of the disease has hitherto been demonstrated. Most of the clinical trials comprised only few patients and often patients with interstitial pancreatitis were included although this condition is self-limiting and does not require specific therapy<sup>[9]</sup>. The largest and best conducted study is a German prospective multicenter study with 302 patients from 32 hospitals with moderate to severe AP randomized to either octreotide or placebo. This study revealed no significant differences between the treatment groups with respect to mortality, rate of complications, surgical interventions or length of hospital stay<sup>[10]</sup>.

Several studies have examined SS or octreotide as prophylaxis to PEP. Various regimes have demonstrated a reduced incidence of PEP compared to placebo: SS administered as a bolus immediately after ERCP (4.4% *vs* 13.3%,  $P = 0.01$ )<sup>[11]</sup>, SS given as a 12-h continuous infusion starting 30 min before ERCP (1.7% *vs* 9.8%,  $P < 0.05$ )<sup>[12]</sup> and octreotide in repeated injections starting 24 h prior to ERCP (2% *vs* 8.9%,  $P = 0.03$ )<sup>[13]</sup>. It should be noted that these studies have a fairly high incidence of PEP in the placebo groups. Andriulli *et al* have performed two similar



large, double blind, multicenter, placebo-controlled trials using SS. They used a dosage of 750 micrograms SS as an infusion starting 30 min prior to ERCP, ending 2 h (SS = 183, placebo = 199) or 6 h (SS = 351, placebo = 395) after ERCP. The incidences of PEP in the placebo groups were 6.5% and 4.8% respectively and no advantageous effect of SS was observed<sup>[14,15]</sup>.

The reports published during the years 2002 to 2006 have been summarized in a meta-analysis, which concluded that SS or octreotide have no effect as prophylaxis prior to ERCP<sup>[16]</sup>. However, this meta-analysis did not include the most recent trial from China with 832 patients. In this study, octreotide was administered as a combination of intravenous infusion and subcutaneous injections and the incidence of PEP in the treatment group ( $n = 414$ ) and the placebo group ( $n = 418$ ) was 2.42% and 5.26% respectively ( $P = 0.046$ )<sup>[17]</sup>.

Octreotide and SS have thus been investigated in several clinical studies and may have an advantageous effect as prophylaxis prior to ERCP. Optimal dosage and cost-effectiveness still need to be elucidated.

### Protease inhibitor-Gabexate mesilate (GM)

The intracellular activation of proteases is a mandatory step in the development of AP and the protease inhibitors could theoretically have an effect in the treatment of AP or as prophylaxis prior to ERCP. The first protease inhibitor, Aprotinin, was widely used in the 1960's but randomized trials could not demonstrate any beneficial effect<sup>[18,19]</sup>. GM is a synthetic protease inhibitor, which improve histology score in animal models of AP<sup>[20]</sup>.

In the 1980's several reports with a varying number of patients with AP ( $n = 42$  to 223) have been published but none showed any advantage of GM<sup>[21-26]</sup>. Conversely Chen *et al* observed a significant improved survival in a randomized trial including 52 patients with severe AP who received GM (mortality 33% *vs* 8%)<sup>[27]</sup>. A meta-analysis later concluded that GM may reduce the mortality in patients with moderate to severe pancreatitis, but the authors also noted that poor quality of the included randomized trials limits the power of this meta-analysis<sup>[28]</sup>.

Several papers from Japan report a reduced mortality rate in patients with necrotizing AP receiving GM as continuous regional arterial infusion (CRAI). However this conclusion is based merely on clinical observations and not placebo-controlled randomized trials<sup>[29,30]</sup>.

Looking at the effect on PEP two large studies by Andriulli *et al* with in total 1172 patients did not reveal any beneficial effect of GM. These results are in conflict with an earlier study by Cavallini *et al*, who in a study of 418 patients observed a PEP incidence of 6% in the GM group and 14% in the placebo group ( $P = 0.009$ )<sup>[31]</sup>. However, a recent meta-analysis concludes that GM does not have an advantageous effect as prophylaxis to PEP<sup>[32]</sup>.

The question continues to be a matter of debate and based on their trial with 608 patients, Manes *et al* argue that high-risk patients may benefit from GM. They administered GM either before or after ERCP compared to a saline solution. The incidence of PEP was 9.4% in the placebo group, and significantly lower ( $P < 0.01$ ) in the GM groups regardless of the time GM was administered

(before ERCP 3.9%, after ERCP 3.4%)<sup>[33]</sup>.

The FDA does not approve GM and the use of GM is not recommended in published guidelines concerning the treatment of AP<sup>[4,2,34]</sup>. However, Japanese national guidelines recommend the use of protease inhibitors either applied intravenously or as CRAI<sup>[35]</sup> and GM is also approved in Italy where it is also used as prophylaxis against PEP<sup>[36]</sup>.

### Antioxidants

Oxidative stress most likely plays a major role in the early development of AP<sup>[37]</sup> and several experimental animal models show a beneficial effect of anti-oxidative drugs<sup>[38-44]</sup>. In humans depletion of antioxidants is observed in AP<sup>[45,46]</sup> correlating to the severity of the disease<sup>[47]</sup>.

Therapy with antioxidants administered intravenously has been investigated in a prospective double-blind placebo controlled randomized trial on patients with predicted severe AP but no effect on mortality could be demonstrated<sup>[48]</sup>. The prophylactic effect on the incidence of PEP was tested in two randomized prospective randomized trials with 256 and 106 patients, respectively. N-acetylcysteine (NAC) was administered before and after ERCP and both studies concluded that NAC was without any preventive effect<sup>[49,50]</sup>. Thus, there is no evidence for the use of NAC.

### NGL

NGL is a donor of nitrogen oxide (NO), which causes vasodilatation and reduces cardiac preload. The main indication for using NGL is angina pectoris<sup>[51]</sup>.

Experimental animal models have shown reduced pancreatic edema when administering NO as an infusion<sup>[52]</sup>, but until now no clinical randomized trials using NGL in the treatment of AP have been published. As prophylaxis prior to ERCP three prospective randomized trials have evaluated NGL. NO induces periampullary sphincter relaxation and dilation of the micro vascular vessels, which hypothetically improve pancreatic circulation and nutrition<sup>[53]</sup>.

Sudhindran *et al* observed in a study of 186 patients randomized to either NGL 2 mg sublingual 5 min prior to ERCP or placebo, an incidence of PEP in the NGL group of 8% compared to 18% ( $P < 0.05$ ) in the placebo group<sup>[54]</sup>. This finding was supported by Moreto *et al* who randomized 144 patients to either NGL as dermal patch or placebo, and found a significant reduction in the incidence of PEP (4% *vs* 16%,  $P < 0.05$ )<sup>[55]</sup>. Both studies have been criticized for having a surprisingly high incidence of PEP in the placebo groups<sup>[56]</sup>. In a recently published prospective randomized trial of 318 patients the overall incidence of PEP was 7.5% and no significant difference between the NGL group and the placebo group was revealed<sup>[57]</sup>.

NGL has the optimal qualities as a prophylactic agent as it is cheap and easy to administer. However further trials are needed to determine its potential use as prophylaxis against PEP.

### Corticosteroids

Corticosteroids are potent unspecific anti-inflammatory

drugs utilized in a variety of inflammatory diseases. Several case reports have suspected steroids of being the etiology to iatrogenic AP but a definitive relationship has not been established<sup>[58-60]</sup>.

In rat models of AP hydrocortisone has reduced mortality and blood cytokine levels<sup>[61,62]</sup>. No human trials using steroids as treatment of AP have been published and attempts to show a beneficial effect of steroids as prophylaxis against PEP in prospective placebo-controlled trials have so far been disappointing. In 1999 De Palma *et al* randomized 539 patients to either placebo ( $n = 266$ ) or hydrocortisone 100 mg ( $n = 273$ ) administered intravenously prior to ERCP. The total incidence of PEP was 5.3% ( $n = 28$ ) and no significant difference between the two groups could be demonstrated<sup>[63]</sup>. In a Polish trial published in 2001, 300 patients received oral prednisone 40 mg, allopurinol 200 mg or placebo 15 h and 3 h prior to ERCP. The total incidence of PEP was 10.7% and no significant difference among the three groups was displayed<sup>[64]</sup>. Sherman *et al* have confirmed these negative findings in an even larger prospective trial with 1115 patients<sup>[65]</sup>.

Although steroids have the potential to inhibit the inflammatory cascade there is no evidence for the use of neither hydrocortisone nor prednisone as prophylaxis against PEP.

### NSAID

NSAID have an analgesic as well as an anti-inflammatory effect. Most NSAID act as non-selective inhibitors of the enzyme COX which catalyses the formation of prostaglandins and thromboxane from arachidonic acid. NSAID are used for virtually every known inflammatory disease.

Salicylic acid and indomethacin have in isolated case reports been related to the development of AP<sup>[66-68]</sup> as has the selective cyclooxygenase (COX)-2 inhibitor celecoxib<sup>[69-72]</sup>.

Experimental animal models studying the effect of NSAID on AP have been contradictory and not revealed any effect on mortality<sup>[73-76]</sup>.

The only randomized human study on the therapeutic effect of NSAID on AP has been conducted by Ebbelhøj *et al* who included 30 patients randomized in two groups receiving either indomethacin suppositories 50 mg twice daily for 7 d or placebo. No difference in serum amylase or calcium was observed but patients in the indomethacin group demanded less opiate as analgesics during hospitalization. Mortality was not registered<sup>[77]</sup>.

Two studies testing the prophylactic effect of indomethacin given prior to ERCP to prevent PEP have been published. Montano *et al* included 117 patients, who received either indomethacin suppositories 100 mg or placebo 2 h prior to ERCP. The incidence of PEP was 2.5% and 6.8% respectively but the difference was not significantly different<sup>[78]</sup>. In a larger study from Iran 490 participants received 100 mg indomethacin suppositories or placebo and an incidence of PEP of 3.2% in the indomethacin group and 6.8% in the placebo group was observed. The difference was only borderline significant different ( $P = 0.06$ ) and a post hoc analysis showed

significant lower incidence of PEP in the subpopulation of patients who underwent pancreatography<sup>[79]</sup>. However this conclusion was hampered by the fact that the post hoc analysis was conducted on 10 subpopulations, which in general reduces the statistical power considerably<sup>[80]</sup>.

Another NSAID, diclofenac, has been investigated in a study including 220 patients receiving either diclofenac suppositories 100 mg or placebo immediately after ERCP. PEP occurred with lower frequency in the group receiving diclofenac compared to the placebo group (6% *vs* 15%,  $P < 0.05$ )<sup>[81]</sup>.

The overall impression from placebo-controlled trials suggests a beneficial effect of NSAID used as prophylaxis against PEP. Both diclofenac and indomethacin can be administered easily as suppositories and are inexpensive drugs. Still, placebo controlled randomized trials with a larger sample size are needed to verify this promising effect.

### IL-10

IL-10 is produced and released by the helper T cells and its primary effect is anti-inflammatory. Clinical observations have shown increased levels of IL-10 in the blood during AP but its role in the treatment of AP remains to be determined<sup>[82,83]</sup>. The effect of IL-10 on AP has been validated in two experimental studies which showed a reduced mortality<sup>[84,85]</sup>.

No human study on the therapeutic effect of IL-10 has been conducted but the prophylactic effect of IL-10 on PEP has been evaluated in two randomized studies. No significant difference among the IL-10 and placebo-treated group was observed in a study with 200 patients receiving either recombinant IL-10 (8 µg/kg) or placebo (9% *vs* 11%,  $P = 0.65$ )<sup>[86]</sup>. A second study randomized 137 patients to placebo or IL-10 (4 µg/kg or 20 µg/kg) administered 30 min prior to ERCP. Overall incidence of PEP was 14% and a significant difference in the incidence among the three groups was noted (24%, 10%, and 7%). However the incidence of PEP in the placebo was remarkable high<sup>[87]</sup>.

The results from these two published studies do not definitively support the use of IL-10 as prophylaxis against PEP.

### TNF-α

During AP the serum level of TNF-α is elevated<sup>[88,89]</sup>. The synthesis and release of TNF-α takes place in macrophages located in the pancreas. SAC may as well release TNF-α and do also express TNF-α receptors during AP<sup>[90-92]</sup>. A possible relationship between genetic polymorphism and severity of AP has been established<sup>[93]</sup>.

Blocking the TNF-α mediated inflammation with anti-TNF-α antibodies or pentoxifylline seems to have a beneficial effect on histology score and mortality in experimental animal models<sup>[94-98]</sup>.

No data on humans has hitherto been published apart from a single case-report concerning a patient with interstitial pancreatitis. In this case, a male patient with severe bloody diarrhea due to segmental Crohn's disease also showed signs of AP. Serum amylase was high and ultrasound and abdominal computer tomography (CT) scans revealed an edematous pancreas. Because of these

findings treatment with steroids and azathioprine was abandoned and instead a single infusion with infliximab 5 mg/kg was administered without complications. The patient's overall condition improved and serum amylase levels normalized<sup>[99]</sup>.

Thus experimental data suggest a potential role of specific TNF- $\alpha$  inhibition in the treatment of AP, but high risk of bacterial infection during AP is a matter of concern. Infliximab has been evaluated in alcoholic hepatitis, another condition associated with a high risk of bacterial infection. The administration of prednisolone 40 mg daily and infliximab 10 mg/kg at wk 0, 2 and 4 showed an increased mortality due to infection and the study was terminated prematurely by the monitoring committee<sup>[100]</sup>.

Hence clinical studies on AP must be carefully designed to evaluate the safety of infliximab or other specific TNF- $\alpha$  inhibitor.

### PAF

PAF was discovered in the 1970's and soon recognized to be an important inflammatory mediator<sup>[101]</sup>. Later studies with experimental pancreatitis revealed that PAF is released during AP<sup>[102]</sup> and induce AP when infused in arteries supplying the pancreas<sup>[103,104]</sup>. Experimental studies have shown a benefit from PAF inhibition with various antagonists on pancreatic edema and systemic inflammation as well as a decreased bacterial translocation<sup>[105-107]</sup>.

As a consequence of the promising results with experimental AP different clinical studies have evaluated the effect of PAF inhibition. The first trial consisted of 83 patients with AP receiving lexipafant ( $n = 42$ ) or placebo ( $n = 41$ ). Lexipafant was administered intravenously (60 mg/d for 3 d) and follow-up was assessed for 5 d by Organ Failure Score (OFS). The investigators reported a greater reduction in OFS in the lexipafant group (0.905 *vs* 0.341,  $P = 0.048$ ), but during the 5 d period mortality was unaffected<sup>[108]</sup>. These findings were confirmed in a second trial including only patients with severe AP. The participants received lexipafant ( $n = 27$ ), 100 mg/d for 5-7 d or placebo ( $n = 23$ ). In the treatment group a larger reduction in OFS was registered (1.42 *vs* 0.17,  $P = 0.003$ ). Overall mortality was 18% with no difference between the groups<sup>[109]</sup>. The last study was published in 2001 and involved 286 patients with severe AP. Lexipafant (100 mg/d,  $n = 148$ ) or placebo ( $n = 138$ ) was administered for 7 d. No positive effect could be shown neither on OFS nor mortality<sup>[110]</sup>. It has been argued that data on the effect of lexipafant on mortality from experimental AP were warranted before initiation of human trials and the sponsor's communication of the result has been questionable<sup>[111]</sup>. After the termination of the clinical trials the lack of effect on mortality in experimental AP was acknowledged<sup>[112]</sup>. Randomized trials on sepsis were also disappointing<sup>[113,114]</sup> and inhibition of PAF in the treatment of AP has thus been abandoned.

### Antibiotics

Antibiotics is used to prevent or treat infected necrosis in

the pancreas and does not have potential to change the pathobiologic course of AP. Infected necrosis in pancreas is a major clinical problem during AP which severely deteriorate the prognosis<sup>[115,116]</sup>. Hence, administration of antibiotics to prevent infection has been evaluated in several randomized trials.

Sainio *et al* randomized 60 patients with necrotizing pancreatitis. The inclusion criteria were C-reactive protein > 120 mg/L and pancreatic necrosis verified by an abdominal CT scan. The treated group received infusion of cefuroxim 1.5 g  $\times$  3 daily, while patients allocated to the control group only received antibiotics in case of clinical signs of infection. A significant higher mortality was registered in the control group compared to the cefuroxim group (23% *vs* 3%,  $P = 0.03$ )<sup>[117]</sup>.

Pederzoli *et al* randomized 74 patients with CT verified pancreatic necrosis to receive either imipenem or placebo but no effect on mortality was registered<sup>[118]</sup>. In another prospective randomized study with 90 patients Nordback *et al* administered imipenem intravenously 1.0 g  $\times$  3 daily and found a reduced incidence of multiorgan failure compared to the control group (28% *vs* 76%,  $P = 0.0003$ ) but no difference in mortality<sup>[119]</sup>.

The studies described above are open-label trials. In 2004 Isenmann *et al* published a controlled double-blind study of 114 patients with CT verified necrotizing AP. The inclusion criteria were C-reactive protein > 150 mg/L and/or CT-verified pancreas necrosis. Placebo was compared to a combination of ciprofloxacin and metronidazole and if any complications occurred the treatment was converted to open conventional treatment. No difference in mortality or incidence of pancreas necrosis could be shown<sup>[120]</sup>.

A Cochrane meta-analysis of 294 patients with CT-verified pancreas necrosis showed a reduced mortality in patients with necrotizing AP when beta-lactams and quinolones were administered intravenously as prophylaxis<sup>[121]</sup>.

The subject continues to be a matter of debate and recommendations from major clinical associations have different approaches to this issue<sup>[4,2,34]</sup>.

Studies on the effect of prophylactic antibiotics given prior to ERCP are limited. In a study by Raty *et al* with 315 patients cephtazidime was administered intravenously prior to ERCP compared with a control group. They found reduced incidence of PEP and cholangitis in the treatment group (3% *vs* 9%,  $P = 0.009$ )<sup>[122]</sup>. However the study was not placebo-controlled and routine administration of antibiotics prior to ERCP cannot be recommended until randomized placebo controlled studies confirm this finding.

### Probiotics

Intestinal permeability is increased during AP, which may facilitate translocation of bacteria from the intestinal lumen. Oral probiotics are living microorganisms that exert health benefits beyond those of inherent basic nutrition<sup>[123]</sup>.

Olah *et al* conducted two prospective placebo controlled double-blinded studies of the therapeutic effect of probiotics to AP. The studies were published in 2002

and 2007 including 45 and 62 patients with interstitial or severe AP. In the latter study reduced mortality in the probiotics groups was observed but the difference was not statistical significant<sup>[124,125]</sup>.

## CONCLUSION

As described in this review we only have limited evidence based pharmacological approaches when treating AP and none of these are curative. Several treatments including animal experimental studies have been tried in order to establish evidence for etiology based medical treatment (Tables 1 and 2). In Italy and Japan gabexate is used routinely with the aim to limit pancreatic auto-digestion, but as reported in this review there is no conclusive evidence for this approach.

Ocreotide may be considered as prophylaxis against PEP but high cost of this peptide hormone limits its potential in clinical practice. A much cheaper alternative is NSAID, which may be considered as prophylaxis against PEP.

Antibiotics are the drugs of choice when infection is evident. However, recommendations regarding the prevention of infected pancreatic necrosis are contradictory.

Various problems are encountered when designing clinical studies of AP. The low incidence of severe necrotizing AP constitutes a major problem, which demands multicenter studies. Another clinical challenge is the resemblance of AP to infection and before initialization of any experimental anti-inflammatory therapy bacterial infection must be refuted. This delays the start-up of the experimental protocol and causes bias as the patients in the meantime receive anti-bacterial treatment. A possible solution could be to administer antibiotics to all patients in combination with specific anti-inflammatory treatment or placebo.

In spite of these challenges the search for pharmacological treatment of AP must be sustained. As we present in this review experimental animal models support a potential effect of several anti-inflammatory drugs, which are candidates for randomized trials. The most interesting of these potential drugs is probably steroids, which are standard treatment of numerous inflammatory diseases but have never been investigated in the treatment of AP.

Because the outcome of the disease depends highly on the involvement of other organs, developing methods that inhibit the inflammatory signaling pathways presents a great potential. As new information regarding the inflammatory pathways continues to emerge from animal and clinical trials, specific treatment targeting these inflammatory processes should be considered. It is our opinion that animal models at this time support clinical trials with anti-TNF- $\alpha$  antibodies although a randomized trial must be designed not forgetting the safety issue.

## REFERENCES

- 1 Lankisch PG, Lerch MM. Pharmacological prevention and treatment of acute pancreatitis: where are we now? *Dig Dis* 2006; **24**: 148-159
- 2 Pandol SJ, Saluja AK, Imrie CW, Banks PA. Acute pancreatitis: bench to the bedside. *Gastroenterology* 2007; **132**: 1127-1151
- 3 Makhija R, Kingsnorth AN. Cytokine storm in acute pancreatitis. *J Hepatobiliary Pancreat Surg* 2002; **9**: 401-410
- 4 Banks PA, Freeman ML. Practice guidelines in acute pancreatitis. *Am J Gastroenterol* 2006; **101**: 2379-2400
- 5 Sherman S, Ruffolo TA, Hawes RH, Lehman GA. Complications of endoscopic sphincterotomy. A prospective series with emphasis on the increased risk associated with sphincter of Oddi dysfunction and nondilated bile ducts. *Gastroenterology* 1991; **101**: 1068-1075
- 6 Freeman ML, DiSario JA, Nelson DB, Fennerty MB, Lee JG, Bjorkman DJ, Overby CS, Aas J, Ryan ME, Bochna GS, Shaw MJ, Snady HW, Erickson RV, Moore JP, Roel JP. Risk factors for post-ERCP pancreatitis: a prospective, multicenter study. *Gastrointest Endosc* 2001; **54**: 425-434
- 7 Cheng CL, Sherman S, Watkins JL, Barnett J, Freeman M, Geenen J, Ryan M, Parker H, Frakes JT, Fogel EL, Silverman WB, Dua KS, Aliperti G, Yakshe P, Uzer M, Jones W, Goff J, Lazzell-Pannell L, Rashdan A, Temkit M, Lehman GA. Risk factors for post-ERCP pancreatitis: a prospective multicenter study. *Am J Gastroenterol* 2006; **101**: 139-147
- 8 Katz MD, Erstad BL. Octreotide, a new somatostatin analogue. *Clin Pharm* 1989; **8**: 255-273
- 9 Cavallini G, Frulloni L. Somatostatin and octreotide in acute pancreatitis: the never-ending story. *Dig Liver Dis* 2001; **33**: 192-201
- 10 Uhl W, Buchler MW, Malfertheiner P, Beger HG, Adler G, Gaus W. A randomised, double blind, multicentre trial of octreotide in moderate to severe acute pancreatitis. *Gut* 1999; **45**: 97-104
- 11 Poon RT, Yeung C, Liu CL, Lam CM, Yuen WK, Lo CM, Tang A, Fan ST. Intravenous bolus somatostatin after diagnostic cholangiopancreatography reduces the incidence of pancreatitis associated with therapeutic endoscopic retrograde cholangiopancreatography procedures: a randomised controlled trial. *Gut* 2003; **52**: 1768-1773
- 12 Arvanitidis D, Anagnostopoulos GK, Giannopoulos D, Pantes A, Agaritsi R, Margantinis G, Tsiakos S, Sakorafas G, Kostopoulos P. Can somatostatin prevent post-ERCP pancreatitis? Results of a randomized controlled trial. *J Gastroenterol Hepatol* 2004; **19**: 278-282
- 13 Thomopoulos KC, Pagoni NA, Vagenas KA, Margaritis VG, Theocharis GI, Nikolopoulou VN. Twenty-four hour prophylaxis with increased dosage of octreotide reduces the incidence of post-ERCP pancreatitis. *Gastrointest Endosc* 2006; **64**: 726-731
- 14 Andriulli A, Clemente R, Solmi L, Terruzzi V, Suriani R, Sigillito A, Leandro G, Leo P, De Maio G, Perri F. Gabexate or somatostatin administration before ERCP in patients at high risk for post-ERCP pancreatitis: a multicenter, placebo-controlled, randomized clinical trial. *Gastrointest Endosc* 2002; **56**: 488-495
- 15 Andriulli A, Solmi L, Loperfido S, Leo P, Festa V, Belmonte A, Spirito F, Silla M, Forte G, Terruzzi V, Marengo G, Ciliberto E, Sabatino A, Monica F, Magnolia MR, Perri F. Prophylaxis of ERCP-related pancreatitis: a randomized, controlled trial of somatostatin and gabexate mesylate. *Clin Gastroenterol Hepatol* 2004; **2**: 713-718
- 16 Andriulli A, Leandro G, Federici T, Ippolito A, Forlano R, Iacobellis A, Annese V. Prophylactic administration of somatostatin or gabexate does not prevent pancreatitis after ERCP: an updated meta-analysis. *Gastrointest Endosc* 2007; **65**: 624-632
- 17 Li ZS, Pan X, Zhang WJ, Gong B, Zhi FC, Guo XG, Li PM, Fan ZN, Sun WS, Shen YZ, Ma SR, Xie WF, Chen MH, Li YQ. Effect of octreotide administration in the prophylaxis of post-ERCP pancreatitis and hyperamylasemia: A multicenter, placebo-controlled, randomized clinical trial. *Am J Gastroenterol* 2007; **102**: 46-51
- 18 Morbidity of acute pancreatitis: the effect of aprotinin and glucagon. *Gut* 1980; **21**: 334-339



- 19 **Trapnell JE**, Rigby CC, Talbot CH, Duncan EH. A controlled trial of Trasylol in the treatment of acute pancreatitis. *Br J Surg* 1974; **61**: 177-182
- 20 **Wisner JR Jr**, Renner IG, Grendell JH, Niederau C, Ferrell LD. Gabexate mesilate (FOY) protects against cerulein-induced acute pancreatitis in the rat. *Pancreas* 1987; **2**: 181-186
- 21 **Tympner F**, Rosch W. [Effect of secretin and gabexate-mesilate (synthetic protease inhibitor) on serum amylase level after ERCP] *Z Gastroenterol* 1982; **20**: 688-693
- 22 **Freise J**, Melzer P, Schmidt FW, Horbach L. [Gabexate mesilate in the treatment of acute pancreatitis. Results of a Hannover multicenter double-blind study with 50 patients] *Z Gastroenterol* 1986; **24**: 200-211
- 23 **Yang CY**, Chang-Chien CS, Liaw YF. Controlled trial of protease inhibitor gabexate mesilate (FOY) in the treatment of acute pancreatitis. *Pancreas* 1987; **2**: 698-700
- 24 **Harada H**, Miyake H, Ochi K, Tanaka J, Kimura I. Clinical trial with a protease inhibitor gabexate mesilate in acute pancreatitis. *Int J Pancreatol* 1991; **9**: 75-79
- 25 **Valderrama R**, Perez-Mateo M, Navarro S, Vazquez N, Sanjose L, Adrian MJ, Estruch J. Multicenter double-blind trial of gabexate mesilate (FOY) in unselected patients with acute pancreatitis. *Digestion* 1992; **51**: 65-70
- 26 **Buchler M**, Malfertheiner P, Uhl W, Scholmerich J, Stockmann F, Adler G, Gaus W, Rolle K, Beger HG. Gabexate mesilate in human acute pancreatitis. German Pancreatitis Study Group. *Gastroenterology* 1993; **104**: 1165-1170
- 27 **Chen HM**, Chen JC, Hwang TL, Jan YY, Chen MF. Prospective and randomized study of gabexate mesilate for the treatment of severe acute pancreatitis with organ dysfunction. *Hepatogastroenterology* 2000; **47**: 1147-1150
- 28 **Seta T**, Noguchi Y, Shimada T, Shikata S, Fukui T. Treatment of acute pancreatitis with protease inhibitors: a meta-analysis. *Eur J Gastroenterol Hepatol* 2004; **16**: 1287-1293
- 29 **Takeda K**, Matsuno S, Sunamura M, Kakugawa Y. Continuous regional arterial infusion of protease inhibitor and antibiotics in acute necrotizing pancreatitis. *Am J Surg* 1996; **171**: 394-398
- 30 **Takeda K**, Yamauchi J, Shibuya K, Sunamura M, Mikami Y, Matsuno S. Benefit of continuous regional arterial infusion of protease inhibitor and antibiotic in the management of acute necrotizing pancreatitis. *Pancreatol* 2001; **1**: 668-673
- 31 **Cavallini G**, Tittobello A, Frulloni L, Masci E, Mariana A, Di Francesco V. Gabexate for the prevention of pancreatic damage related to endoscopic retrograde cholangiopancreatography. Gabexate in digestive endoscopy--Italian Group. *N Engl J Med* 1996; **335**: 919-923
- 32 **Zheng M**, Chen Y, Yang X, Li J, Zhang Y, Zeng Q. Gabexate in the prophylaxis of post-ERCP pancreatitis: a meta-analysis of randomized controlled trials. *BMC Gastroenterol* 2007; **7**: 6
- 33 **Manes G**, Ardizzone S, Lombardi G, Uomo G, Pieramico O, Porro GB. Efficacy of postprocedure administration of gabexate mesilate in the prevention of post-ERCP pancreatitis: a randomized, controlled, multicenter study. *Gastrointest Endosc* 2007; **65**: 982-987
- 34 **Whitcomb DC**. Clinical practice. Acute pancreatitis. *N Engl J Med* 2006; **354**: 2142-2150
- 35 **Otsuki M**, Hirota M, Arata S, Koizumi M, Kawa S, Kamisawa T, Takeda K, Mayumi T, Kitagawa M, Ito T, Inui K, Shimosegawa T, Tanaka S, Kataoka K, Saisho H, Okazaki K, Kuroda Y, Sawabu N, Takeyama Y. Consensus of primary care in acute pancreatitis in Japan. *World J Gastroenterol* 2006; **12**: 3314-3323
- 36 **Pelagotti F**, Cecchi M, Messori A. Use of gabexate mesilate in Italian hospitals: a multicentre observational study. *J Clin Pharm Ther* 2003; **28**: 191-196
- 37 **Sweiry JH**, Mann GE. Role of oxidative stress in the pathogenesis of acute pancreatitis. *Scand J Gastroenterol Suppl* 1996; **219**: 10-15
- 38 **Neuschwander-Tetri BA**, Ferrell LD, Sukhabote RJ, Grendell JH. Glutathione monoethyl ester ameliorates cerulein-induced pancreatitis in the mouse. *J Clin Invest* 1992; **89**: 109-116
- 39 **Demols A**, Van Laethem JL, Quertinmont E, Legros F, Louis H, Le Moine O, Deviere J. N-acetylcysteine decreases severity of acute pancreatitis in mice. *Pancreas* 2000; **20**: 161-169
- 40 **Sevillano S**, De la Mano AM, De Dios I, Ramudo L, Manso MA. Major pathological mechanisms of acute pancreatitis are prevented by N-acetylcysteine. *Digestion* 2003; **68**: 34-40
- 41 **Mumcu S**, Alhan E, Turkiylmaz S, Kural BV, Ercin C, Kalyoncu NI. Effects of N-acetylcysteine on acute necrotizing pancreatitis in rats. *Eur Surg Res* 2005; **37**: 173-178
- 42 **Ramudo L**, Manso MA, Vicente S, De Dios I. Pro- and anti-inflammatory response of acinar cells during acute pancreatitis. Effect of N-acetyl cysteine. *Cytokine* 2005; **32**: 125-131
- 43 **Esrefoglu M**, Gul M, Ates B, Yilmaz I. Ultrastructural clues for the protective effect of ascorbic acid and N-acetylcysteine against oxidative damage on cerulein-induced pancreatitis. *Pancreatol* 2006; **6**: 477-485
- 44 **Manso MA**, Ramudo L, De Dios I. Extrapancreatic organ impairment during acute pancreatitis induced by bile-pancreatic duct obstruction. Effect of N-acetylcysteine. *Int J Exp Pathol* 2007; **88**: 343-349
- 45 **Scott P**, Bruce C, Schofield D, Shiel N, Braganza JM, McCloy RF. Vitamin C status in patients with acute pancreatitis. *Br J Surg* 1993; **80**: 750-754
- 46 **Braganza JM**, Scott P, Bilton D, Schofield D, Chaloner C, Shiel N, Hunt LP, Bottiglieri T. Evidence for early oxidative stress in acute pancreatitis. Clues for correction. *Int J Pancreatol* 1995; **17**: 69-81
- 47 **Bonham MJ**, Abu-Zidan FM, Simovic MO, Sluis KB, Wilkinson A, Winterbourn CC, Windsor JA. Early ascorbic acid depletion is related to the severity of acute pancreatitis. *Br J Surg* 1999; **86**: 1296-1301
- 48 **Siriwardena AK**, Mason JM, Balachandra S, Bagul A, Galloway S, Formela L, Hardman JG, Jamdar S. Randomised, double blind, placebo controlled trial of intravenous antioxidant (n-acetylcysteine, selenium, vitamin C) therapy in severe acute pancreatitis. *Gut* 2007; **56**: 1439-1444
- 49 **Katsinelos P**, Kountouras J, Paroutoglou G, Beltsis A, Mimidis K, Zavos C. Intravenous N-acetylcysteine does not prevent post-ERCP pancreatitis. *Gastrointest Endosc* 2005; **62**: 105-111
- 50 **Milewski J**, Rydzewska G, Degowska M, Kierzkiewicz M, Rydzewski A. N-acetylcysteine does not prevent post-endoscopic retrograde cholangiopancreatography hyperamylasemia and acute pancreatitis. *World J Gastroenterol* 2006; **12**: 3751-3755
- 51 **Sorkin EM**, Brogden RN, Romankiewicz JA. Intravenous glyceryl trinitrate (nitroglycerin). A review of its pharmacological properties and therapeutic efficacy. *Drugs* 1984; **27**: 45-80
- 52 **Werner J**, Rivera J, Fernandez-del Castillo C, Lewandrowski K, Adrie C, Rattner DW, Warshaw AL. Differing roles of nitric oxide in the pathogenesis of acute edematous versus necrotizing pancreatitis. *Surgery* 1997; **121**: 23-30
- 53 **Wehrmann T**, Schmitt T, Stergiou N, Caspary WF, Seifert H. Topical application of nitrates onto the papilla of Vater: manometric and clinical results. *Endoscopy* 2001; **33**: 323-328
- 54 **Sudhindran S**, Bromwich E, Edwards PR. Prospective randomized double-blind placebo-controlled trial of glyceryl trinitrate in endoscopic retrograde cholangiopancreatography-induced pancreatitis. *Br J Surg* 2001; **88**: 1178-1182
- 55 **Moreto M**, Zaballa M. Prospective randomized double-blind placebo-controlled trial of glyceryl trinitrate in endoscopic retrograde cholangiopancreatography-induced pancreatitis. *Br J Surg* 2002; **89**: 628; author reply 629
- 56 **Muralidharan V**, Jamidar P. Pharmacologic prevention of post-ERCP pancreatitis: is nitroglycerin a sangreal? *Gastrointest Endosc* 2006; **64**: 358-360
- 57 **Kaffes AJ**, Bourke MJ, Ding S, Alrubaie A, Kwan V, Williams SJ. A prospective, randomized, placebo-controlled trial of transdermal glyceryl trinitrate in ERCP: effects on technical success and post-ERCP pancreatitis. *Gastrointest Endosc* 2006; **64**: 351-357
- 58 **Bourne MS**, Dawson H. Acute pancreatitis complicating prednisolone therapy. *Lancet* 1958; **2**: 1209-1210
- 59 **Boruchowicz A**, Gallon P, Foissey D, Gower P, Gamblin C, Cuingnet P, Maunoury V, Cortot A, Colombel JF. [Acute

- pancreatitis associated with corticosteroid treatment in Crohn's disease] *Gastroenterol Clin Biol* 2003; **27**: 560-561
- 60 **Khanna S**, Kumar A. Acute pancreatitis due to hydrocortisone in a patient with ulcerative colitis. *J Gastroenterol Hepatol* 2003; **18**: 1110-1111
  - 61 **Gloor B**, Uhl W, Tcholakov O, Roggo A, Muller CA, Worni M, Buchler MW. Hydrocortisone treatment of early SIRS in acute experimental pancreatitis. *Dig Dis Sci* 2001; **46**: 2154-2161
  - 62 **Lazar G Jr**, Varga J, Lazar G, Duda E, Takacs T, Balogh A, Lonovics J. The effects of glucocorticoids and a glucocorticoid antagonist (RU 38486) on experimental acute pancreatitis in rat. *Acta Chir Hung* 1997; **36**: 190-191
  - 63 **De Palma GD**, Catanzano C. Use of corticosteroids in the prevention of post-ERCP pancreatitis: results of a controlled prospective study. *Am J Gastroenterol* 1999; **94**: 982-985
  - 64 **Budzynska A**, Marek T, Nowak A, Kaczor R, Nowakowska-Dulawa E. A prospective, randomized, placebo-controlled trial of prednisone and allopurinol in the prevention of ERCP-induced pancreatitis. *Endoscopy* 2001; **33**: 766-772
  - 65 **Sherman S**, Blaut U, Watkins JL, Barnett J, Freeman M, Geenen J, Ryan M, Parker H, Frakes JT, Fogel EL, Silverman WB, Dua KS, Aliperti G, Yakshe P, Uzer M, Jones W, Goff J, Earle D, Temkit M, Lehman GA. Does prophylactic administration of corticosteroid reduce the risk and severity of post-ERCP pancreatitis: a randomized, prospective, multicenter study. *Gastrointest Endosc* 2003; **58**: 23-29
  - 66 **Sussman S**. Severe salicylism and acute pancreatitis. *Calif Med* 1963; **99**: 29-32
  - 67 **Guerra M**. Toxicity of indomethacin. Report of a case of acute pancreatitis. *JAMA* 1967; **200**: 552-553
  - 68 **Memis D**, Akalin E, Yucel T. Indomethacin-induced pancreatitis: a case report. *JOP* 2005; **6**: 344-347
  - 69 **Amaravadi RK**, Jacobson BC, Solomon DH, Fischer MA. Acute pancreatitis associated with rofecoxib. *Am J Gastroenterol* 2002; **97**: 1077-1078
  - 70 **Nind G**, Selby W. Acute pancreatitis: a rare complication of celecoxib. *Intern Med J* 2002; **32**: 624-625
  - 71 **Baciewicz AM**, Sokos DR, King TJ. Acute pancreatitis associated with celecoxib. *Ann Intern Med* 2000; **132**: 680
  - 72 **Carrillo-Jimenez R**, Nurnberger M. Celecoxib-induced acute pancreatitis and hepatitis: a case report. *Arch Intern Med* 2000; **160**: 553-554
  - 73 **Alhan E**, Kalyoncu NI, Erinc C, Kural BV. Effects of the celecoxib on the acute necrotizing pancreatitis in rats. *Inflammation* 2004; **28**: 303-309
  - 74 **Coelle EF**, Adham N, Elashoff J, Lewin K, Taylor IL. Effects of prostaglandin and indomethacin on diet-induced acute pancreatitis in mice. *Gastroenterology* 1983; **85**: 1307-1312
  - 75 **de Almeida JL**, Jukemura J, Coelho AM, Patzina RA, Machado MC, da Cunha JE. Inhibition of cyclooxygenase-2 in experimental severe acute pancreatitis. *Clinics* 2006; **61**: 301-306
  - 76 **Foitzik T**, Hotz HG, Hotz B, Wittig F, Buhr HJ. Selective inhibition of cyclooxygenase-2 (COX-2) reduces prostaglandin E2 production and attenuates systemic disease sequelae in experimental pancreatitis. *Hepatogastroenterology* 2003; **50**: 1159-1162
  - 77 **Ebbehoj N**, Friis J, Svendsen LB, Bulow S, Madsen P. Indomethacin treatment of acute pancreatitis. A controlled double-blind trial. *Scand J Gastroenterol* 1985; **20**: 798-800
  - 78 **Montano LA**, Garcia CJ, Gonzalez OA, Fuentes OC, Davalos CC, Rodriguez L, X. [Prevention of hyperamylasemia and pancreatitis after endoscopic retrograde cholangiopancreatography with rectal administration of indomethacin] *Rev Gastroenterol Mex* 2006; **71**: 262-268
  - 79 **Sotoudehmanesh R**, Khatibian M, Kolahdoozan S, Ainechi S, Malboosbaf R, Nouraei M. Indomethacin may reduce the incidence and severity of acute pancreatitis after ERCP. *Am J Gastroenterol* 2007; **102**: 978-983
  - 80 **Wagh MS**, Sherman S. Indomethacin for post-ERCP pancreatitis prophylaxis: another attempt at the Holy Grail. *Am J Gastroenterol* 2007; **102**: 984-986
  - 81 **Murray B**, Carter R, Imrie C, Evans S, O'Suilleabhain C. Diclofenac reduces the incidence of acute pancreatitis after endoscopic retrograde cholangiopancreatography. *Gastroenterology* 2003; **124**: 1786-1791
  - 82 **Berney T**, Gasche Y, Robert J, Jenny A, Mensi N, Grau G, Vermeulen B, Morel P. Serum profiles of interleukin-6, interleukin-8, and interleukin-10 in patients with severe and mild acute pancreatitis. *Pancreas* 1999; **18**: 371-377
  - 83 **Pezzilli R**, Billi P, Miniero R, Barakat B. Serum interleukin-10 in human acute pancreatitis. *Dig Dis Sci* 1997; **42**: 1469-1472
  - 84 **Kusske AM**, Rongione AJ, Ashley SW, McFadden DW, Reber HA. Interleukin-10 prevents death in lethal necrotizing pancreatitis in mice. *Surgery* 1996; **120**: 284-288; discussion 289
  - 85 **Rongione AJ**, Kusske AM, Reber HA, Ashley SW, McFadden DW. Interleukin-10 reduces circulating levels of serum cytokines in experimental pancreatitis. *J Gastrointest Surg* 1997; **1**: 159-165; discussion 165-166
  - 86 **Dumot JA**, Conwell DL, Zuccaro G Jr, Vargo JJ, Shay SS, Easley KA, Ponsky JL. A randomized, double blind study of interleukin 10 for the prevention of ERCP-induced pancreatitis. *Am J Gastroenterol* 2001; **96**: 2098-2102
  - 87 **Devriere J**, Le Moine O, Van Laethem JL, Eisendrath P, Ghilain A, Severs N, Cohard M. Interleukin 10 reduces the incidence of pancreatitis after therapeutic endoscopic retrograde cholangiopancreatography. *Gastroenterology* 2001; **120**: 498-505
  - 88 **Kaufmann P**, Tilz GP, Lueger A, Demel U. Elevated plasma levels of soluble tumor necrosis factor receptor (sTNFRp60) reflect severity of acute pancreatitis. *Intensive Care Med* 1997; **23**: 841-848
  - 89 **Brivet FG**, Emilie D, Galanaud P. Pro- and anti-inflammatory cytokines during acute severe pancreatitis: an early and sustained response, although unpredictable of death. Parisian Study Group on Acute Pancreatitis. *Crit Care Med* 1999; **27**: 749-755
  - 90 **Vaccaro MI**, Ropolo A, Grasso D, Calvo EL, Ferreria M, Iovanna JL, Lanosa G. Pancreatic acinar cells submitted to stress activate TNF-alpha gene expression. *Biochem Biophys Res Commun* 2000; **268**: 485-490
  - 91 **Gukovskaya AS**, Gukovsky I, Zaninovic V, Song M, Sandoval D, Gukovsky S, Pandol SJ. Pancreatic acinar cells produce, release, and respond to tumor necrosis factor-alpha. Role in regulating cell death and pancreatitis. *J Clin Invest* 1997; **100**: 1853-1862
  - 92 **Ramudo L**, Manso MA, Sevillano S, de Dios I. Kinetic study of TNF-alpha production and its regulatory mechanisms in acinar cells during acute pancreatitis induced by bile-pancreatic duct obstruction. *J Pathol* 2005; **206**: 9-16
  - 93 **Balog A**, Gyulai Z, Boros LG, Farkas G, Takacs T, Lonovics J, Mandi Y. Polymorphism of the TNF-alpha, HSP70-2, and CD14 genes increases susceptibility to severe acute pancreatitis. *Pancreas* 2005; **30**: e46-e50
  - 94 **Hughes CB**, Gaber LW, Mohey el-Din AB, Grewal HP, Kotb M, Mann L, Gaber AO. Inhibition of TNF alpha improves survival in an experimental model of acute pancreatitis. *Am Surg* 1996; **62**: 8-13
  - 95 **Chen D**, Wang W, Wang J. Influence of anti-TNF alpha monoclonal antibody on intestinal barrier in rats with acute pancreatitis. *Chin Med Sci J* 2000; **15**: 257
  - 96 **Malleo G**, Mazzon E, Genovese T, Di Paola R, Muia C, Centorrino T, Siriwardena AK, Cuzzocrea S. Etanercept attenuates the development of cerulein-induced acute pancreatitis in mice: a comparison with TNF-alpha genetic deletion. *Shock* 2007; **27**: 542-551
  - 97 **Ramudo L**, Manso MA, Sevillano S, de Dios I. Kinetic study of TNF-alpha production and its regulatory mechanisms in acinar cells during acute pancreatitis induced by bile-pancreatic duct obstruction. *J Pathol* 2005; **206**: 9-16
  - 98 **Pereda J**, Sabater L, Cassinello N, Gomez-Cambronero L, Closa D, Folch-Puy E, Aparisi L, Calvete J, Cerda M, Lledo S, Vina J, Sastre J. Effect of simultaneous inhibition of TNF-alpha production and xanthine oxidase in experimental acute pancreatitis: the role of mitogen activated protein kinases. *Ann Surg* 2004; **240**: 108-116
  - 99 **Triantafyllidis JK**, Cheracakis P, Hereti IA, Argyros N, Karra

- E. Acute idiopathic pancreatitis complicating active Crohn's disease: favorable response to infliximab treatment. *Am J Gastroenterol* 2000; **95**: 3334-3336
- 100 **Naveau S**, Chollet-Martin S, Dharancy S, Mathurin P, Jouet P, Piquet MA, Davion T, Oberti F, Broet P, Emilie D. A double-blind randomized controlled trial of infliximab associated with prednisolone in acute alcoholic hepatitis. *Hepatology* 2004; **39**: 1390-1397
  - 101 **Benveniste J**, Henson PM, Cochrane CG. Leukocyte-dependent histamine release from rabbit platelets. The role of IgE, basophils, and a platelet-activating factor. *J Exp Med* 1972; **136**: 1356-1377
  - 102 **Kald B**, Kald A, Ihse I, Tagesson C. Release of platelet-activating factor in acute experimental pancreatitis. *Pancreas* 1993; **8**: 440-442
  - 103 **Emanuelli G**, Montrucchio G, Gaia E, Dughera L, Corvetto G, Gubetta L. Experimental acute pancreatitis induced by platelet activating factor in rabbits. *Am J Pathol* 1989; **134**: 315-326
  - 104 **Yotsumoto F**, Manabe T, Kyogoku T, Hirano T, Ohshio G, Yamamoto M, Imamura T, Yoshitomi S. Platelet-activating factor involvement in the aggravation of acute pancreatitis in rabbits. *Digestion* 1994; **55**: 260-267
  - 105 **Jancar S**, De Giaccobi G, Mariano M, Mencia-Huerta JM, Sirois P, Braquet P. Immune complex induced pancreatitis: effect of BN 52021, a selective antagonist of platelet-activating factor. *Prostaglandins* 1988; **35**: 757-770
  - 106 **Dabrowski A**, Gabryelewicz A, Chyczewski L. The effect of platelet activating factor antagonist (BN 52021) on cerulein-induced acute pancreatitis with reference to oxygen radicals. *Int J Pancreatol* 1991; **8**: 1-11
  - 107 **Tomaszewska R**, Dembinski A, Warzecha Z, Banas M, Konturek SJ, Stachura J. Platelet activating factor (PAF) inhibitor (TCV-309) reduces caerulein- and PAF-induced pancreatitis. A morphologic and functional study in the rat. *J Physiol Pharmacol* 1992; **43**: 345-352
  - 108 **Kingsnorth AN**, Galloway SW, Formela LJ. Randomized, double-blind phase II trial of Lexipafant, a platelet-activating factor antagonist, in human acute pancreatitis. *Br J Surg* 1995; **82**: 1414-1420
  - 109 **McKay CJ**, Curran F, Sharples C, Baxter JN, Imrie CW. Prospective placebo-controlled randomized trial of lexipafant in predicted severe acute pancreatitis. *Br J Surg* 1997; **84**: 1239-1243
  - 110 **Johnson CD**, Kingsnorth AN, Imrie CW, McMahon MJ, Neoptolemos JP, McKay C, Toh SK, Skaife P, Leeder PC, Wilson P, Larvin M, Curtis LD. Double blind, randomised, placebo controlled study of a platelet activating factor antagonist, lexipafant, in the treatment and prevention of organ failure in predicted severe acute pancreatitis. *Gut* 2001; **48**: 62-69
  - 111 **Abu-Zidan FM**, Windsor JA. Lexipafant and acute pancreatitis: a critical appraisal of the clinical trials. *Eur J Surg* 2002; **168**: 215-219
  - 112 **Rivera JA**, Werner J, Warshaw AL, Lewandrowski KB, Rattner DW, Fernandez del Castillo C. Lexipafant fails to improve survival in severe necrotizing pancreatitis in rats. *Int J Pancreatol* 1998; **23**: 101-106
  - 113 **Suputtamongkol Y**, Intaranongpai S, Smith MD, Angus B, Chaowagul W, Permpikul C, Simpson JA, Leelarasamee A, Curtis L, White NJ. A double-blind placebo-controlled study of an infusion of lexipafant (Platelet-activating factor receptor antagonist) in patients with severe sepsis. *Antimicrob Agents Chemother* 2000; **44**: 693-696
  - 114 **Vincent JL**, Spapen H, Bakker J, Webster NR, Curtis L. Phase II multicenter clinical study of the platelet-activating factor receptor antagonist BB-882 in the treatment of sepsis. *Crit Care Med* 2000; **28**: 638-642
  - 115 **Beger HG**, Bittner R, Block S, Buchler M. Bacterial contamination of pancreatic necrosis. A prospective clinical study. *Gastroenterology* 1986; **91**: 433-438
  - 116 **Isenmann R**, Rau B, Beger HG. Bacterial infection and extent of necrosis are determinants of organ failure in patients with acute necrotizing pancreatitis. *Br J Surg* 1999; **86**: 1020-1024
  - 117 **Sainio V**, Kempainen E, Puolakkainen P, Taavitsainen M, Kivisaari L, Valtonen V, Haapiainen R, Schroder T, Kivilaakso E. Early antibiotic treatment in acute necrotising pancreatitis. *Lancet* 1995; **346**: 663-667
  - 118 **Pederzoli P**, Bassi C, Vesentini S, Campedelli A. A randomized multicenter clinical trial of antibiotic prophylaxis of septic complications in acute necrotizing pancreatitis with imipenem. *Surg Gynecol Obstet* 1993; **176**: 480-483
  - 119 **Nordback I**, Sand J, Saaristo R, Pajanen H. Early treatment with antibiotics reduces the need for surgery in acute necrotizing pancreatitis--a single-center randomized study. *J Gastrointest Surg* 2001; **5**: 113-118; discussion 118-120
  - 120 **Isenmann R**, Runzi M, Kron M, Kahl S, Kraus D, Jung N, Maier L, Malfertheiner P, Goebell H, Beger HG. Prophylactic antibiotic treatment in patients with predicted severe acute pancreatitis: a placebo-controlled, double-blind trial. *Gastroenterology* 2004; **126**: 997-1004
  - 121 **Villatoro E**, Bassi C, Larvin M. Antibiotic therapy for prophylaxis against infection of pancreatic necrosis in acute pancreatitis. *Cochrane Database Syst Rev* 2006: CD002941
  - 122 **Raty S**, Sand J, Pulkkinen M, Matikainen M, Nordback I. Post-ERCP pancreatitis: reduction by routine antibiotics. *J Gastrointest Surg* 2001; **5**: 339-345; discussion 345
  - 123 **Guarner F**, Malagelada JR. Gut flora in health and disease. *Lancet* 2003; **361**: 512-519
  - 124 **Olah A**, Belagyi T, Issekutz A, Gamal ME, Bengmark S. Randomized clinical trial of specific lactobacillus and fibre supplement to early enteral nutrition in patients with acute pancreatitis. *Br J Surg* 2002; **89**: 1103-1107
  - 125 **Olah A**, Belagyi T, Poto L, Romics L Jr, Bengmark S. Synbiotic control of inflammation and infection in severe acute pancreatitis: a prospective, randomized, double blind study. *Hepatogastroenterology* 2007; **54**: 590-594

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Hugh James Freeman, MD, FRCPC, FACP, Series Editor

## Intraductal papillary mucinous neoplasms and other pancreatic cystic lesions

Hugh James Freeman

Hugh James Freeman, Department of Medicine (Gastroenterology), University of British Columbia, Vancouver V6T 1W5, Canada

Author contribution: Freeman HJ contributed all to this paper.

Correspondence to: Dr. Hugh James Freeman, MD, FRCPC, FACP, Department of Medicine (Gastroenterology), University of British Columbia Hospital, 2211 Wesbrook Mall, Vancouver V6T 1W5, Canada. [hugfree@shaw.ca](mailto:hugfree@shaw.ca)

Telephone: +1-604-8227216 Fax: +1-604-8227236

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### Abstract

Pancreatic cystic neoplasms are being increasingly recognized, even in the absence of symptoms, in large part, due to markedly improved imaging modalities such as magnetic resonance imaging (MRI)/magnetic resonance cholangio pancreatography (MRCP) and computer tomography (CT) scanning. During the past 2 decades, better imaging of these cystic lesions has resulted in definition of different types, including pancreatic intraductal papillary mucinous neoplasms (IPMN). While IPMN represent only a distinct minority of all pancreatic cancers, they appear to be a relatively frequent neoplastic form of pancreatic cystic neoplasm. Moreover, IPMN have a much better outcome and prognosis compared to pancreatic ductal adenocarcinomas. Therefore, recognition of this entity is exceedingly important for the clinician involved in diagnosis and further evaluation of a potentially curable form of pancreatic cancer.

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**Key words:** Pancreatic cancer; Pancreatic intraductal papillary mucinous neoplasms; Mucinous cystic neoplasm of pancreas; Serous cystadenoma; Pancreatic cystic lesions

**Peer reviewer:** Michael Steer, Professor, Department of Surgery, Tufts-Nemc, 860 Washington St, Boston, Ma 02111, United States

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### INTRODUCTION

Due to increasing precision of modern imaging modalities, particularly computer tomography (CT) scanning (with contrast enhancement) and magnetic resonance imaging (MRI)/magnetic resonance cholangio pancreatography (MRCP), pancreatic cystic lesions are commonly being detected. This has been reported in up to 25% of patients, particularly with increasing age<sup>[1,2]</sup>. From a pathological rather than imaging perspective, however, a cyst is formally defined as a fluid-filled and closed cavity with an epithelial lining. In the pancreas (as opposed to liver and spleen), cyst-like lesions have special significance as different neoplasms in the pancreas are true cysts, or alternatively, may appear cystic either from dilation of a tumor obstructed or stenosed pancreatic duct or from necrotic changes and degeneration within a solid neoplastic lesion, possibly from rapid tumor growth that outstrips its blood supply.

Most commonly, a pancreatic cystic lesion defined by imaging represents a pseudocyst, usually due to alcoholic pancreatitis. Pseudocysts are distributed evenly throughout the pancreas and have no evident risk of malignancy. In a pseudocyst, no epithelial lining is present. As such, the pathological criteria for a true cyst (despite its cystic imaging appearance) are not satisfied. Most other true cysts (with the exception of congenital pancreatic cysts) are neoplastic and, therefore, these represent a potentially significant clinical finding<sup>[3,4]</sup>. A number of different neoplastic cystic lesions in the pancreas have been identified and labeled as capitalized abbreviations (Table 1)<sup>[4]</sup>. As prognosis for each type of cystic neoplasm may differ, precise definition of any imaged cystic lesion, even if asymptomatic or incidentally detected, is crucial<sup>[5]</sup>.

### NEOPLASTIC PANCREATIC CYSTIC LESIONS

Most neoplastic cystic lesions of the pancreas occur in young or middle-aged females [serous cystadenoma (SCA), mucinous cystic neoplasia (MCN), and solid pseudo-papillary neoplasia (SPN)], however, intraductal papillary mucinous neoplasms (IPMN) are most often detected in elderly males (more so than females)<sup>[4]</sup>. Most cystic neoplasms are evenly distributed throughout the pancreas, however, the pancreatic head and uncinate process are



Table 1 Types of pancreatic cystic neoplasms

Types of pancreatic cystic neoplasms
Serous cystadenoma, or SCA
Mucinous cystic neoplasm, or MCN
Intraductal papillary mucinous neoplasm, or IPMN
Solid pseudopapillary neoplasm (with degeneration), or SPN
Cystic necrosis (endocrine tumor, ductal adenocarcinoma)

most common sites for IPMN while the body and tail are most common sites for MCN<sup>[4]</sup>. For most cystic types, malignant potential appears to be low, except for MCN and rapidly growing pancreatic ductal adenocarcinomas or even more rare endocrine neoplasms<sup>[4]</sup>. In contrast, most IPMN are slow growing and have low malignant potential, with a much better prognosis, especially if compared to pancreatic ductal adenocarcinoma<sup>[4]</sup>. Thus, their recognition is significant because an opportunity may be present at the time of recognition to resect surgically this type of pancreatic cystic neoplasm.

## INTRADUCTAL PAPILLARY MUCINOUS NEOPLASMS

IPMN appears to be quite a unique type of neoplasm, representing a broad spectrum of mucin-producing lesions of the exocrine pancreas classified as benign adenomas to invasive carcinoma. IPMN appear to arise from the epithelium of the main duct or its branches, often with variable duct dilation and IPMN are also believed by some to follow the so-called “adenoma-carcinoma” sequence. Neoplastic cells are most often papillary, although flat epithelium may also be defined.

IPMN of pancreas represents up to one-third of all pancreatic cystic neoplasms, but only about 1% of all pancreatic cancers<sup>[3,6]</sup>. Of particular note, IPMN also represents about 25% of all surgically resected pancreatic neoplasms, again likely emphasizing the critical significance of early recognition<sup>[6]</sup>. IPMN was first described by Ohhashi and colleagues in 1982 followed by a notation by the same group of a very prolonged survival (over a quarter century) of a case of IPMN<sup>[7]</sup>. The first series of North American patients were described only in 1990<sup>[8]</sup>. Since then, interest in this neoplasm appears to have increased exponentially as reflected in the annual number of publications in Medline from 1994 to 2006 related to IPMN noted elsewhere<sup>[6]</sup>. Interestingly, IPMN has also been documented to be multifocal and there is a higher occurrence rate of synchronous extrapancreatic malignancies (compared to pancreatic ductal adenocarcinoma)<sup>[9]</sup>. Finally, independent synchronous or metachronous pancreatic ductal adenocarcinomas<sup>[10]</sup> as well as endocrine tumors<sup>[11]</sup> with IPMN have also been described.

Based on anatomic involvement of the pancreatic duct, IPMN of the pancreas may predominantly involve the main pancreatic duct (“main duct type”), secondary ducts (“branch duct type”) or both (“mixed type”). Branch duct type IPMN are usually smaller, less papillary and

tend to occur in the periphery of the pancreatic head or in the distal pancreas. Malignant transformations occur less often in the branch duct type. Evidence suggests that asymptomatic branch duct IPMN less than 3 cm in size without mural nodules, thickened septa or high grade dysplasia have a relatively benign biological behavior and should be considered optimal candidates for surgical treatment<sup>[6]</sup>.

Diagnosis may be enhanced by MRCP combined with dynamic imaging since this improves localization, staging and, most important, the potential for surgical resectability. A contrast-enhanced CT scan may also yield added information regarding invasion, but also the relationship of the lesion to contiguous organs and vessels. Finally, endoscopic visualization of a patulous or so-called “fish-eye” papilla of Vater, especially at the time of ERCP may be helpful, if not pathognomonic. ERCP also permits an opportunity for added sampling of ductal content for cytology. Endoscopic ultrasonograph (EUS) may also provide added imaging information and permit fine needle aspiration biopsy although some concerns have been raised regarding the risk of seeding and dissemination of malignant cells<sup>[4]</sup>. Positron emission tomographic scanning may be helpful but its role needs to be better defined. Finally, serological studies including CA 19-9 and CEA may serve some value but it is limited since these are reported to be elevated in less than 20% of IPMN<sup>[4]</sup>. For pre-operative evaluation, all of these investigations may provide useful information, but are most important if surgical resection is being contemplated. Unfortunately, some patients are older with other concomitant health issues. In these, surgical treatment and resection may not lead to a significant positive long-term result and, as in these patients, even a more judicious approach to invasive evaluative investigations may be reasonable. If surgical excision is complete, recurrence may still occur, usually at a distant site, but this rate of recurrence is limited, and the overall 5-year survival rate has been reported to exceed 80% for noninvasive IPMN and approximately 50% for malignant invasive IPMN. Thus, accurate evaluation of IPMN is exceedingly important as a recent analysis<sup>[4]</sup> has suggested that this is one of the few surgically curable pancreatic neoplasms.

## REFERENCES

- 1 Zhang XM, Mitchell DG, Dohke M, Holland GA, Parker L. Pancreatic cysts: depiction on single-shot fast spin-echo MR images. *Radiology* 2002; **223**: 547-553
- 2 Kimura W, Nagai H, Kuroda A, Muto T, Esaki Y. Analysis of small cystic lesions of the pancreas. *Int J Pancreatol* 1995; **18**: 197-206
- 3 Brugge WR, Lauwers GY, Sahani D, Fernandez-del Castillo C, Warshaw AL. Cystic neoplasms of the pancreas. *N Engl J Med* 2004; **351**: 1218-1226
- 4 Oh HC, Kim MH, Hwang CY, Lee TY, Lee SS, Seo DW, Lee SK. Cystic lesions of the pancreas: challenging issues in clinical practice. *Am J Gastroenterol* 2008; **103**: 229-239; quiz 228, 240
- 5 Fernandez-del Castillo C, Targarona J, Thayer SP, Rattner DW, Brugge WR, Warshaw AL. Incidental pancreatic cysts: clinicopathologic characteristics and comparison with symptomatic patients. *Arch Surg* 2003; **138**: 427-423; discussion 433-434
- 6 Belyaev O, Seelig MH, Muller CA, Tannapfel A, Schmidt

- WE, Uhl W. Intraductal papillary mucinous neoplasms of the pancreas. *J Clin Gastroenterol* 2008; **42**: 284-294
- 7 **Shimizu Y**, Yasui K, Morimoto T, Torii A, Yamao K, Ohhashi K. Case of intraductal papillary mucinous tumor (noninvasive adenocarcinoma) of the pancreas resected 27 years after onset. *Int J Pancreatol* 1999; **26**: 93-98
- 8 **Warshaw AL**, Compton CC, Lewandrowski K, Cardenosa G, Mueller PR. Cystic tumors of the pancreas. New clinical, radiologic, and pathologic observations in 67 patients. *Ann Surg* 1990; **212**: 432-443; discussion 444-445
- 9 **Sugiyama M**, Atomi Y. Extrapancreatic neoplasms occur with unusual frequency in patients with intraductal papillary mucinous tumors of the pancreas. *Am J Gastroenterol* 1999; **94**: 470-473
- 10 **Proshin S**, Yamaguchi K, Wada T, Miyagi T. Modulation of neuritogenesis by ganglioside-specific sialidase (Neu 3) in human neuroblastoma NB-1 cells. *Neurochem Res* 2002; **27**: 841-846
- 11 **Marrache F**, Cazals-Hatem D, Kianmanesh R, Palazzo L, Couvelard A, O'Toole D, Maire F, Hammel P, Levy P, Sauvanet A, Ruszniewski P. Endocrine tumor and intraductal papillary mucinous neoplasm of the pancreas: a fortuitous association? *Pancreas* 2005; **31**: 79-83

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REVIEW

## Proton pump inhibitors in cirrhosis: Tradition or evidence based practice?

Francesca Lodato, Francesco Azzaroli, Maria Di Girolamo, Valentina Feletti, Paolo Cecinato, Andrea Lisotti, Davide Festi, Enrico Roda, Giuseppe Mazzella

Francesca Lodato, Francesco Azzaroli, Maria Di Girolamo, Valentina Feletti, Paolo Cecinato, Andrea Lisotti, Davide Festi, Enrico Roda, Giuseppe Mazzella, Department of Internal Medicine and Gastroenterology, Gastroenterology Unit, University of Bologna, Bologna 40138, Italy

**Author contributions:** Lodato F, Azzaroli F and Mazzella G wrote the paper; Di Girolamo M, Feletti V, Cecinato P, Lisotti A did the bibliographic research; Festi D and Roda E contributed in writing and reviewing the paper.

**Correspondence to:** Francesca Lodato, Dr, Dipartimento di Medicina Interna e Gastroenterologia, U.O. di Gastroenterologia, Via Massarenti 9, Bologna 40138,

Italy. [francesca.lodato@unibo.it](mailto:francesca.lodato@unibo.it)

Telephone: +39-51-6364120 Fax: +39-51-6364120

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### Abstract

Proton Pump Inhibitors (PPI) are very effective in inhibiting acid secretion and are extensively used in many acid related diseases. They are also often used in patients with cirrhosis sometimes in the absence of a specific acid related disease, with the aim of preventing peptic complications in patients with variceal or hypertensive gastropathic bleeding receiving multidrug treatment. Contradicting reports support their use in cirrhosis and evidence of their efficacy in this condition is poor. Moreover there are convincing papers suggesting that acid secretion is reduced in patients with liver cirrhosis. With regard to *Helicobacter pylori* (*H. pylori*) infection, its prevalence in patients with cirrhosis is largely variable among different studies, and it seems that *H. pylori* eradication does not prevent gastro-duodenal ulcer formation and bleeding. With regard to the prevention and treatment of oesophageal complications after banding or sclerotherapy of oesophageal varices, there is little evidence for a protective role of PPI. Moreover, due to liver metabolism of PPI, the dose of most available PPIs should be reduced in cirrhotics. In conclusion, the use of this class of drugs seems more habit related than evidence-based eventually leading to an increase in health costs.

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**Key words:** Proton pump inhibitors; Cirrhosis; *Helicobacter pylori*; Peptic ulcer; CYP P450

**Peer reviewer:** Katja Breitskopf, Dr, Department of Medicine II, University Hospital Mannheim, University of Heidelberg,

Theodor-Kutzer-Ufer 1-3, Mannheim 68167, Germany

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### INTRODUCTION

Proton Pump Inhibitors (PPI) are extensively used in different acid related diseases. Their efficacy in inhibiting acid secretion is well known<sup>[1-4]</sup>, and the use of this class of drugs has increased worldwide. They act through inhibition of the H<sup>+</sup>/K<sup>+</sup> ATPase of parietal cells producing the so called “inhibitory complex” and blocking HCl secretion<sup>[5]</sup>. They are metabolized in the liver by the CYP450 cytochrome<sup>[6]</sup>.

PPI are also often used in patients with liver cirrhosis sometimes in the absence of a specific acid related disease, with the aim of preventing peptic complications in patients with variceal or hypertensive gastropathic bleeding receiving multidrug treatment.

The aim of this editorial is to revise the efficacy and safety profile of PPI in patients with liver cirrhosis.

### GASTRIC ACID SECRETION AND LIVER CIRRHOSIS

The role of gastric secretion in cirrhosis is controversial. Some studies report reduced acid production<sup>[7-10]</sup> while others reported normal production<sup>[11-15]</sup>. The evaluation of 24-h acidity by gastric pH-metry in 49 patients with cirrhosis showed a marked hypoacidity in patients with cirrhosis compared to controls, mainly during the night hours<sup>[16]</sup>. This may depend on hemodynamic alterations consequent to portal hypertension and is supported by experimental studies showing reduced gastric acid secretion in animals with portal hypertension<sup>[17,18]</sup>. These observations rule out the relevance of gastric acid in the pathogenesis of ulcers in cirrhotics.

Gastrin, the gastric hormone whose secretion is regulated by intragastric pH, and that regulates the production of HCl and pepsin, is partially metabolized

**Table 1** Prevalence of peptic ulcer in patients with liver cirrhosis

Investigator	Number of patients	Gastric ulcer prevalence (%)
Siringo, 1995	324	4.6
Chen, 1996	245	20.8
Tsai, 1998	130	16.1
Kirk, 1980	163	14.7
Rabinovitz, 1990	216	7.8

by the liver and mainly by the kidneys. Gastrin is elevated in serum of patients with *Helicobacter pylori* (*H. pylori*) infection or atrophic gastritis. Few studies have evaluated gastrin levels in cirrhosis, and their contribution towards understanding the pathophysiology of gastric acid secretion is very limited. Avgerinos *et al*<sup>[19]</sup> evaluated the urinary gastrin output in patients with cirrhosis with and without hepato-renal syndrome. Serum gastrin levels were higher in cirrhotics compared to controls; and in cirrhotics with hepato-renal syndrome the difference was greater suggesting that impaired urinary gastrin secretion may contribute to their hypergastrinemia. The same results were found by Lo *et al*<sup>[11]</sup> who also showed a significantly lower maximal pepsin output in cirrhotics compared to controls.

Progastrin and gastrin serum levels have been reported to be significantly higher in patients with cirrhosis of any Child-Pugh class compared to controls while there are no differences between controls and patients with chronic hepatitis B or C<sup>[20]</sup>. Indeed, it is important to note that in this study, the prevalence of *H. pylori* infection in cirrhotic patients was 83% *versus* 50% in controls. Therefore, it is not clear whether the difference in progastrin and gastrin level was due to reduced liver metabolism, to *H. pylori* infection, or both. In summary, gastrin increase in patients with liver cirrhosis could be related to: (1) impaired hepatic gastrin catabolism; (2) impaired renal function, at least in those with HRS; (3) gastric mucosal alteration due to gastropathy-related cirrhosis.

## PEPTIC ULCERS AND LIVER CIRRHOSIS

Many authors reported an increased prevalence of peptic ulcers in patients with cirrhosis<sup>[21,22]</sup> and it was shown that cirrhotics have an increased risk of developing gastric or duodenal ulcers during an interval of one year compared to non cirrhotics<sup>[23]</sup>. The prevalence of peptic ulcers ranges between 4.6% and 21% in patients with cirrhosis<sup>[21,22,24-26,39]</sup> (Table 1). However, the pathogenesis of this finding is far from being elucidated and different factors have been proposed in relation to increased ulcer prevalence in patients with cirrhosis. Furthermore the prevalence of duodenal and gastric ulcers in patients with liver cirrhosis increases with disease progression<sup>[27]</sup> (Table 2). Several theories have been postulated. It has been demonstrated that the gastric mucosa in rats with portal hypertension is more susceptible to aggressive agents such as bile acids, aspirin and alcohol<sup>[28]</sup>. Some investigators have attributed to portal hypertension itself the increased risk of peptic ulcer<sup>[29]</sup>, nevertheless no

**Table 2** Gastric and duodenal ulcer in patients with liver cirrhosis according to the severity of portal hypertension (from Wu *et al* 1995)

	Controls (n = 60)		Compensated cirrhosis (n = 60)		Decompensated cirrhosis (n = 60)		P
	n	%	n	%	n	%	
Duodenal ulcer	2	3.3	10	16.7	8	13.3	0.046
Gastric ulcer	1	1.7	2	3.3	9	15.0	0.006
All ulcers	3	5.0	12	20.0	17	28.3	0.003

study has clarified the pathogenesis of peptic ulceration in cirrhosis.

## H PYLORI IN PATIENTS WITH LIVER CIRRHOSIS

The prevalence of *H. pylori* in patients with cirrhosis has been investigated in many epidemiological studies with values ranging from 27% to 89%<sup>[24,27,30-33]</sup>. This large variability may be due to the test used to evaluate *H. pylori* infection. In the study with the largest prevalence of *H. pylori* infection, values were obtained by titration of serum IgG, against *H. pylori*. The tests usually used for evaluating the presence of *H. pylori* should be revised since haemodynamic alterations in cirrhosis could impair the results of urea 13C BT, and hypergammaglobulinemia typical of cirrhosis, might produce a false positive test<sup>[34-38]</sup>. Italian studies generally and sometimes significantly showed a higher prevalence than in non cirrhotic patients, while studies from Taiwan failed to show a similar trend. When evaluating the prevalence of *H. pylori* infection in cirrhotics there seems to be no relationship between the aetiology of cirrhosis and the prevalence of *H. pylori* evaluated by determination of serum IgG<sup>[24]</sup>. The role of *H. pylori* in determining peptic ulceration in cirrhosis is controversial: some authors conclude that the increased risk of gastroduodenal ulcer is not related to *H. pylori* infection, whilst others conclude that peptic disease and non-ulcer dyspepsia are firmly linked to *H. pylori* infection<sup>[32,39-41]</sup>. A meta-analysis showed an increased risk of ulcers developing in patients with *H. pylori* infection and cirrhosis<sup>[42]</sup>.

If *H. pylori* infection were an etiopathological factor implicated in digestive bleeding in cirrhosis, eradication of infection would decrease the risk of ulcer recurrence. However a study aiming to investigate the role of *H. pylori* eradication in cirrhotics demonstrated a similar recurrence rate between cirrhotics with successful *H. pylori* eradication and those with active *H. pylori* infection<sup>[43]</sup>. In conclusion, the role of *H. pylori* infection in the occurrence of gastric or duodenal ulcers or in determining digestive bleeding in the setting of liver cirrhosis is still unclear.

## ESOPHAGEAL DISORDERS AND LIVER CIRRHOSIS

It has been postulated in the past, that gastro-esophageal reflux may contribute to oesophagitis and variceal



bleeding in cirrhotic patients<sup>[44]</sup>, and acid reflux could be exacerbated by the presence of ascites and water retention<sup>[45]</sup>. More recent papers do not confirm these hypotheses<sup>[46,47]</sup> and report a high incidence of gastro-esophageal reflux only in patients with alcoholic cirrhosis, though the presence of reflux did not correlate with disease severity or bleeding episodes<sup>[48]</sup>. Functional studies showed decreased lower esophageal sphincter function with low amplitude of primary peristalsis and acid clearance in patients with large varices<sup>[49-51]</sup>. These phenomenon could also be due to a mechanical effect of the presence of varices. In conclusion, it is unclear whether the presence of cirrhosis itself could predispose to the onset of gastro-esophageal reflux. It seems that the presence of varices is related to reflux episodes, although it is not clear whether these might contribute to bleeding from varices.

Another more studied point is the fact that endoscopic treatment for variceal bleeding or prevention of bleeding varices, may produce oesophageal motility dysfunction. Several studies evaluated the effect of endoscopic variceal sclerotherapy (EVS) on gastro-oesophageal reflux. Some authors suggest that endoscopic treatment produces an acute impairment of oesophageal motility which is partially restored after days or weeks<sup>[52-54]</sup>, others suggest that sclerotherapy produce a chemical esophagitis that impairs oesophageal motility and in turn may favour acid related reflux esophagitis<sup>[55]</sup>. It seems that endoscopic variceal ligation (EVL) is safer in terms of oesophageal dysmotility induction when compared to EVS<sup>[56-59]</sup>. The reason for this finding is unclear. Autoptical studies after EVS show the presence of obliteration of the submucosal vascular channels, fibrosis and oesophagitis<sup>[60]</sup> reflecting the necrosis induced by the sclerosing agent. The inflammation caused by EVS may justify motor dysfunctions and acid reflux. Avgerinos *et al*<sup>[61]</sup> showed that EVL produces a higher early increase in lower oesophageal sphincter pressure, and this might prevent gastro-oesophageal reflux.

Apart from the pathogenesis of motor dysfunction following EVS and EVL, these procedures are related to local complications such as oesophageal ulcerations, strictures and perforations<sup>[62,63]</sup>, although from this point of view, EVL seem to be safer than EVS<sup>[64,65]</sup>. Uncontrolled non randomized studies, showed that PPI may have a role in the prevention and healing of post-EVS ulcerations<sup>[66-69]</sup> although this was not confirmed by other authors<sup>[70]</sup>. With regard to post EVL ulcers, the incidence is between 2% to 5%<sup>[71,72]</sup>. Pantoprazole has been shown to reduce the size of ulcers in patients undergoing elective band ligation, but not the rate of occurrence or the symptoms<sup>[73]</sup>. Given the relatively benign nature of the intervention, the authors conclude that PPI treatment is advisable in patients undergoing elective EVL.

In summary, expert opinion based on evidence of scarce value, advise PPI use in cirrhotic patients undergoing endoscopic treatment for varices, especially when treatment is performed by EVS, to prevent gastro-esophageal reflux which may worsen the procedure related inflammation or ulceration.

## PPI SAFETY IN CIRRHOTIC PATIENTS

Acute hepatitis due to PPI use is described in the literature for most PPIs available on the market<sup>[74-79]</sup>. All PPIs are metabolized in the liver by cytochrome CYP450; two isoenzymes are involved in PPI metabolism (CYP2C19 and CYP3A4)<sup>[6]</sup>. CYP2C19 is the main metabolic pathway while CYP3A4 is activated only when the other enzyme is saturated<sup>[80]</sup>. Nevertheless, the affinity of each isoenzyme for different PPIs is different and rabeprazole is metabolized mainly by a non enzymatic pathway. There are two CYP2C19 phenotypes: extensive and poor metabolisers<sup>[81-83]</sup>. The poor phenotype is present in 2%-6% of Caucasians and 20% of the Asian population. Poor metabolisers have higher plasma levels of PPI, which could lead to higher efficacy but also to potential adverse events. The effects of these genotypes varies according to the specific PPI used and in general is greater when using omeprazole decreasing progressively to lansoprazole, esomeprazole, pantoprazole and finally rabeprazole<sup>[6,83]</sup>.

PPI are metabolized in the liver and secreted by the kidney. Renal impairment has minimal effect on PPI clearance, and therefore there is no need to reduce PPI dosage in patients with renal diseases<sup>[80,84]</sup>. This is not the case for liver impairment in which the Area under the Curve (AUC) of PPIs increases and their half-life becomes 4 h to 8 h greater<sup>[80]</sup> with increasing risk of accumulation. This effect was also seen with rabeprazole<sup>[85]</sup> although a dose reduction seems to be unnecessary with a 20 mg, once daily dose in patients with mild to moderate liver cirrhosis. When using other PPIs or rabeprazole at 40 mg/d dose, dose reduction in patients with cirrhosis is advisable.

## CONCLUSION

PPI drugs are extensively used in clinical practice in cirrhotic patients. Besides habit, the evidence that PPI are necessary in most indications is very weak. First of all, there is convincing evidence that acid secretion is reduced in patients with liver cirrhosis. This is mainly due to the presence of hypertensive gastropathy for which there is no evidence of any efficacy of PPI. With regard to *H. pylori* infection, its prevalence in patients with cirrhosis is largely variable among different studies, probably as a result of different diagnostic tests used. We believe that the condition of hypochloridemia of cirrhotics makes it more probable that its prevalence is lower than in the general population. Nevertheless, it seems that *H. pylori* eradication does not prevent from gastro-duodenal ulcer formation and bleeding.

It is probable that the main reason for PPI use in cirrhosis might be the prevention and treatment of oesophageal complications after banding or sclerotherapy of oesophageal varices. However even in this case evidence for a protective role of PPI are scarce. When using PPI in cirrhotic patients, the dose should be reduced in consideration of the increased half-life of these drugs in this group of patients. Dose adjustment does not seem necessary when using rabeprazole at a 20 mg, once daily

dose. The use of this class of drugs seems more habit-related than evidence-based, eventually leading to an increase in health costs.

## REFERENCES

- Bamberg P, Caswell CM, Frame MH, Lam SK, Wong EC. A meta-analysis comparing the efficacy of omeprazole with H<sub>2</sub>-receptor antagonists for acute treatment of duodenal ulcer in Asian patients. *J Gastroenterol Hepatol* 1992; **7**: 577-585
- Eriksson S, Langstrom G, Rikner L, Carlsson R, Naesdal J. Omeprazole and H<sub>2</sub>-receptor antagonists in the acute treatment of duodenal ulcer, gastric ulcer and reflux oesophagitis: a meta-analysis. *Eur J Gastroenterol Hepatol* 1995; **7**: 467-475
- Gisbert JP, Gonzalez L, Calvet X, Roque M, Gabriel R, Pajares JM. Proton pump inhibitors versus H<sub>2</sub>-antagonists: a meta-analysis of their efficacy in treating bleeding peptic ulcer. *Aliment Pharmacol Ther* 2001; **15**: 917-926
- Gisbert JP, Khorrami S, Calvet X, Gabriel R, Carballo F, Pajares JM. Meta-analysis: proton pump inhibitors vs. H<sub>2</sub>-receptor antagonists--their efficacy with antibiotics in *Helicobacter pylori* eradication. *Aliment Pharmacol Ther* 2003; **18**: 757-766
- Sachs G, Shin JM, Briving C, Wallmark B, Hersey S. The pharmacology of the gastric acid pump: the H<sup>+</sup>, K<sup>+</sup> ATPase. *Annu Rev Pharmacol Toxicol* 1995; **35**: 277-305
- Andersson T, Cederberg C, Edvardsson G, Heggelund A, Lundborg P. Effect of omeprazole treatment on diazepam plasma levels in slow versus normal rapid metabolizers of omeprazole. *Clin Pharmacol Ther* 1990; **47**: 79-85
- Fraser AG, Pounder RE, Burroughs AK. Gastric secretion and peptic ulceration in cirrhosis. *J Hepatol* 1993; **19**: 171-182
- Scobie BA, Summerskill WH. Reduced Gastric Acid Output in Cirrhosis: Quantitation and Relationships. *Gut* 1964; **5**: 422-428
- Lam SK. Hypergastrinaemia in cirrhosis of liver. *Gut* 1976; **17**: 700-708
- Gaur SK, Vij JC, Sarin SK, Anand BS. Gastric secretion in cirrhosis and non-cirrhotic portal fibrosis. *Digestion* 1988; **39**: 151-155
- Lo WC, Lin HJ, Wang K, Lee FY, Perng CL, Lin HC, Lee SD. Gastric secretion in Chinese patients with cirrhosis. *J Clin Gastroenterol* 1996; **23**: 256-260
- Mazzacca G, Budillon G, De Marco F, De Ritis F. Serum gastrin in patients with cirrhosis of the liver. *Digestion* 1974; **11**: 232-239
- Tabaqchali S, Dawson AM. Peptic Ulcer and Gastric Secretion in Patients with Liver Disease. *Gut* 1964; **5**: 417-421
- Orloff MJ, Chandler JG, Alderman SJ, Keiter JE, Rosen H. Gastric secretion and peptic ulcer following portacaval shunt in man. *Ann Surg* 1969; **170**: 515-527
- Lenz HJ, Struck T, Greten H, Koss MA, Eysselein VE, Walsh JH, Isenberg JI. Increased sensitivity of gastric acid secretion to gastrin in cirrhotic patients with portacaval shunt. *J Clin Invest* 1987; **79**: 1120-1124
- Savarino V, Mela GS, Zentilin P, Mansi C, Mele MR, Vigneri S, Cutela P, Vassallo A, Dallorto E, Celle G. Evaluation of 24-hour gastric acidity in patients with hepatic cirrhosis. *J Hepatol* 1996; **25**: 152-157
- Kaur S, Kaur U, Agnihotri N, Tandon CD, Majumdar S. Modulation of acid secretion in common bile duct ligation-related gastropathy in Wistar rats. *J Gastroenterol Hepatol* 2001; **16**: 755-762
- Agnihotri N, Kaur U, Dhawan V, Dilawari JB. Extrahepatic portal hypertensive gastropathy in Wistar rats: modulation of acid secretion in isolated parietal cells. *Dig Dis Sci* 1998; **43**: 56-66
- Avgerinos A, Dimitriou-Voudri Y, Adamopoulos A, Papadimitriou N, Voudris B, Rekoumis G, Raptis S. Urinary gastrin output and serum gastrin in patients with liver cirrhosis. *Urinary gastrin output in cirrhosis. Hepatogastroenterology* 1994; **41**: 445-448
- Konturek SJ, Gonciarz M, Gonciarz Z, Bielanski W, Mazur W, Mularczyk A, Konturek PC, Goetze JP, Rehfeld JF. Progastrin and its products from patients with chronic viral hepatitis and liver cirrhosis. *Scand J Gastroenterol* 2003; **38**: 643-647
- Kirk AP, Dooley JS, Hunt RH. Peptic ulceration in patients with chronic liver disease. *Dig Dis Sci* 1980; **25**: 756-760
- Rabinovitz M, Schade RR, Dindzans V, Van Thiel DH, Gavalier JS. Prevalence of duodenal ulcer in cirrhotic males referred for liver transplantation. Does the etiology of cirrhosis make a difference? *Dig Dis Sci* 1990; **35**: 321-326
- Siringo S, Burroughs AK, Bolondi L, Muia A, Di Febo G, Miglioli M, Cavalli G, Barbara L. Peptic ulcer and its course in cirrhosis: an endoscopic and clinical prospective study. *J Hepatol* 1995; **22**: 633-641
- Siringo S, Vaira D, Menegatti M, Piscaglia F, Sofia S, Gaetani M, Miglioli M, Corinaldesi R, Bolondi L. High prevalence of *Helicobacter pylori* in liver cirrhosis: relationship with clinical and endoscopic features and the risk of peptic ulcer. *Dig Dis Sci* 1997; **42**: 2024-2030
- Chen LS, Lin HC, Lee FY, Hou MC, Lee SD. Prevalence of duodenal ulcer in cirrhotic patients and its relation to *Helicobacter pylori* and portal hypertension. *Zhonghua Yixue Zazhi (Taipei)* 1995; **56**: 226-231
- Sacchetti C, Capello M, Rebecchi P, Roncucci L, Zanghieri G, Tripodi A, Ponz de Leon M. Frequency of upper gastrointestinal lesions in patients with liver cirrhosis. *Dig Dis Sci* 1988; **33**: 1218-1222
- Wu CS, Lin CY, Liaw YF. *Helicobacter pylori* in cirrhotic patients with peptic ulcer disease: a prospective, case controlled study. *Gastrointest Endosc* 1995; **42**: 424-427
- Sarfeh IJ, Tarnawski A, Malki A, Mason GR, Mach T, Ivey KJ. Portal hypertension and gastric mucosal injury in rats. Effects of alcohol. *Gastroenterology* 1983; **84**: 987-993
- Chen LS, Lin HC, Hwang SJ, Lee FY, Hou MC, Lee SD. Prevalence of gastric ulcer in cirrhotic patients and its relation to portal hypertension. *J Gastroenterol Hepatol* 1996; **11**: 59-64
- Nam YJ, Kim SJ, Shin WC, Lee JH, Choi WC, Kim KY, Han TH. [Gastric pH and *Helicobacter pylori* infection in patients with liver cirrhosis] *Korean J Hepatol* 2004; **10**: 216-222
- Pellicano R, Leone N, Berrutti M, Cutufia MA, Fiorentino M, Rizzetto M, Ponzetto A. *Helicobacter pylori* seroprevalence in hepatitis C virus positive patients with cirrhosis. *J Hepatol* 2000; **33**: 648-650
- Zullo A, Rinaldi V, Meddi P, Folino S, Lauria V, Diana F, Winn S, Attili AF. *Helicobacter pylori* infection in dyspeptic cirrhotic patients. *Hepatogastroenterology* 1999; **46**: 395-400
- Chen JJ, Changchien CS, Tai DI, Chiou SS, Lee CM, Kuo CH. Role of *Helicobacter pylori* in cirrhotic patients with peptic ulcer. A serological study. *Dig Dis Sci* 1994; **39**: 1565-1568
- Nishiguchi S, Kuroki T, Ueda T, Fukuda K, Takeda T, Nakajima S, Shiomi S, Kobayashi K, Otani S, Hayashi N. Detection of hepatitis C virus antibody in the absence of viral RNA in patients with autoimmune hepatitis. *Ann Intern Med* 1992; **116**: 21-25
- Theilmann L, Blazek M, Goeser T, Gmelin K, Kommerell B, Fiehn W. False-positive anti-HCV tests in rheumatoid arthritis. *Lancet* 1990; **335**: 1346
- Rivera J, Garcia-Monforte A, Pineda A, Millan Nunez-Cortes J. Arthritis in patients with chronic hepatitis C virus infection. *J Rheumatol* 1999; **26**: 420-424
- Maillefert JF, Muller G, Falgarone G, Bour JB, Ratovohery D, Dougados M, Tavernier C, Breban M. Prevalence of hepatitis C virus infection in patients with rheumatoid arthritis. *Ann Rheum Dis* 2002; **61**: 635-637
- Borque L, Elena A, Maside C, Rus A, Del Cura J. Rheumatoid arthritis and hepatitis C virus antibodies. *Clin Exp Rheumatol* 1991; **9**: 617-619
- Tsai CJ. *Helicobacter pylori* infection and peptic ulcer disease in cirrhosis. *Dig Dis Sci* 1998; **43**: 1219-1225
- Calvet X, Navarro M, Gil M, Lafont A, Sanfeliu I, Brullet E,

- Campo R, Dalmau B, Rivero E, Mas P. Epidemiology of peptic ulcer disease in cirrhotic patients: role of *Helicobacter pylori* infection. *Am J Gastroenterol* 1998; **93**: 2501-2507
- 41 Dore MP, Mura D, Deledda S, Maragkoudakis E, Pironti A, Realdi G. Active peptic ulcer disease in patients with hepatitis C virus-related cirrhosis: the role of *Helicobacter pylori* infection and portal hypertensive gastropathy. *Can J Gastroenterol* 2004; **18**: 521-524
  - 42 Vergara M, Calvet X, Roque M. *Helicobacter pylori* is a risk factor for peptic ulcer disease in cirrhotic patients. A meta-analysis. *Eur J Gastroenterol Hepatol* 2002; **14**: 717-722
  - 43 Lo GH, Yu HC, Chan YC, Chen WC, Hsu PI, Lin CK, Lai KH. The effects of eradication of *Helicobacter pylori* on the recurrence of duodenal ulcers in patients with cirrhosis. *Gastrointest Endosc* 2005; **62**: 350-356
  - 44 Ahmed AM, al Karawi MA, Shariq S, Mohamed AE. Frequency of gastroesophageal reflux in patients with liver cirrhosis. *Hepatogastroenterology* 1993; **40**: 478-480
  - 45 Simpson JA, Conn HO. Role of ascites in gastroesophageal reflux with comments on the pathogenesis of bleeding esophageal varices. *Gastroenterology* 1968; **55**: 17-25
  - 46 Van Thiel DH, Strempel JF. Lower esophageal sphincter pressure in cirrhotic men with ascites: before and after diuresis. *Gastroenterology* 1977; **72**: 842-844
  - 47 Eckardt VF, Grace ND, Kantrowitz PA. Does lower esophageal sphincter incompetency contribute to esophageal bleeding? *Gastroenterology* 1976; **71**: 185-189
  - 48 Arsene D, Bruley des Varannes S, Galmiche JP, Denis P, Chayvialle JA, Hellot MF, Ducrotte P, Colin R. Gastroesophageal reflux and alcoholic cirrhosis. A reappraisal. *J Hepatol* 1987; **4**: 250-258
  - 49 Iwakiri K, Kobayashi M, Sesoko M, Nomura T. Gastroesophageal reflux and esophageal motility in patients with esophageal varices. *Gastroenterol Jpn* 1993; **28**: 477-482
  - 50 Flores PP, Lemme EM, Coelho HS. [Esophageal motor disorders in cirrhotic patients with esophageal varices non-submitted to endoscopic treatment] *Arq Gastroenterol* 2005; **42**: 213-220
  - 51 Passaretti S, Mazzotti G, de Franchis R, Cipolla M, Testoni PA, Tittobello A. Esophageal motility in cirrhotics with and without esophageal varices. *Scand J Gastroenterol* 1989; **24**: 334-338
  - 52 Grande L, Planas R, Lacima G, Boix J, Ros E, Esteve M, Morillas R, Gasull MA. Sequential esophageal motility studies after endoscopic injection sclerotherapy: a prospective investigation. *Am J Gastroenterol* 1991; **86**: 36-40
  - 53 Snady H, Korsten MA. Esophageal acid-clearance and motility after endoscopic sclerotherapy of esophageal varices. *Am J Gastroenterol* 1986; **81**: 419-422
  - 54 Sauerbruch T, Wirsching R, Leisner B, Weinzierl M, Pfahler M, Paumgartner G. Esophageal function after sclerotherapy of bleeding varices. *Scand J Gastroenterol* 1982; **17**: 745-751
  - 55 Reilly JJ Jr, Schade RR, Van Thiel DS. Esophageal function after injection sclerotherapy: pathogenesis of esophageal stricture. *Am J Surg* 1984; **147**: 85-88
  - 56 Viazis N, Armonis A, Vlachogiannakos J, Rekoumis G, Stefanidis G, Papadimitriou N, Manolakopoulos S, Avgerinos A. Effects of endoscopic variceal treatment on esophageal function: a prospective, randomized study. *Eur J Gastroenterol Hepatol* 2002; **14**: 263-269
  - 57 Goff JS, Reveille RM, Van Stiegmann G. Endoscopic sclerotherapy versus endoscopic variceal ligation: esophageal symptoms, complications, and motility. *Am J Gastroenterol* 1988; **83**: 1240-1244
  - 58 Berner JS, Gaing AA, Sharma R, Almenoff PL, Muhlfelder T, Korsten MA. Sequelae after esophageal variceal ligation and sclerotherapy: a prospective randomized study. *Am J Gastroenterol* 1994; **89**: 852-858
  - 59 Hou MC, Yen TC, Lin HC, Kuo BI, Chen CH, Lee FY, Liu RS, Chang FY, Lee SD. Sequential changes of esophageal motility after endoscopic injection sclerotherapy or variceal ligation for esophageal variceal bleeding: a scintigraphic study. *Am J Gastroenterol* 1997; **92**: 1875-1878
  - 60 Papadimos D, Kerlin P, Harris OD. Endoscopic sclerotherapy: lessons from a necropsy study. *Gastrointest Endosc* 1986; **32**: 269-273
  - 61 Avgerinos A, Viazis N, Armonis A, Vlachogiannakos J, Rekoumis G, Stefanidis G, Papadimitriou N, Manolakopoulos S, Raptis SA. Early increase of lower esophageal sphincter pressure after band ligation of esophageal varices in cirrhotics: an intriguing phenomenon. *Eur J Gastroenterol Hepatol* 2002; **14**: 1319-1323
  - 62 Stiegmann GV. Evolution of endoscopic therapy for esophageal varices. *Surg Endosc* 2006; **20** Suppl 2: S467-S470
  - 63 Krige JE, Bornman PC, Shaw JM, Apostolou C. Complications of endoscopic variceal therapy. *S Afr J Surg* 2005; **43**: 177-188, 190-194
  - 64 Schmitz RJ, Sharma P, Badr AS, Qamar MT, Weston AP. Incidence and management of esophageal stricture formation, ulcer bleeding, perforation, and massive hematoma formation from sclerotherapy versus band ligation. *Am J Gastroenterol* 2001; **96**: 437-441
  - 65 Stiegmann GV, Goff JS, Michaletz-Onody PA, Korula J, Lieberman D, Saeed ZA, Reveille RM, Sun JH, Lowenstein SR. Endoscopic sclerotherapy as compared with endoscopic ligation for bleeding esophageal varices. *N Engl J Med* 1992; **326**: 1527-1532
  - 66 Jaspersen D, Korner T, Schorr W, Hammar CH. Omeprazole in the management of sclerotherapy-induced esophageal ulcers resistant to H2 blocker treatment. *J Gastroenterol* 1995; **30**: 128-130
  - 67 Gimson A, Polson R, Westaby D, Williams R. Omeprazole in the management of intractable esophageal ulceration following injection sclerotherapy. *Gastroenterology* 1990; **99**: 1829-1831
  - 68 Shephard H, Barkin JS. Omeprazole heals mucosal ulcers associated with endoscopic injection sclerotherapy. *Gastrointest Endosc* 1993; **39**: 474-475
  - 69 Johlin FC, Labrecque DR, Neil GA. Omeprazole heals mucosal ulcers associated with endoscopic injection sclerotherapy. *Dig Dis Sci* 1992; **37**: 1373-1376
  - 70 Garg PK, Sidhu SS, Bhargava DK. Role of omeprazole in prevention and treatment of postendoscopic variceal sclerotherapy esophageal complications. Double-blind randomized study. *Dig Dis Sci* 1995; **40**: 1569-1574
  - 71 Laine L, el-Newihi HM, Migikovsky B, Sloane R, Garcia F. Endoscopic ligation compared with sclerotherapy for the treatment of bleeding esophageal varices. *Ann Intern Med* 1993; **119**: 1-7
  - 72 Gimson AE, Ramage JK, Panos MZ, Hayllar K, Harrison PM, Williams R, Westaby D. Randomised trial of variceal banding ligation versus injection sclerotherapy for bleeding esophageal varices. *Lancet* 1993; **342**: 391-394
  - 73 Shaheen NJ, Stuart E, Schmitz SM, Mitchell KL, Fried MW, Zacks S, Russo MW, Galanko J, Shrestha R. Pantoprazole reduces the size of postbanding ulcers after variceal band ligation: a randomized, controlled trial. *Hepatology* 2005; **41**: 588-594
  - 74 Koury SI, Stone CK, La Charite DD. Omeprazole and the development of acute hepatitis. *Eur J Emerg Med* 1998; **5**: 467-469
  - 75 Romero-Gomez M, Otero MA, Suarez-Garcia E, Garcia Diaz E, Fobelo MJ, Castro-Fernandez M. Acute hepatitis related to omeprazole. *Am J Gastroenterol* 1999; **94**: 1119-1120
  - 76 Viana de Miguel C, Alvarez Garcia M, Sanchez Sanchez A, Carvajal Garcia-Pando A. [Lansoprazole-induced hepatitis] *Med Clin (Barc)* 1997; **108**: 599
  - 77 Cordes A, Vogt W, Maier KP. [Pantoprazole-induced hepatitis] *Dtsch Med Wochenschr* 2003; **128**: 611-614
  - 78 Garcia-Cortes M, Lucena MI, Andrade RJ, Romero-Gomez M, Fernandez MC. Lansoprazole-induced hepatic dysfunction. *Ann Pharmacother* 2003; **37**: 1731
  - 79 Darabi K. Proton-pump-inhibitor-induced hepatitis. *South Med J* 2005; **98**: 844-845

- 80 **Thjodleifsson B**. Treatment of acid-related diseases in the elderly with emphasis on the use of proton pump inhibitors. *Drugs Aging* 2002; **19**: 911-927
- 81 **Horai Y**, Ishizaki T. Pharmacogenetics and its clinical implications. Part II. Oxidation polymorphism. *Ration Drug Ther* 1988; **22**: 1-8
- 82 **Kupfer A**, Preisig R. Pharmacogenetics of mephenytoin: a new drug hydroxylation polymorphism in man. *Eur J Clin Pharmacol* 1984; **26**: 753-759
- 83 **Ishizaki T**, Horai Y. Review article: cytochrome P450 and the metabolism of proton pump inhibitors--emphasis on rabeprazole. *Aliment Pharmacol Ther* 1999; **13** Suppl 3: 27-36
- 84 **Keane WF**, Swan SK, Grimes I, Humphries TJ. Rabeprazole: pharmacokinetics and tolerability in patients with stable, end-stage renal failure. *J Clin Pharmacol* 1999; **39**: 927-933
- 85 **Hoyumpa AM**, Trevino-Alanis H, Grimes I, Humphries TJ. Rabeprazole: pharmacokinetics in patients with stable, compensated cirrhosis. *Clin Ther* 1999; **21**: 691-701

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## TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, Professor; Sharon DeMorrow, Assistant Professor, Series Editor

# Mechanisms of biliary carcinogenesis and growth

Candace Wise, Metaneeya Pilanthananond, Benjamin F Perry, Gianfranco Alpini, Michael McNeal, Shannon S Glaser

Candace Wise, Gianfranco Alpini, Shannon S Glaser, Systems Biology and Translational Medicine, Scott and White Hospital and The Texas A&M University System Health Science Center, Temple, TX 76504, United States

Metaneeya Pilanthananond, Gianfranco Alpini, Shannon S Glaser, Department of Medicine, Scott and White Hospital and The Texas A&M University System Health Science Center, Temple, TX 76504, United States

Benjamin F Perry, Michael McNeal, The Texas A&M University System Health Science Center, College of Medicine, Temple, TX 76504, United States

Gianfranco Alpini, Central Texas Veterans Health Care System, Temple, TX 76504, United States

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Correspondence to: Shannon S Glaser, Assistant Professor, Department of Medicine, Scott and White Hospital, Medical Research Building Room 316B, 702 SW HK Dodgen Loop, Temple, Texas 76504, United States. [sglaser@medicine.tamhsc.edu](mailto:sglaser@medicine.tamhsc.edu)  
Telephone: +1-254-7427058 Fax: +1-254-7245944

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## Abstract

Cholangiocarcinoma is a rare cancer originating from the neoplastic transformation of the epithelial cells (i.e. cholangiocytes) that line the biliary tract. The prognosis for patients with cholangiocarcinoma is grim due to lack of viable treatment options. The increase in world-wide incidence and mortality from cholangiocarcinoma highlights the importance of understanding the intracellular mechanisms that trigger the neoplastic transformation of cholangiocytes and the growth of biliary cancers. The purpose of the following review is to address what has been learned over the past decade concerning the molecular basis of cholangiocarcinogenesis. The material presented is divided into two sections: (1) mechanisms regulating neoplastic transformation of cholangiocytes; and (2) factors regulating cholangiocarcinoma growth. An understanding of the growth regulatory mechanisms of cholangiocarcinoma will lead to the identification of therapeutic targets for this devastating cancer.

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**Key words:** Biliary carcinogenesis; Cholangiocarcinomas; Primary biliary cirrhosis; Primary sclerosing cholangitis

**Peer reviewer:** Giammarco Fava, MD, Department of Gastroenterology, Università Politecnica delle Marche, Ancona, via

Gervasoni 12, 60129 Ancona, Italy

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## INTRODUCTION

Cholangiocytes are simple epithelial cells that line the intrahepatic biliary tract, which is a three-dimensional network of interconnecting ducts. The primary physiological function of cholangiocytes is the modification of bile of canalicular origin and drainage of bile from the liver<sup>[1]</sup>. In addition to their role in the modification of ductal bile, cholangiocytes also participate in the detoxification of xenobiotics<sup>[1]</sup>. In recent years, interest in the study of cholangiocytes has increased dramatically due to a rise in the incidence of cholestatic liver diseases and biliary tract cancers (i.e. cholangiocarcinoma) in patients worldwide<sup>[2-5]</sup>.

In diseases of the biliary tree (e.g. primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), liver allograft rejection and graft-versus-host disease), cholangiocytes are the primary target cells<sup>[1]</sup>. These cholangiopathies cause morbidity and mortality and are a major indication for liver transplantation<sup>[1]</sup>. In fact, these diseases contribute to 20% of the liver transplants in adults and 50% of those in pediatric patients<sup>[6]</sup>. Proliferation of cholangiocytes is critical for the maintenance of biliary mass and secretory function during the pathogenesis of chronic cholestatic liver diseases, such as primary biliary cirrhosis (PBC), and primary sclerosing cholangitis (PSC). Previous studies have demonstrated that proliferating cholangiocytes serve as a neuroendocrine compartment during liver disease pathogenesis and as such secrete and respond to a number of hormones and neuropeptides contributing to the autocrine and paracrine pathways that modulate liver inflammation and fibrosis, which are predicted to play key roles in cholangiocarcinogenesis<sup>[7]</sup>.

Cholangiocarcinomas are adenocarcinomas that arise from the neoplastic transformation of cholangiocytes. Cholangiocarcinoma occurs in approximately 2 per 100 000 people and account for approximately 13% of primary liver cancers<sup>[4]</sup>. Cholangiocarcinomas can be divided into three categories based upon anatomic location: (1) intrahepatic

cholangiocarcinoma, occurring in the bile ducts residing within the liver; (2) hilar cholangiocarcinoma, occurring at the confluence of the right and left hepatic ducts; and (3) distal extrahepatic bile duct cancers<sup>[8]</sup>. The prognosis for cholangiocarcinoma is grim due to lack of early diagnostic modalities and effective treatment paradigms. Cholangiocarcinomas are slow growing, metastasize late during the cancer's progression, and present with symptoms of cholestasis due to the blockage of the bile duct by tumor growth<sup>[8]</sup>. In most cases, the tumors are well advanced at the time of diagnosis, which results in limited treatment options<sup>[9]</sup>. Many of these tumors are too advanced to be removed surgically and chemotherapy and radiation therapy usually are not effective, which indicates the dire need to understand the mechanisms that activate the neoplastic transformation of cholangiocytes and control the growth of cholangiocarcinomas.

## RISK FACTORS FOR CHOLANGIOCARCINOMA

Several recent studies have shown that there is an increasing incidence of cholangiocarcinoma world-wide and in particular in Western countries, such as the United States, the United Kingdom, and Australia<sup>[4,10-12]</sup>. Although most patients do not have identifiable risk factors for the disease, several risk factors have been established for cholangiocarcinoma<sup>[13]</sup>. The list of risk factors includes: gallstones or gallbladder inflammation, chronic ulcerative colitis, chronic infection of liver flukes, *Clonorchis sinensis* and *Opisthorchis viverrini*, and primary sclerosing cholangitis (PSC)<sup>[14]</sup>. In addition, a recent study revealed several novel risk factors for intrahepatic cholangiocarcinoma<sup>[15]</sup>. Hepatitis C virus infection, non-alcoholic fatty liver disease, obesity and smoking are all associated with intrahepatic cholangiocarcinoma<sup>[15]</sup>. The majority of these risk factors have the common features of chronic liver inflammation, cholestasis, and increased cholangiocyte turnover<sup>[16,17]</sup>.

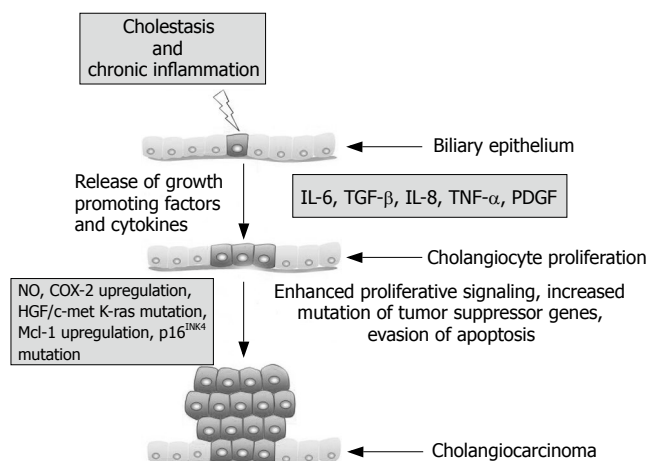
## MOLECULAR MECHANISMS CONTRIBUTING TO CHOLANGIOCARCINOGENESIS

A common and important contributor to the malignant transformation of cholangiocytes is chronic inflammation of the liver. This inflammation is often coupled with the injury of bile duct epithelium and obstruction of bile flow, which increases cholangiocyte turnover (i.e. dysregulation in the balance of cholangiocyte proliferation and apoptosis)<sup>[9,18]</sup>. Persistent inflammation is thought to promote carcinogenesis by causing DNA damage, activating tissue reparative proliferation, and by creating a local environment that is enriched with cytokines and other growth factors<sup>[19]</sup>. Thereby, chronic inflammation creates local environmental conditions that are primed for cells to develop autonomous proliferative signaling by constitutive activation of pro-proliferative intracellular signaling pathways and enhanced production of mitogenic

factors. Indeed recent studies have demonstrated that cholangiocytes release cytokines, such as interleukin 6 (IL-6), transforming growth factor- $\beta$  (TGF- $\beta$ ), IL-8, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) and platelet-derived growth factor (PDGF). These factors can interact with cholangiocytes in an autocrine/paracrine fashion to regulate cholangiocyte intracellular signaling responses, which are thought to be altered during cholangiocarcinogenesis<sup>[20]</sup>.

Cytokines activate inducible nitric oxide synthase (iNOS) in cholangiocytes. This results in the generation of nitric oxide, which along with other reactive oxygen species, may alter DNA bases, result in direct DNA damage and trigger the downregulation of DNA repair mechanisms<sup>[18]</sup>. Nitric oxide can directly or through the formation of peroxynitrite species can lead to the deamination of guanine and DNA adduct formation thereby promoting DNA mutations<sup>[18,21]</sup>. The resultant DNA damage leads to an increased mutation rate and alteration of genes critical to cell proliferation control. Consistent with this line of thought, activating mutations and the overexpression of EGFR, erb-2, K-ras, BRAF and hepatocyte growth factor/c-met (HGF) have been reported for cholangiocarcinoma. In addition, the proto-oncogene c-erbB-2 is activated in patients with cholangiocarcinoma<sup>[22,23]</sup>. Mutations affecting the promoter of the tumor suppressor p16<sup>INK4a</sup> that result in loss of transcription occur in both PSC and PSC-associated cholangiocarcinoma<sup>[24]</sup>. Alterations of p53, APC, and DPC4 tumor suppressor genes by a combination of chromosomal deletion, mutation, or methylation; and infrequently microsatellite instability have also been linked to cholangiocarcinoma<sup>[25]</sup>. In addition to increased ability of cholangiocytes to escape from senescence, activation of iNOS promotes the upregulation of COX-2 in immortalized mouse cholangiocytes suggesting that COX-2 and COX-2 derived prostanoids might play a key role in cholangiocarcinogenesis<sup>[26,27]</sup>. COX-2 also upregulated the expression of Notch, which has been implicated in other cancer types<sup>[27]</sup>.

Cytokines, such as IL-6, appear to play an important role in cholangiocyte evasion of apoptosis. During the pathogenesis of cancer, the activation of evasion of apoptosis pathways helps to prevent cells with accumulating DNA damage from undergoing cell death pathways that normally eliminate such dysfunctional cells. Cholangiocarcinoma cells have been shown to secrete IL-6 and in an autocrine fashion IL-6 activates the pro-survival p38 mitogen activated protein kinase<sup>[28]</sup>. In fact, IL-6 upregulated the expression of myeloid cell leukemia-1 (Mcl-1) expression through STAT3 and AKT signaling pathways<sup>[29,30]</sup>. Mcl-1 is an anti-apoptotic protein in the Bcl-2 family of apoptotic proteins. Recently, the cellular expression of Mcl-1 has been shown to be controlled by the small endogenous RNA molecule, mir-29, which is down regulated in malignant cells consistent with Mcl-1 overexpression<sup>[31]</sup>. Enforced expression of mir-29 reduced Mcl-1 expression the malignant human cholangiocytes, KMCH<sup>[31]</sup>. Modulation of expression small endogenous RNAs such as mir-29 might represent a therapeutic paradigm for cholangiocarcinomas.



**Figure 1** Summary of key mechanisms regulating cholangiocarcinogenesis.

## FACTORS REGULATING CHOLANGIOCARCINOMA GROWTH

A number of recent studies have shown that the nervous system, neuropeptides and neuroendocrine hormones play a key role in the modulation of cholangiocarcinoma growth<sup>[32]</sup>. We have demonstrated that proliferating cholangiocytes serve as a neuroendocrine compartment during liver disease pathogenesis and as such secrete and respond to a number of hormones and neuropeptides contributing to the autocrine and paracrine pathways that modulate liver inflammation and fibrosis<sup>[7]</sup>. The sympathetic nervous system has been shown to have a role in the negative regulation of cholangiocarcinoma growth<sup>[33]</sup>. The cholangiocarcinoma cell lines, Mz-ChA-1 and TFK-1, express the  $\alpha_{2A}$ -,  $\alpha_{2B}$ -, and  $\alpha_{2C}$ -adrenergic receptor subtypes<sup>[33]</sup>. Stimulation of the  $\alpha_2$  receptors induced an upregulation of intracellular cAMP, which inhibited EGF-induced MAPK activity through an increase in Raf-1 and the sustained activation of B-raf, which resulted in the subsequent reduction of cholangiocarcinoma proliferation<sup>[33]</sup>. Although muscarinic acetylcholine receptor are expressed by cholangiocytes and play a role in regulating secretin-induced bile flow in rodents, the role of the parasympathetic nervous system has not been evaluated in cholangiocarcinoma and warrants consideration as a factor regulating cholangiocarcinoma growth<sup>[34,35]</sup>.

Other neuroendocrine hormones and neurotransmitters have also been shown to play a role in the regulation of cholangiocarcinoma growth. The cholangiocarcinoma cell lines, Mz-ChA-1, HuH-28 and TFK-1, express the gastrin/CCK-B receptor and gastrin inhibits the proliferation and induces the activation of apoptosis in these cholangiocarcinomas through the activation of  $Ca^{2+}$ -dependent PKC- $\alpha$  signaling. Most recently, Fava *et al* have shown that  $\gamma$ -aminobutyric acid (GABA) inhibits cholangiocarcinoma cell proliferation and migration<sup>[36]</sup>. This effect was also evident *in vivo* with GABA significantly decreasing the growth of cholangiocarcinoma tumor xenografts in nude mice<sup>[36]</sup>.

Female steroid hormones, such as estrogens, have also been shown to play a role in the promoting of

cholangiocarcinoma cell growth. 17- $\beta$  estradiol stimulated the proliferation of human cholangiocarcinoma cells *in vitro*, which was blocked by tamoxifen<sup>[37]</sup>. Alvaro *et al* have demonstrated that human intrahepatic cholangiocarcinomas express the receptors for both estrogens and insulin-like growth factor (IGF-1)<sup>[38]</sup>. Their study indicates that estrogens and IGF-1 coordinately regulate cholangiocarcinoma growth and apoptosis<sup>[38]</sup>.

Modulation of the endocannabinoid system is currently being targeted to develop possible therapeutic strategies for other cancer types. We recently demonstrated the novel finding that the two major endocannabinoids, anandamide and 2-arachidonylglycerol, have opposing actions on cholangiocarcinoma growth<sup>[39]</sup>. Interestingly, anandamide was found to be antiproliferative and to promote apoptosis, while, in contrast, 2-arachidonylglycerol stimulated cholangiocarcinoma cell growth<sup>[39]</sup>. Anandamide was shown to recruited Fas and Fas ligand into lipid rafts resulting in the activation of death receptor pathways and apoptosis in the cholangiocarcinoma cells<sup>[39]</sup>.

## CONCLUSION

Cholangiocarcinoma is a devastating neoplasm of the biliary tract that is increasing in incidence. While treatment options are limited, our knowledge base of the factors controlling cholangiocarcinogenesis and cholangiocarcinoma growth has greatly expanded in the past decade. These studies have clearly demonstrated that, during the course of chronic cholestasis and associated liver inflammation, a number of factors are released into the local environment that set into motion a series of events compound genomic damage leading to autonomous proliferation and escape from apoptosis (Figure 1). Several potential areas seem promising for the development of prevention and treatment strategies for cholangiocarcinoma. In particular, inhibition of the COX-2 pathway during PSC warrants further investigation. Also, modulation of cholangiocarcinoma growth by the regulating neural input or the endocannabinoid system might prove fruitful. Understanding the molecular mechanisms triggering biliary tract cancers will be key for the development of new treatments and diagnostic tools for cholangiocarcinoma.

## REFERENCES

- 1 Alpini G, Prall RT, LaRusso NF. The pathobiology of biliary epithelia. *The Liver; Biology & Pathobiology*, 4E I M Arias, Boyer JL, Chisari FV, Fausto N, Jakoby W, Schachter D, and Shafritz D. Philadelphia: Lippincott Williams & Wilkins, 2001: 421-435
- 2 Roberts SK, Ludwig J, Larusso NF. The pathobiology of biliary epithelia. *Gastroenterology* 1997; **112**: 269-279
- 3 Ahrendt SA, Nakeeb A, Pitt HA. Cholangiocarcinoma. *Clin Liver Dis* 2001; **5**: 191-218
- 4 Patel T. Worldwide trends in mortality from biliary tract malignancies. *BMC Cancer* 2002; **2**: 10
- 5 Patel T. Cholangiocarcinoma. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 33-42
- 6 Annual Report of the US Organ Procurement. Transplantation Network and the Scientific Registry for Transplant Recipients: Transplant Data 1991-2000
- 7 Alvaro D, Mancino MG, Glaser S, Gaudio E, Marziani

- M, Francis H, Alpini G. Proliferating cholangiocytes: a neuroendocrine compartment in the diseased liver. *Gastroenterology* 2007; **132**: 415-431
- 8 **Malhi H**, Gores GJ. Cholangiocarcinoma: modern advances in understanding a deadly old disease. *J Hepatol* 2006; **45**: 856-867
  - 9 **Lazaridis KN**, Gores GJ. Cholangiocarcinoma. *Gastroenterology* 2005; **128**: 1655-1667
  - 10 **Taylor-Robinson SD**, Toledano MB, Arora S, Keegan TJ, Hargreaves S, Beck A, Khan SA, Elliott P, Thomas HC. Increase in mortality rates from intrahepatic cholangiocarcinoma in England and Wales 1968-1998. *Gut* 2001; **48**: 816-820
  - 11 **Khan SA**, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P, Thomas HC. Changing international trends in mortality rates for liver, biliary and pancreatic tumours. *J Hepatol* 2002; **37**: 806-813
  - 12 **Davila JA**, El-Serag HB. Cholangiocarcinoma: the "other" liver cancer on the rise. *Am J Gastroenterol* 2002; **97**: 3199-200
  - 13 **Gores GJ**. Cholangiocarcinoma: current concepts and insights. *Hepatology* 2003; **37**: 961-969
  - 14 **Khan SA**, Davidson BR, Goldin R, Pereira SP, Rosenberg WM, Taylor-Robinson SD, Thillainayagam AV, Thomas HC, Thursz MR, Wasan H. Guidelines for the diagnosis and treatment of cholangiocarcinoma: consensus document. *Gut* 2002; **51** Suppl 6: VII-VI9
  - 15 **Welzel TM**, Graubard BI, El-Serag HB, Shaib YH, Hsing AW, Davila JA, McGlynn KA. Risk factors for intrahepatic and extrahepatic cholangiocarcinoma in the United States: a population-based case-control study. *Clin Gastroenterol Hepatol* 2007; **5**: 1221-1228
  - 16 **Okuda K**, Nakanuma Y, Miyazaki M. Cholangiocarcinoma: recent progress. Part 2: molecular pathology and treatment. *J Gastroenterol Hepatol* 2002; **17**: 1056-1063
  - 17 **Okuda K**, Nakanuma Y, Miyazaki M. Cholangiocarcinoma: recent progress. Part 1: epidemiology and etiology. *J Gastroenterol Hepatol* 2002; **17**: 1049-1055
  - 18 **Jaiswal M**, LaRusso NF, Burgart LJ, Gores GJ. Inflammatory cytokines induce DNA damage and inhibit DNA repair in cholangiocarcinoma cells by a nitric oxide-dependent mechanism. *Cancer Res* 2000; **60**: 184-190
  - 19 **Schottenfeld D**, Beebe-Dimmer J. Chronic inflammation: a common and important factor in the pathogenesis of neoplasia. *CA Cancer J Clin* 2006; **56**: 69-83
  - 20 **Berthiaume EP**, Wands J. The molecular pathogenesis of cholangiocarcinoma. *Semin Liver Dis* 2004; **24**: 127-137
  - 21 **Jaiswal M**, LaRusso NF, Shapiro RA, Billiar TR, Gores GJ. Nitric oxide-mediated inhibition of DNA repair potentiates oxidative DNA damage in cholangiocytes. *Gastroenterology* 2001; **120**: 190-199
  - 22 **Endo K**, Yoon BI, Pairojkul C, Demetris AJ, Sirica AE. ERBB-2 overexpression and cyclooxygenase-2 up-regulation in human cholangiocarcinoma and risk conditions. *Hepatology* 2002; **36**: 439-450
  - 23 **Lai GH**, Zhang Z, Shen XN, Ward DJ, Dewitt JL, Holt SE, Rozich RA, Hixson DC, Sirica AE. erbB-2/neu transformed rat cholangiocytes recapitulate key cellular and molecular features of human bile duct cancer. *Gastroenterology* 2005; **129**: 2047-2057
  - 24 **Taniai M**, Higuchi H, Burgart LJ, Gores GJ. p16INK4a promoter mutations are frequent in primary sclerosing cholangitis (PSC) and PSC-associated cholangiocarcinoma. *Gastroenterology* 2002; **123**: 1090-1098
  - 25 **Rashid A**. Cellular and molecular biology of biliary tract cancers. *Surg Oncol Clin N Am* 2002; **11**: 995-1009
  - 26 **Ishimura N**, Bronk SF, Gores GJ. Inducible nitric oxide synthase upregulates cyclooxygenase-2 in mouse cholangiocytes promoting cell growth. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G88-G95
  - 27 **Ishimura N**, Bronk SF, Gores GJ. Inducible nitric oxide synthase up-regulates Notch-1 in mouse cholangiocytes: implications for carcinogenesis. *Gastroenterology* 2005; **128**: 1354-1368
  - 28 **Park J**, Tadlock L, Gores GJ, Patel T. Inhibition of interleukin 6-mediated mitogen-activated protein kinase activation attenuates growth of a cholangiocarcinoma cell line. *Hepatology* 1999; **30**: 1128-1133
  - 29 **Isomoto H**, Kobayashi S, Werneburg NW, Bronk SF, Guicciardi ME, Frank DA, Gores GJ. Interleukin 6 upregulates myeloid cell leukemia-1 expression through a STAT3 pathway in cholangiocarcinoma cells. *Hepatology* 2005; **42**: 1329-1338
  - 30 **Kobayashi S**, Werneburg NW, Bronk SF, Kaufmann SH, Gores GJ. Interleukin-6 contributes to Mcl-1 up-regulation and TRAIL resistance via an Akt-signaling pathway in cholangiocarcinoma cells. *Gastroenterology* 2005; **128**: 2054-2065
  - 31 **Mott JL**, Kobayashi S, Bronk SF, Gores GJ. mir-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene* 2007; **26**: 6133-6140
  - 32 **Marzioni M**, Fava G, Benedetti A. Nervous and Neuroendocrine regulation of the pathophysiology of cholestasis and of biliary carcinogenesis. *World J Gastroenterol* 2006; **12**: 3471-3480
  - 33 **Kanno N**, Lesage G, Phinizz JL, Glaser S, Francis H, Alpini G. Stimulation of alpha2-adrenergic receptor inhibits cholangiocarcinoma growth through modulation of Raf-1 and B-Raf activities. *Hepatology* 2002; **35**: 1329-1340
  - 34 **Alvaro D**, Alpini G, Jezequel AM, Bassotti C, Francia C, Fraioli F, Romeo R, Marucci L, Le Sage G, Glaser SS, Benedetti A. Role and mechanisms of action of acetylcholine in the regulation of rat cholangiocyte secretory functions. *J Clin Invest* 1997; **100**: 1349-1362
  - 35 **LeSage G**, Alvaro D, Benedetti A, Glaser S, Marucci L, Baiocchi L, Eisel W, Caligiuri A, Phinizz JL, Rodgers R, Francis H, Alpini G. Cholinergic system modulates growth, apoptosis, and secretion of cholangiocytes from bile duct-ligated rats. *Gastroenterology* 1999; **117**: 191-199
  - 36 **Fava G**, Marucci L, Glaser S, Francis H, De Morrow S, Benedetti A, Alvaro D, Venter J, Meininger C, Patel T, Taffetani S, Marzioni M, Summers R, Reichenbach R, Alpini G. gamma-Aminobutyric acid inhibits cholangiocarcinoma growth by cyclic AMP-dependent regulation of the protein kinase A/extracellular signal-regulated kinase 1/2 pathway. *Cancer Res* 2005; **65**: 11437-11446
  - 37 **Sampson LK**, Vickers SM, Ying W, Phillips JO. Tamoxifen-mediated growth inhibition of human cholangiocarcinoma. *Cancer Res* 1997; **57**: 1743-1749
  - 38 **Alvaro D**, Barbaro B, Franchitto A, Onori P, Glaser SS, Alpini G, Francis H, Marucci L, Sterpetti P, Ginanni-Corradini S, Onetti Muda A, Dostal DE, De Santis A, Attili AF, Benedetti A, Gaudio E. Estrogens and insulin-like growth factor 1 modulate neoplastic cell growth in human cholangiocarcinoma. *Am J Pathol* 2006; **169**: 877-888
  - 39 **DeMorrow S**, Glaser S, Francis H, Venter J, Vaculin B, Vaculin S, Alpini G. Opposing actions of endocannabinoids on cholangiocarcinoma growth: recruitment of Fas and Fas ligand to lipid rafts. *J Biol Chem* 2007; **282**: 13098-13113

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## TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, Professor; Sharon DeMorrow, Assistant Professor, Series Editor

# c-Met targeted therapy of cholangiocarcinoma

Matei P Socoteanu, Frank Mott, Gianfranco Alpini, Arthur E Frankel

Matei P Socoteanu, Frank Mott, Gianfranco Alpini, Arthur E Frankel, Scott & White Hospital and Clinics, 5701 S, Airport Rd, Temple, Texas 76502, United States

Author contributions: Socoteanu MP wrote the article; Frankel AE helped write the article; Mott F and Alpini G oversaw and reviewed the article.

Correspondence to: Arthur E Frankel, Director of Scott & White Cancer Research Institute, 5701 S, Airport Rd, Temple, Texas 76502, United States. [afrankel@swmail.sw.org](mailto:afrankel@swmail.sw.org)

Telephone: +1-254-7240094 Fax: +1-254-7247682

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## Abstract

Cholangiocarcinoma continues to be a challenging disease to treat. Systemic therapy is used in unresectable disease, disease progression after surgery, and in the palliative setting. Unfortunately, results of multiple phase II trials have rarely yielded positive results. As data on the molecular carcinogenesis of cholangiocarcinoma is developing, we are more able to understand the disease process and can use this understanding to create unique targeted therapies. We reviewed the role of c-Met/hepatocyte growth factor (HGF) in the development of cholangiocarcinoma. Furthermore, we explored the use of the c-Met guided cascade as a target to treat cholangiocarcinoma. We reviewed the current use and options for future development of c-Met agents to treat this disease.

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**Key words:** Cholangiocarcinoma; c-Met; Chemotherapy; Target therapy

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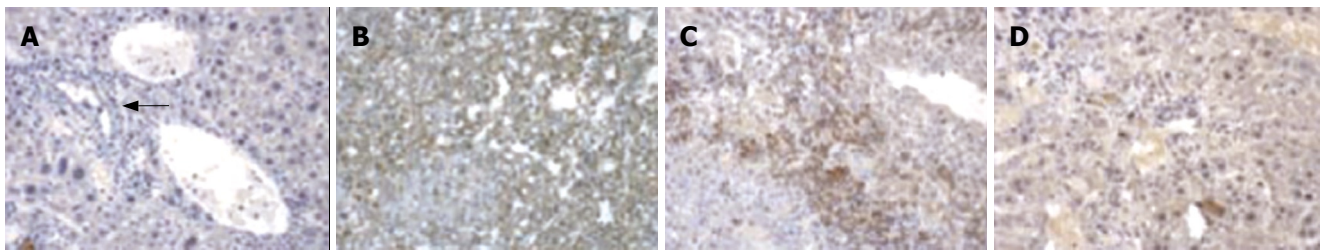
## CHOLANGIOCARCINOMA

Cholangiocarcinoma continues to be a challenging

disease to treat. The only curative option remains surgical resection. Recent trends have allowed previously inoperable patients to undergo potentially curative surgery. Most recently, liver transplantation has been used in locally unresectable tumors with variable results<sup>[1-3]</sup>. Becker *et al*<sup>[4]</sup> reported outcome analysis for 280 patients treated at multiple centers over an 18-year period. Their data shows 5 and 10 years survivals of 74% and 38% respectively. Unfortunately, relatively few patients are diagnosed with limited stage disease that is amenable to surgical intervention (either resection or transplantation). Systemic therapy has been used in unresectable disease, disease progression after surgery, and in the palliative setting. Results of multiple phase II trials have rarely yielded positive results<sup>[5]</sup>. Average median survival remains less than one year and response rates are generally under 30%. Although the benefit is minimal, the most efficacious and clinically utilized chemotherapy regimens have been either gemcitabine or 5-fluorouracil (5FU)-based. Alberts *et al*<sup>[6]</sup> conducted a Phase II trial of gemcitabine, 5FU, and Leucovorin in advanced biliary disease. This study delivered 4 wk cycles of gemcitabine/5FU/Leucovorin on d 1, 8 and 15. The study enrolled carcinoma of the gallbladder and cholangiocarcinoma. For our scope, we will focus on their cholangiocarcinoma data. The study enrolled 28 patients with biliary cancer at multiple centers. Using the Response Evaluation Criteria in Solid Tumors (RECIST)<sup>[7]</sup> criteria two patients with biliary tract cancer achieved a partial response. The median time to disease progression was 4.6 mo and median survival was 9.9 mo. The primary endpoint of the study was successfully achieved, namely, to determine 6 mo survival. The overall data is fairly consistent with the previously reported data for single agent 5FU or gemcitabine. Although it is clear that 5FU and gemcitabine are active in cholangiocarcinoma, this study showed that the combination of the two most potent agents failed to produce a greater response rate or duration of response than either agent alone. This study and others like it increases suspicion that traditional chemotherapy is unlikely to make tangible progress in this devastating disease.

## MOLECULAR BIOLOGY OF CHOLANGIOCARCINOMA

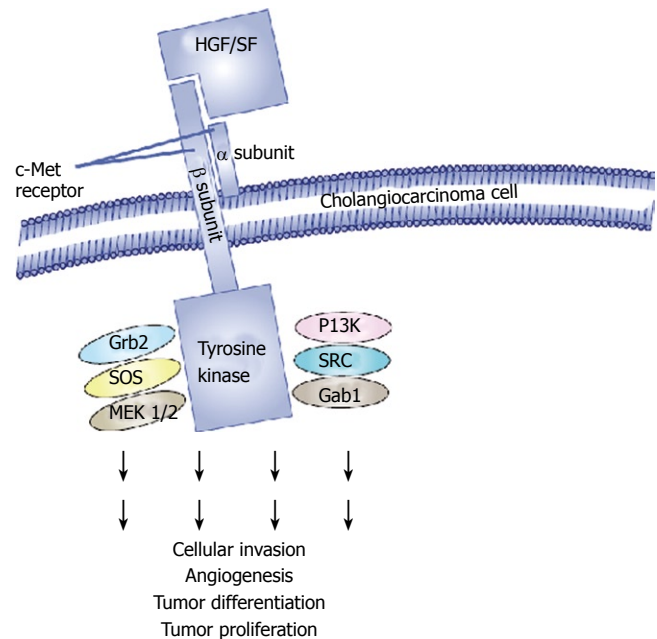
Like many other malignancies, cholangiocarcinoma cells over-express epidermal growth factor receptors (EGFR)<sup>[8-10]</sup>. This observation led several investigators to postulate that Erlotinib (an oral inhibitor of EGFR/



**Figure 1** c-Met immunohistochemistry performed on: **A:** Normal liver; **B:** Cholangiocarcinoma; **C:** Early stage cholangiocarcinoma; **D:** Bile duct hyperplasia reproduced from Fazari (19) with permission.

HER1 tyrosine kinase) would show activity against cholangiocarcinoma. This interest intensified when activity was demonstrated against other malignancies such as lung<sup>[11]</sup> and pancreatic cancer<sup>[12]</sup>. Philip *et al.*<sup>[13]</sup> reported a Phase II trial in 42 patients with advanced biliary cancer. Using the Response Evaluation Criteria in Solid Tumors (RECIST) criteria three patients achieved a partial response (7%). Median time to disease progression was only 2.6 mo, and median overall survival was 7.5 mo. Interestingly, there was a certain subgroup (17%) that seemed to achieve prolonged (greater than 24 wk) disease stability. There did not appear to be a correlation between *EGFR/HER1* gene over-expression and response. This was not unexpected since *EGFR* mutations, *K-Ras* mutations, *p-AKT* levels, and proteomic signatures are also important predictors of erlotinib response and signal pathway dependence is difficult to predict from gene expression alone<sup>[11,14,15]</sup>. Although these results are promising, clinicians are left searching for better treatment options. Further advancement in the treatment of cholangiocarcinoma begins with a better understanding of the molecular mechanisms of carcinogenesis.

Data on the molecular carcinogenesis of cholangiocarcinoma is developing rapidly<sup>[16,17]</sup>. As in most cancers, multiple genes have been implicated in the molecular transformation of normally functioning tissue to malignant cells. These genetic changes cause a cascade of effects that include activation of oncogenes, inactivation of tumor suppressor genes, alterations in cell signaling, resistance to apoptosis, and direct induction of DNA damage. These genetic alterations affect all phases of the cell cycle and work in concert to transform bile secreting cells into an aggressive carcinoma. A detailed description of all of these mutations and their specific role in cholangiocarcinogenesis is beyond the scope of this publication. Here, we focus on the role of c-Met/hepatocyte growth factor (HGF) and its possible therapeutic implications. It has been reported that c-Met is over-expressed in more than half of biliary carcinomas<sup>[18]</sup>. As shown in Figure 1, Farazi *et al.*<sup>[19]</sup> demonstrated c-met over-expression in 80% of humanoid murine intrahepatic cholangiocarcinoma. Radaeva *et al.*<sup>[20]</sup> confirmed that cholangiocarcinoma expressed strong cell-surface immunoreactivity for c-Met. *c-Met* is a proto-oncogene located on chromosome 7q that codes for a tyrosine kinase growth factor receptor called HGF receptor<sup>[21]</sup>. HGF (also known as scatter factor) binds to c-Met and initiates autophosphorylation of an intracellular tyrosine kinase on the beta-subunit of the receptor. This activation allows the binding and ultimate activation of



**Figure 2** Schema of c-Met signaling pathways.

multiple signaling molecules such as Src, P13K, Gab1, SOS, Grb2, and MEK1/2 (Figure 2). The interaction of this multi-faceted activation system ultimately results in cellular alterations that contribute to carcinogenesis. It has been suggested in multiple studies that over-expression of c-Met is linked to cell invasion, angiogenesis, and tumor differentiation/proliferation<sup>[22-24]</sup> ([www.vai.org/met](http://www.vai.org/met)). Although the data is not conclusive, several researchers have suggested that c-Met behaves differently in intrahepatic and extrahepatic cholangiocarcinoma<sup>[25,26]</sup>. Leelawat *et al.*<sup>[27]</sup> demonstrated that stimulated over-expression of the *c-Met* gene in cholangiocarcinoma cells resulted in increased cell migration and invasion. Conversely, inhibition of *c-Met* expression decreased cellular phosphorylation and ultimately reduced cellular invasiveness. The presence of the *c-Met* oncogene and its unique cell signaling pathway provides one of many avenues by which specific cell targeting can be used to achieve better tumor control in cholangiocarcinoma<sup>[28]</sup>.

## C-MET THERAPIES

There are multiple focal points for interrupting c-Met activity with clinical compounds<sup>[29]</sup>. The earliest target in the cascade focuses on inhibition of the interaction

between HGF and the c-met receptor. Blocking the binding of the HGF to the transmembranous c-Met receptor works to halt c-Met signaling at the earliest point. Ultimately, c-Met fails to dimerize and tyrosine kinase activation does not occur. The alteration of this HGF/c-Met interaction can occur via multiple modalities including small interference RNAs (siRNA) which block c-Met expression, monoclonal antibodies against c-Met or HGF, and soluble c-Met fragment which can block HGF binding. Another target in the c-Met system is the direct tyrosine kinase inhibition. Similar to the tyrosine kinase inhibitors in chronicmyeloidleukemia (CML) and other tumors, designer compounds that are specific to the *c-Met* gene tyrosine kinase are administered. Although the interaction between HGF and the c-Met receptor is preserved, the cascade is halted by the selective binding of the inhibitor to the tyrosine kinase. Theoretically, all of these mechanisms would function to reduce cellular invasion, migration, angiogenesis, and ultimately, halt the process of carcinogenesis.

## C-MET THERAPIES FOR CHOLANGIOCARCINOMA

To date, only one study has reported c-Met targeted therapy in an animal model of cholangiocarcinoma<sup>[27]</sup>. This study showed that inhibition in c-Met expression or its downstream target MEK1/2 through specific targeted therapy is effective in halting disease progression *in vivo*. Inhibition was achieved through two molecular strategies. First, c-Met expression was altered through a *c-Met* specific small interfering RNA (siRNA) binding to the c-Met coded receptor. Second, siRNA specific binding to the *c-Met* downhill cascade product, MEK1/2, resulted in blunting of the cellular invasiveness of cholangiocarcinoma cells.

A number of c-Met and HGF antibody directed therapies receptor interaction have been shown biological activity in non-biliary cancer animal models<sup>[30,31]</sup> and human studies<sup>[32]</sup>. AMG102 is a fully human IgG2 monoclonal antibody against HGF. This compound has completed both preclinical trials and phase I dosing trials<sup>[33]</sup>. Although, the dose-escalating trials were performed on a variety of solid tumors, there is not current data on cholangiocarcinoma. A one-armed c-Met antibody has shown activity in preclinical studies<sup>[34]</sup>. Again, patients with cholangiocarcinoma have not been treated. Decoy met<sup>[35]</sup> is a soluble met receptor that interferes with HGF binding. It has been shown *in vivo* to have multiple anti-malignant properties including inhibiting angiogenesis, suppressing metastasis, and halting cellular proliferation. Decoy met functions to block the c-Met receptor as well as altering met dimerization. Decoy met has several properties that may make it more desirable than standard antibody directed therapies. For example, decoy met has a logarithmically greater affinity for the c-met receptor.

A series of c-Met tyrosine kinase inhibitors have been examined. XL880 is an oral c-Met tyrosine kinase inhibitor that is completing Phase I trials and beginning Phase II trials in humans. XL880 is a multi-kinase inhibitor that affects both the HGF/c-Met receptor family and the VEGF receptor family. The most common side effect

Table 1 Target sites of c-Met therapies

Target	Example	Current phase
HGF/c-met monoclonal antibody	AMG102 <sup>[33]</sup>	Phase II
Soluble c-met receptor	Decoy met <sup>[35]</sup>	Phase I
Tyrosine kinase inhibition	ARQ 197 <sup>[42]</sup>	Phase II
	XL880 <sup>[36,37]</sup>	Phase II
	PHA665752 <sup>[38]</sup>	Animal testing

of XL880 is hypertension. Although XL880-induced hypertension is very common, in phase I testing, it was manageable with anti-hypertensive medications<sup>[36]</sup>. XL880 is currently undergoing phase II clinical trials in a number of cancers. Early Phase II data on renal cell cancer has been positive<sup>[37]</sup>. It has shown activity in lung cancer (both small cell and non-small cell) xenografts in immunocompromised mice<sup>[38]</sup>. Additional tyrosine kinase inhibitors that are specific to the c-Met receptor have been developed<sup>[39-41]</sup>. ARQ 197 is a c-Met specific receptor tyrosine kinase inhibitor. This compound has completed Phase I dose escalation and has reached the recommended phase II dose. Partial responses and durable long term disease control have been achieved in several malignancies<sup>[42]</sup>. PHA665752 is a selective small molecule tyrosine kinase inhibitor of c-Met<sup>[38,43]</sup>. It has been shown to inhibit angiogenesis and induce apoptosis and cell cycle arrest. Interestingly, PHA665752 has been shown to have a cooperative effect when administered with rapamycin<sup>[43]</sup>. No current data on PHA665752 in humans is available.

## CLINICAL TRIALS WITH C-MET THERAPIES

There is scant data for any of the compounds in patients with cholangiocarcinomas (Table 1). The previously mentioned XL880 Phase I trial included 1 patient with cholangiocarcinoma. The slides presented at the 2007 ASCO meeting indicated that there was as 5 mo duration of response in Phase I testing. Unfortunately, the XL880 trial did not select for tumors over-expressing c-Met. Progress is rapidly being made through inhibition of the c-Met cascade. Hopefully, this will result in treatment advances in cholangiocarcinoma. Furthermore, other molecular mechanisms exist for using c-Met to target cellular death in cholangiocarcinoma. The possibility of using HGF or a monoclonal antibody to c-Met for immunotoxin construction should also be explored<sup>[45]</sup>. This would result in preferential introduction of deadly toxins into the cholangiocarcinoma cellular environment sparing normal cells (non c-Met expressing). As treatments directed against aggressive incurable cancers develop, their success will likely depend on their ability to deliver tumor selective, highly toxic treatments to carcinoma cells while sparing normal tissue. The c-Met cascade and others like it provide such an opportunity. Through these rapid developments, researchers, clinicians, and patients have hope of better treatments in the future.

## REFERENCES

- 1 Shimoda M, Farmer DG, Colquhoun SD, Rosove M, Ghobrial RM, Yersiz H, Chen P, Busuttil RW. Liver transplantation



- for cholangiocellular carcinoma: analysis of a single-center experience and review of the literature. *Liver Transpl* 2001; **7**: 1023-1033
- 2 **Iwatsuki S**, Todo S, Marsh JW, Madariaga JR, Lee RG, Dvorchik I, Fung JJ, Starzl TE. Treatment of hilar cholangiocarcinoma (Klatskin tumors) with hepatic resection or transplantation. *J Am Coll Surg* 1998; **187**: 358-364
  - 3 **Goldstein RM**, Stone M, Tillery GW, Senzer N, Levy M, Husberg BS, Gonwa T, Klintmalm G. Is liver transplantation indicated for cholangiocarcinoma? *Am J Surg* 1993; **166**: 768-771; discussion 771-772
  - 4 **Becker NS**, Rodriguez JA, Barshes NR, O'Mahony CA, Goss JA, Aloia TA. Outcomes Analysis for 280 Patients with Cholangiocarcinoma Treated with Liver Transplantation Over an 18-year Period. *J Gastrointest Surg* 2008; **12**: 117-122
  - 5 **Thongprasert S**. The role of chemotherapy in cholangiocarcinoma. *Ann Oncol* 2005; **16** Suppl 2: ii93-ii96
  - 6 **Alberts SR**, Al-Khatib H, Mahoney MR, Burgart L, Cera PJ, Flynn PJ, Finch TR, Levitt R, Windschitl HE, Knost JA, Tschetter LK. Gemcitabine, 5-fluorouracil, and leucovorin in advanced biliary tract and gallbladder carcinoma: a North Central Cancer Treatment Group phase II trial. *Cancer* 2005; **103**: 111-118
  - 7 **Therasse P**, Arbuck SG, Eisenhauer EA, Wanders J, Kaplan RS, Rubinstein L, Verweij J, Van Glabbeke M, van Oosterom AT, Christian MC, Gwyther SG. New guidelines to evaluate the response to treatment in solid tumors. European Organization for Research and Treatment of Cancer, National Cancer Institute of the United States, National Cancer Institute of Canada. *J Natl Cancer Inst* 2000; **92**: 205-216
  - 8 **Lee CS**, Pirdas A. Epidermal growth factor receptor immunoreactivity in gallbladder and extrahepatic biliary tract tumours. *Pathol Res Pract* 1995; **191**: 1087-1091
  - 9 **Yoon JH**, Gwak GY, Lee HS, Bronk SF, Werneburg NW, Gores GJ. Enhanced epidermal growth factor receptor activation in human cholangiocarcinoma cells. *J Hepatol* 2004; **41**: 808-814
  - 10 **Werneburg NW**, Yoon JH, Higuchi H, Gores GJ. Bile acids activate EGF receptor via a TGF- $\alpha$ -dependent mechanism in human cholangiocyte cell lines. *Am J Physiol Gastrointest Liver Physiol* 2003; **285**: G31-G36
  - 11 **Shepherd FA**, Rodrigues Pereira J, Ciuleanu T, Tan EH, Hirsh V, Thongprasert S, Campos D, Maoleekoonpiroj S, Smylie M, Martins R, van Kooten M, Dediu M, Findlay B, Tu D, Johnston D, Bezjak A, Clark G, Santabarbara P, Seymour L. Erlotinib in previously treated non-small-cell lung cancer. *N Engl J Med* 2005; **353**: 123-132
  - 12 **Moore MJ**, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark G, Voskoglou-Nomikos T, Ptasynski M, Parulekar W. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 2007; **25**: 1960-1966
  - 13 **Philip PA**, Mahoney MR, Allmer C, Thomas J, Pitot HC, Kim G, Donehower RC, Fitch T, Picus J, Erlichman C. Phase II study of erlotinib in patients with advanced biliary cancer. *J Clin Oncol* 2006; **24**: 3069-3074
  - 14 **Tsao MS**, Sakurada A, Cutz JC, Zhu CQ, Kamel-Reid S, Squire J, Lorimer I, Zhang T, Liu N, Daneshmand M, Marrano P, da Cunha Santos G, Lagarde A, Richardson F, Seymour L, Whitehead M, Ding K, Pater J, Shepherd FA. Erlotinib in lung cancer - molecular and clinical predictors of outcome. *N Engl J Med* 2005; **353**: 133-144
  - 15 **Tsao MS**, Liu G, Shepherd FA. Serum proteomic classifier for predicting response to epidermal growth factor receptor inhibitor therapy: have we built a better mousetrap? *J Natl Cancer Inst* 2007; **99**: 826-827
  - 16 **Olnes MJ**, Erlich R. A review and update on cholangiocarcinoma. *Oncology* 2004; **66**: 167-179
  - 17 **Rashid A**. Cellular and molecular biology of biliary tract cancers. *Surg Oncol Clin N Am* 2002; **11**: 995-1009
  - 18 **Terada T**, Nakanuma Y, Sirica AE. Immunohistochemical demonstration of MET overexpression in human intrahepatic cholangiocarcinoma and in hepatolithiasis. *Hum Pathol* 1998; **29**: 175-180
  - 19 **Farazi PA**, Zeisberg M, Glickman J, Zhang Y, Kalluri R, DePinho RA. Chronic bile duct injury associated with fibrotic matrix microenvironment provokes cholangiocarcinoma in p53-deficient mice. *Cancer Res* 2006; **66**: 6622-6627
  - 20 **Radaeva S**, Ferreira-Gonzalez A, Sirica AE. Overexpression of C-NEU and C-MET during rat liver cholangiocarcinogenesis: A link between biliary intestinal metaplasia and mucin-producing cholangiocarcinoma. *Hepatology* 1999; **29**: 1453-1462
  - 21 **Furge KA**, Zhang YW, Vande Woude GF. Met receptor tyrosine kinase: enhanced signaling through adapter proteins. *Oncogene* 2000; **19**: 5582-5589
  - 22 **Gao CF**, Vande Woude GF. HGF/SF-Met signaling in tumor progression. *Cell Res* 2005; **15**: 49-51
  - 23 **Birchmeier C**, Birchmeier W, Gherardi E, Vande Woude GF. Met, metastasis, motility and more. *Nat Rev Mol Cell Biol* 2003; **4**: 915-925
  - 24 **Zhang YW**, Vande Woude GF. HGF/SF-met signaling in the control of branching morphogenesis and invasion. *J Cell Biochem* 2003; **88**: 408-417
  - 25 **Hida Y**, Morita T, Fujita M, Miyasaka Y, Horita S, Fujioka Y, Nagashima K, Katoh H. Clinical significance of hepatocyte growth factor and c-Met expression in extrahepatic biliary tract cancers. *Oncol Rep* 1999; **6**: 1051-1056
  - 26 **Aishima SI**, Taguchi KI, Sugimachi K, Shimada M, Sugimachi K, Tsuneyoshi M. c-erbB-2 and c-Met expression relates to cholangiocarcinogenesis and progression of intrahepatic cholangiocarcinoma. *Histopathology* 2002; **40**: 269-278
  - 27 **Leelawat K**, Leelawat S, Tepaksorn P, Rattanasinganchan P, Leungchaweng A, Tohtong R, Sobhon P. Involvement of c-Met/hepatocyte growth factor pathway in cholangiocarcinoma cell invasion and its therapeutic inhibition with small interfering RNA specific for c-Met. *J Surg Res* 2006; **136**: 78-84
  - 28 **Sirica AE**. Cholangiocarcinoma: molecular targeting strategies for chemoprevention and therapy. *Hepatology* 2005; **41**: 5-15
  - 29 **Peruzzi B**, Bottaro DP. Targeting the c-Met signaling pathway in cancer. *Clin Cancer Res* 2006; **12**: 3657-3660
  - 30 **Cao B**, Su Y, Oskarsson M, Zhao P, Kort EJ, Fisher RJ, Wang LM, Vande Woude GF. Neutralizing monoclonal antibodies to hepatocyte growth factor/scatter factor (HGF/SF) display antitumor activity in animal models. *Proc Natl Acad Sci USA* 2001; **98**: 7443-7448
  - 31 **Kim KJ**, Wang L, Su YC, Gillespie GY, Salhotra A, Lal B, Lateral J. Systemic anti-hepatocyte growth factor monoclonal antibody therapy induces the regression of intracranial glioma xenografts. *Clin Cancer Res* 2006; **12**: 1292-1298
  - 32 **Burgess T**, Coxon A, Meyer S, Sun J, Rex K, Tsuruda T, Chen Q, Ho SY, Li L, Kaufman S, McDorman K, Cattley RC, Sun J, Elliott G, Zhang K, Feng X, Jia XC, Green L, Radinsky R, Kendall R. Fully human monoclonal antibodies to hepatocyte growth factor with therapeutic potential against hepatocyte growth factor/c-Met-dependent human tumors. *Cancer Res* 2006; **66**: 1721-1729
  - 33 **Kakkar T**, Ma M, Zhuang Y, Patton A, Hu Z, Mounho B. Pharmacokinetics and safety of a fully human hepatocyte growth factor antibody, AMG 102, in cynomolgus monkeys. *Pharm Res* 2007; **24**: 1910-1918
  - 34 **Martens T**, Schmidt NO, Eckerich C, Fillbrandt R, Merchant M, Schwall R, Westphal M, Lamszus K. A novel one-armed anti-c-Met antibody inhibits glioblastoma growth in vivo. *Clin Cancer Res* 2006; **12**: 6144-6152
  - 35 **Michieli P**, Mazzone M, Basilico C, Cavassa S, Sottile A, Naldini L, Comoglio PM. Targeting the tumor and its microenvironment by a dual-function decoy Met receptor. *Cancer Cell* 2004; **6**: 61-73
  - 36 **Eder JP**, Heath E, Appleman L, Shapiro G, Wang D, Malburg L, Zhu AX, Leader T, Wolanski A, LoRusso P. Phase I experience with c-MET inhibitor XL880 administered orally to patients (pts) with solid tumors. *ASCO (Meeting Abstracts)* 2007; **25**: 3526
  - 37 **Ross RW**, Stein M, Sarantopoulos J, Eisenberg P, Logan T, Srinivas S, Rosenberg J, Vaishampayan U. A phase II study of



- the c-Met RTK inhibitor XL880 in patients (pts) with papillary renal-cell carcinoma (PRC). *ASCO (Meeting Abstracts)* 2007; **25**: 15601
- 38 **Puri N**, Khramtsov A, Ahmed S, Nallasura V, Hetzel JT, Jagadeeswaran R, Karczmar G, Salgia R. A selective small molecule inhibitor of c-Met, PHA665752, inhibits tumorigenicity and angiogenesis in mouse lung cancer xenografts. *Cancer Res* 2007; **67**: 3529-3534
- 39 **Christensen JG**, Schreck R, Burrows J, Kuruganti P, Chan E, Le P, Chen J, Wang X, Ruslim L, Blake R, Lipson KE, Ramphal J, Do S, Cui JJ, Cherrington JM, Mendel DB. A selective small molecule inhibitor of c-Met kinase inhibits c-Met-dependent phenotypes in vitro and exhibits cytoreductive antitumor activity in vivo. *Cancer Res* 2003; **63**: 7345-7355
- 40 **Wang X**, Le P, Liang C, Chan J, Kiewlich D, Miller T, Harris D, Sun L, Rice A, Vasile S, Blake RA, Howlett AR, Patel N, McMahon G, Lipson KE. Potent and selective inhibitors of the Met [hepatocyte growth factor/scatter factor (HGF/SF) receptor] tyrosine kinase block HGF/SF-induced tumor cell growth and invasion. *Mol Cancer Ther* 2003; **2**: 1085-1092
- 41 **Sattler M**, Pride YB, Ma P, Gramlich JL, Chu SC, Quinnan LA, Shirazian S, Liang C, Podar K, Christensen JG, Salgia R. A novel small molecule met inhibitor induces apoptosis in cells transformed by the oncogenic TPR-MET tyrosine kinase. *Cancer Res* 2003; **63**: 5462-5469
- 42 **Garcia A**, Rosen L, C. C. Cunningham, J. Nemunaitis, C. Li, N. Rulewski, A. Dovholuk, R. Savage, T. Chan, R. Bukowski and T. Mekhail. Phase 1 study of ARQ 197, a selective inhibitor of the c-Met RTK in patients with metastatic solid tumors reaches recommended phase 2 dose. *ASCO Annual Meeting Proceedings (Post-Meeting Edition)* 2007; **25**: 3525
- 43 **Smolen GA**, Sordella R, Muir B, Mohapatra G, Barmettler A, Archibald H, Kim WJ, Okimoto RA, Bell DW, Sgroi DC, Christensen JG, Settleman J, Haber DA. Amplification of MET may identify a subset of cancers with extreme sensitivity to the selective tyrosine kinase inhibitor PHA-665752. *Proc Natl Acad Sci USA* 2006; **103**: 2316-2321
- 44 **Ma PC**, Schaefer E, Christensen JG, Salgia R. A selective small molecule c-MET Inhibitor, PHA665752, cooperates with rapamycin. *Clin Cancer Res* 2005; **11**: 2312-2319
- 45 **Wong L**, Suh DY, Frankel AE. Toxin conjugate therapy of cancer. *Semin Oncol* 2005; **32**: 591-595

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## Review of endoscopic techniques in the diagnosis and management of cholangiocarcinoma

Katherine Nguyen, James T Sing Jr

Katherine Nguyen, James T Sing Jr, Scott & White Clinic, Texas A&M University Health Science Center College of Medicine, Temple, Texas 76508, United States

Correspondence to: Katherine Nguyen, MD, Scott & White Clinic, Texas A & M University Health Science Center College of Medicine, Temple, Texas 76508,

United States. [knguyen@swmail.sw.org](mailto:knguyen@swmail.sw.org)

Telephone: +1-254-7242274 Fax: +1-254-7247210

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### Abstract

Cholangiocarcinoma is a rare malignancy of the biliary tract. Key factors in determining therapeutic options include knowledge of tumor extent, anatomy and obtaining tissue diagnosis. Endoscopically, there are three modalities available to make the diagnosis of cholangiocarcinoma. These include endoscopic retrograde cholangiopancreatography, endoscopic ultrasound with fine needle aspiration and cholangioscopy. Management of cholangiocarcinoma endoscopically is typically confined to stent placement for palliative purposes or as a bridge to surgery. In this article, we will review the endoscopic techniques available for the diagnosis and management of cholangiocarcinoma.

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**Key words:** Cholangiocarcinoma; Endoscopic ultrasound; Endoscopic cholangiopancreatography; Cholangioscopy; Diagnosis; Management

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### INTRODUCTION

Cholangiocarcinomas are rare malignancies involving the biliary tract. They can be divided into three anatomic groups: intrahepatic, perihilar, and distal extrahepatic. Perihilar tumors, also known as Klastkin tumors, involve the hepatic duct bifurcation. They are the most common, accounting for about 60%-80% of cholangiocarcinomas. Intrahepatic

tumors are least common<sup>[1]</sup>. The Bismuth classification is used to describe the biliary tract involvement and is helpful in planning surgical intervention. Type I tumors are found below the bifurcation of the left and right hepatic ducts. Type II tumors involve the bifurcation. Type IIIa and IIIb tumors occlude the common hepatic duct and either the right or left hepatic duct, respectively. Type IV tumors are multicentric, or they involve the bifurcation and both the right and left hepatic ducts (Figure 1). The incidence rates for cholangiocarcinomas vary depending on geographic location with the highest rates found in Southeast Asia. In the United States, between 4000 and 5000 cases are found annually. For unknown reason, the incidence of and mortality rates for intrahepatic cholangiocarcinomas have been increasing in recent years while the incidence of extrahepatic cholangiocarcinomas have been decreasing<sup>[2]</sup>. Accurate knowledge of tumor extent and anatomy as well as obtaining a tissue diagnosis is important in determining therapeutic options. In this article, we will review the endoscopic modalities available in the diagnosis and management of cholangiocarcinomas.

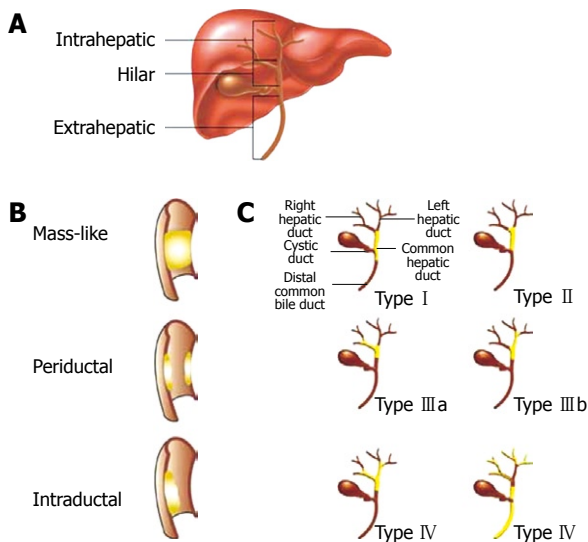
### DIAGNOSIS

The etiology of biliary strictures can often be difficult to establish. The differential diagnosis of biliary strictures is extensive and includes, but is not limited to, primary sclerosing cholangitis, gallbladder carcinoma, pancreatic carcinoma, intraductal papillary mucinous tumor, or benign biliary strictures from causes such as pancreatitis.

Cholangiocarcinomas often pose a diagnostic challenge due to difficulties in obtaining an adequate specimen for cytology. Tissue diagnosis is important in certain subgroups of patients such as those who are borderline surgical candidates, those with indeterminate strictures (e.g. patients with primary sclerosing cholangitis), or before chemotherapy and radiation therapy.

#### Magnetic resonance cholangiopancreatography

Magnetic resonance cholangiopancreatography (MRCP) can be a useful noninvasive adjunct to current techniques. It has the ability to define the proximal and distal extent of strictures as well as to evaluate for any intrahepatic mass lesion. One series evaluated the role of MRCP in patients with bile duct obstruction. Of 126 patients, 14 had bile duct malignancy. Of those 14, 12 patients were diagnosed by MRCP, with a sensitivity of 86% and specificity of 98%<sup>[3]</sup>. Another study by Rosch *et al*<sup>[4]</sup> had lower specificity for malignant obstructions. This study compared endoscopic retrograde cholangiopancreatography



**Figure 1** Classification of cholangiocarcinoma. **A:** The classification of cholangiocarcinoma can be based on anatomic location, intrahepatic, hilar or extrahepatic; **B:** Nonhilar lesions can be described as mass-like, periductal or intraductal; **C:** Bismuth classification for hilar lesions.

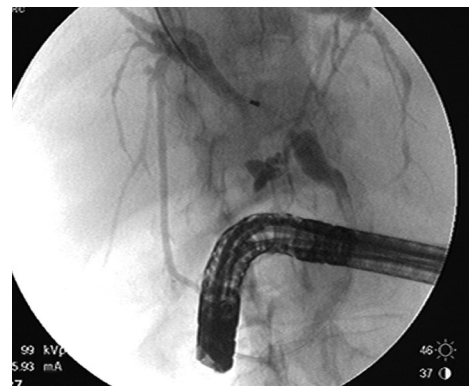
(ERCP), MRCP, computed tomography (CT) and endoscopic ultrasound (EUS) in the evaluation of biliary strictures. The specificity and sensitivity for MRCP to detect malignancy was 77% and 63%, respectively. Although MRCP provides the same imaging information as ERCP, many times ERCP is still required to provide a tissue diagnosis.

### ERCP

ERCP is useful in both the diagnosis and management of cholangiocarcinomas. It can delineate the anatomy of the biliary system and determine the extent of bile duct involvement which is important in determining resectability and surgical management (Figure 2). On cholangiography, the appearance of a stricture can suggest malignancy, but is not conclusive. Some characteristics suggestive of malignancy include a length greater than 10 mm, irregular margins, and an abrupt transition from normal duct to stricture, also known as shouldering<sup>[5]</sup>. Hilar strictures should also raise the suspicion for malignancy. Although, the appearance and location of a biliary stricture can suggest malignancy, tissue confirmation is usually needed in the majority of patients. Tissue for cytology may be obtained during ERCP by brushing, biopsy, bile aspiration or a combination of these. When necessary, therapeutic procedures can be performed, such as the placement of a biliary stent for treatment of obstructive jaundice.

Brush cytology has a high specificity of nearly 100%, but sensitivity is much lower, ranging from 18%-60% in most series<sup>[6,7]</sup>. The low sensitivity is likely related to low cellularity of these tumors and the desmoplastic reaction that is present.

Stricture manipulation by dilation theoretically should increase the availability of malignant cells for cytological examination. However, studies by deBellis *et al*<sup>[8]</sup> did not show a statistically significant difference in sensitivity before and after dilation. Patients underwent dilation with either a graduated dilating catheter or a dilating balloon. However, when the results of the pre- and post-dilation



**Figure 2** Hilar lesion causing bilateral strictures at the bifurcation of the left and right hepatic ducts with proximal dilation.

brushings were combined, the diagnostic yield increased from 35% to 44% ( $P = 0.001$ ). This indicates that repeated brushing, not necessarily the stricture manipulation, should increase the diagnostic yield.

Further studies compared different brush lengths and stiffness<sup>[6]</sup>. A standard cytology brush, 1.5 cm long with soft bristles, was compared to the Cytolong brush, 5 cm long with rigid bristles. Detection rates were not increased with usage of the longer cytology brush.

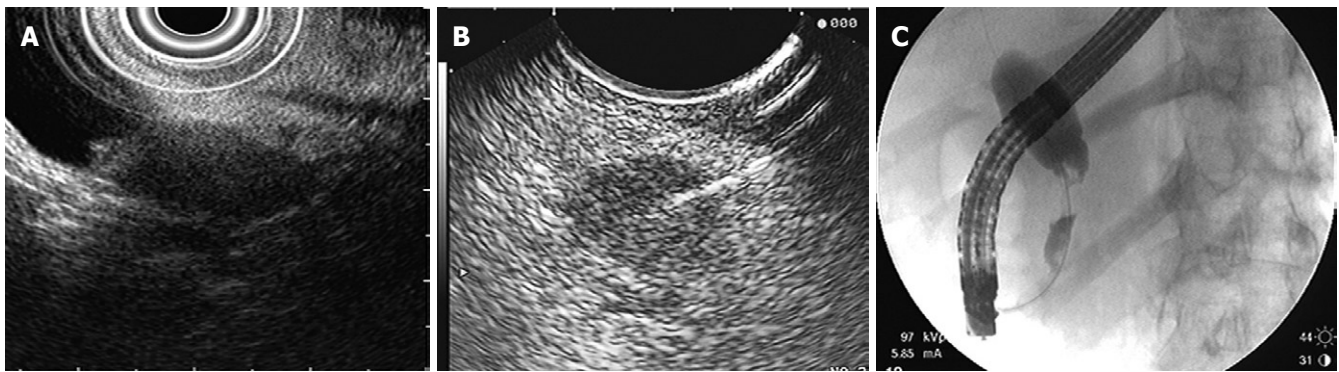
Advances in diagnostic methods have increased the diagnostic yield of brush cytology. Digital image analysis (DIA) is useful in specimens with limited cellularity as it looks at the DNA content of individual cells. DIA uses spectrophotometric methods to quantify DNA content, chromatin distribution, and nuclear morphology. Aneuploidy, or the presence of increased amounts of DNA, is quantitated and, if present, suggests malignancy. DIA increases the sensitivity of routine brush cytology from 18%-40%, but decreased the specificity from 98%-77%<sup>[9]</sup>.

Fluorescence *in-situ* hybridization (FISH) uses a commercial probe set to assess for polysomy of chromosomes 3, 7, 17, and 9p21. FISH increased the sensitivity from 15%-34%, and increased the specificity from 91%-98%<sup>[10]</sup>.

Of special note, when performing ERCP in patients with biliary strictures, there is a risk of cholangitis due to the injection of contrast and possible bacteria into an obstructed biliary system. Therefore, it is important to obtain adequate drainage across the biliary obstruction with placement of a stent to decrease this risk. In patients with a stent that had been previously placed, the removed biliary stent can be sent for cytology in order to increase diagnostic yield.

### EUS

Another modality that has become useful in diagnosing hilar cholangiocarcinomas is EUS with fine needle aspiration (FNA). CT or percutaneous ultrasound guided fine needle aspiration are not routinely used because these tumors are small and isoechoic to the liver, making them more difficult to assess. EUS has high-resolution imaging and can visualize lesions of 3 mm or greater. Although ERCP is the conventional test for evaluating biliary strictures, as we have discussed, the sensitivity remains low. In patients with ERCPs that are indeterminate or non-diagnostic for ma-



**Figure 3** A: Distal common bile duct lesion with proximal biliary dilation; B: Fine needle aspiration of common bile duct lesion; C: ERCP shows distal common bile duct stricture consistent with findings on EUS.

lignancy, EUS with fine needle aspiration is a useful tool (Figure 3).

EUS images, alone without FNA, are not reliable in evaluating hilar lesions. Criteria such as echotexture, size of mass, contour abnormalities, and the shape and borders of the stenosis do not reliably differentiate malignant from benign lesions. EUS also provides visualization of hilar, celiac axis and para-aortic lymph nodes to determine local and distant metastasis. Fine needle aspiration of these lymph nodes is the most accurate way to diagnose cholangiocarcinoma and also allows for staging. In addition, EUS can evaluate the pancreas for causes of biliary strictures such as pancreatic masses or changes of chronic pancreatitis.

In a study by Fritscher-Ravens *et al*<sup>[11]</sup>, patients with hilar strictures and inconclusive tissue diagnosis by ERCP, underwent EUS with fine needle aspiration. Of 44 patients, lesions at the hilum were noted in all the patients, and adequate material was obtained in 43 patients. Cytology revealed hilar cholangiocarcinoma in 59% of patients, with an accuracy of 91%, sensitivity of 89% and specificity of 100%. Accurate diagnosis changed the management in more than half of these patients that previously had non-diagnostic ERCPs.

In 2004, Eloubeidi *et al*<sup>[12]</sup> evaluated 28 patients in a prospective study to assess how EUS-FNA impacted patient management. Of the 28 patients, 3 were excluded because the lesion could not be identified by EUS. The sensitivity, specificity, and accuracy were 86%, 100%, and 88%, respectively, with numbers similar to the study by Fritscher-Ravens *et al*. A positive impact was made in 84% of patients. In 10 patients, surgery was prevented in patients with inoperable disease, 8 patients had surgery facilitated as they had unidentifiable cancer by other modalities, and 4 patients with benign disease avoided surgery. Prior studies have shown that 13%-24% of patients with suspected cholangiocarcinomas had benign disease at the time of surgery. By performing EUS with FNA in these patients with indeterminate strictures, surgical treatment could be tailored and appropriate management decisions be made.

A further modification of ultrasound technology allows the placement of a high frequency intraductal ultrasound probe (IDUS). Although several features such

as irregular wall thickening can be highly suggestive of malignancy, IDUS as yet has no associated capability for tissue acquisition.

### Peroral cholangioscopy and spyglass

During ERCP, miniature cholangioscopes can be used to directly visualize the bile ducts and any strictures or filling defects seen during ERCP. Directed tissue biopsies can also be obtained with miniature cholangioscopic biopsy forceps. Shah *et al*<sup>[13]</sup> in 2006 evaluated 62 patients with suspected pancreatic or biliary malignancy that had prior nondiagnostic studies. Cholangioscopy with either cholangioscopy-directed or assisted biopsies performed when applicable. Sixty-two patients underwent 72 examinations and 53 lesions were seen on cholangioscopy. Twenty-nine patients had either cholangioscopy-directed or assisted biopsies and 24 had both. Cholangiocarcinoma was identified in 14 patients. Two patients with intrahepatic cholangiocarcinomas were missed by cholangioscopy. In this study, the sensitivity and specificity for cholangioscopy to detect malignancy was 89% and 96%, respectively.

More recently, a single-operator peroral cholangio-pancreatography system known as Spyglass has been developed<sup>[14,15]</sup>. Older cholangioscopes were fragile, had limited tip deflection, and had limited ability to clean the lens and visual field. In addition, they required two endoscopists, one to operate the duodenoscope and another to operate the cholangioscope. With the Spyglass system, a single operator can control both scopes, there is 4-way deflected steering, and there are separate irrigation channels. A single operator system allows tight coordination of the duodenoscope and cholangioscope. Mastering the use of the system does require experience and advanced skills. The increased maneuverability of the Spyglass system allows for 4 quadrant biopsies. In bench stimulations, the Spyglass system had 100% success rates in obtaining target quadrant biopsies compared to 50% in conventional choledochoduodenoscopes. A feasibility study was performed with 35 patients, 22 of whom had indeterminate strictures. The procedure was successful in 91% of patients. Spyglass-directed biopsies were performed in 20 patients, and 19 had adequate tissue for examination. The preliminary sensitivity and specificity of Spyglass to detect malignancy were 71% and 100%, respectively. In this study, 2 patients



(6%) developed complications; one developed ascending cholangitis and the other intrahepatic abscess. Both patients recovered without sequelae. Currently, prospective multicenter clinical trials are ongoing.

## MANAGEMENT

Cholangiocarcinomas have a very poor prognosis with an average five-year survival of only 5%-10%. The only curative therapy for cholangiocarcinomas is surgical resection. If patients are not candidates for surgical resection, their median survival is 6.7-11.6 mo compared to 37.4-42.9 mo for patients who undergo surgical resection<sup>[16,17]</sup>. Distal cholangiocarcinomas have the highest resectability rates of about 91% while perihilar tumors have the lowest at 56%. Based on the experience at Johns Hopkins Hospital over 23 years, distal, intrahepatic, perihilar cholangiocarcinomas after resection have five-year survival rates of 28%, 44%, and 11%, respectively<sup>[18]</sup>.

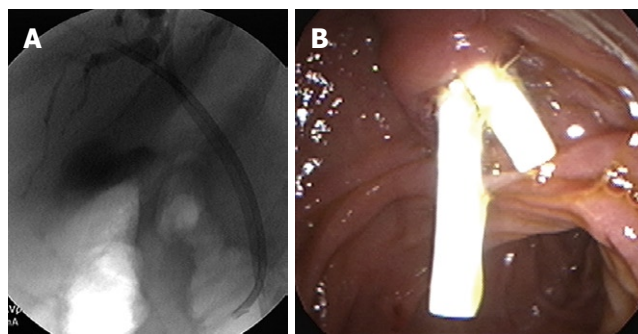
Biliary decompression by placing a stent prior to surgery is a controversial issue. A biliary stent may make it difficult to assess the proximal extent of the tumor intraoperatively and may increase the risk of infections postoperatively. However, elevated bilirubin levels and liver dysfunction are factors that adversely affect postoperative morbidity. Indications for biliary stent placement preoperatively include cholangitis or prevention of cholangitis after a diagnostic ERCP is performed or if surgery is to be delayed for an extended amount of time<sup>[19,20]</sup>.

Only about 10%-20% of patients are candidates for surgery at the time of diagnosis secondary to advanced disease or overall poor medical health. In these patients with unresectable disease, the survival is very poor and there is rapid progression with biliary obstruction. Biliary decompression for palliative purposes can be accomplished surgically, radiologically or endoscopically.

### Unilateral versus bilateral stents

In order to provide palliation and relieve jaundice, only 25% of the liver needs to be adequately drained. Therefore, unilateral stents of either the left or the right system are typically sufficient. In a randomized controlled prospective trial, De Palma *et al*<sup>[21]</sup> evaluated 157 patients with malignant hilar biliary obstruction due to cholangiocarcinoma, gallbladder cancer or periportal metastatic lymphadenopathy. In patients with unilateral stenting, there was a higher success rate for stent insertion (89% *vs* 77%) and drainage (81% *vs* 73%) and, therefore, a lower early complication rate (19% *vs* 27%) when compared to bilateral stenting of both hepatic lobes. Early complications included cholangitis and stent occlusion. No differences were found in survival or procedure-related mortality.

In order to decrease the risk of cholangitis during an ERCP, it is important not to inject contrast above the level of a stricture unless adequate drainage can be ensured. Selective cannulization with a guidewire above the level of the stricture should be performed. Following that, the catheter should be passed above the stricture before injecting contrast. With the guidewire in place, a stent can be placed in the proper position ensuring that the contaminated segment will be properly drained (Figure 4).



**Figure 4** A: In this patient with a hilar mass, double stents were placed within the right and left hepatic systems to allow adequate drainage of contrast after cholangiogram was performed to decrease the risk of cholangitis; B: Endoscopic view of bilateral stents placed.

### Plastic versus metal stents

Both plastic and metal biliary stents are available. Numerous studies have compared plastic *versus* metal stents with regards to cost, complication rates, and survival<sup>[22-24]</sup>. There are no differences in survival with the use of either stents. Plastic stents have a higher risk of occlusion, with 30% occlusion rates after 3 mo and 70% after 6 mo<sup>[23]</sup>. In order to prevent problems with occlusion and cholangitis, they need to be exchanged every 3 mo. Metal stents have a longer patency of approximately 12 mo due to the fact that they have larger diameters compared to plastic stents (10 mm *vs* 3.8 mm). However, once placed they are very difficult to manipulate or remove. As far as cost effectiveness, the initial cost of a metal biliary stent is higher. However, with plastic stents, there are subsequent costs due to the need for repeat procedures for stent exchange and hospitalization for complications. Overall, there is no significant difference in the cost between metal and plastic stents. The decision to place a plastic *versus* metal stent should take into consideration the patients' overall health, expected length of survival, quality of life and local expertise. Often, a plastic stent is placed initially while further diagnostic workup is underway. Once the diagnosis is made and the patient has unresectable disease and a life expectancy of more than 6 mo, then the plastic stent can be replaced with a metal stent. Placement of a metal stent eliminates the need for repeated procedures and their associated risks.

## CONCLUSION

The diagnosis of cholangiocarcinomas is often challenging. Multiple endoscopic modalities are available to evaluate strictures or masses of indeterminate origin. ERCP with brush cytology using FISH or DIA technology along with EUS with FNA and cholangioscopy are available. Oftentimes, repeated procedures and a combination of these different techniques are necessary to achieve a tissue diagnosis. Having a cytologic diagnosis as well as knowing the stage of the disease plays an important role in decisions regarding management. Surgery is curative if the disease is detected at an early stage. When there is metastatic or advanced disease, endoscopic drainage plays a central role in providing palliation and improving quality of life. Placement of a unilateral stent is sufficient in providing adequate drainage and has lower morbidity than bilateral stents. In patients who require short-

term drainage, plastic stents are a good option. Because long term survival is so poor, metal stents should be considered if patients are not surgical candidates.

## REFERENCES

- 1 **Ahrendt SA**, Nakeeb A, Pitt HA. Cholangiocarcinoma. *Clin Liver Dis* 2001; **5**: 191-218
- 2 **Patel T**. Cholangiocarcinoma. *Nat Clin Pract Gastroenterol Hepatol* 2006; **3**: 33-42
- 3 **Guibaud L**, Bret PM, Reinhold C, Atri M, Barkun AN. Bile duct obstruction and choledocholithiasis: diagnosis with MR cholangiography. *Radiology* 1995; **197**: 109-115
- 4 **Rosch T**, Meining A, Fruhmorgen S, Zillinger C, Schusdziarra V, Hellerhoff K, Classen M, Helmberger H. A prospective comparison of the diagnostic accuracy of ERCP, MRCP, CT, and EUS in biliary strictures. *Gastrointest Endosc* 2002; **55**: 870-876
- 5 **Park MS**, Kim TK, Kim KW, Park SW, Lee JK, Kim JS, Lee JH, Kim KA, Kim AY, Kim PN, Lee MG, Ha HK. Differentiation of extrahepatic bile duct cholangiocarcinoma from benign stricture: findings at MRCP versus ERCP. *Radiology* 2004; **233**: 234-240
- 6 **Fogel EL**, deBellis M, McHenry L, Watkins JL, Chappo J, Cramer H, Schmidt S, Lazzell-Pannell L, Sherman S, Lehman GA. Effectiveness of a new long cytology brush in the evaluation of malignant biliary obstruction: a prospective study. *Gastrointest Endosc* 2006; **63**: 71-77
- 7 **de Bellis M**, Sherman S, Fogel EL, Cramer H, Chappo J, McHenry L Jr, Watkins JL, Lehman GA. Tissue sampling at ERCP in suspected malignant biliary strictures (Part 2). *Gastrointest Endosc* 2002; **56**: 720-730
- 8 **de Bellis M**, Fogel EL, Sherman S, Watkins JL, Chappo J, Younger C, Cramer H, Lehman GA. Influence of stricture dilation and repeat brushing on the cancer detection rate of brush cytology in the evaluation of malignant biliary obstruction. *Gastrointest Endosc* 2003; **58**: 176-182
- 9 **Baron TH**, Harewood GC, Rumalla A, Pochron NL, Stadheim LM, Gores GJ, Therneau TM, De Groen PC, Sebo TJ, Salomao DR, Kipp BR. A prospective comparison of digital image analysis and routine cytology for the identification of malignancy in biliary tract strictures. *Clin Gastroenterol Hepatol* 2004; **2**: 214-219
- 10 **Patel T**, Singh P. Cholangiocarcinoma: emerging approaches to a challenging cancer. *Curr Opin Gastroenterol* 2007; **23**: 317-323
- 11 **Fritscher-Ravens A**, Broering DC, Knoefel WT, Rogiers X, Swain P, Thonke F, Bobrowski C, Topalidis T, Soehendra N. EUS-guided fine-needle aspiration of suspected hilar cholangiocarcinoma in potentially operable patients with negative brush cytology. *Am J Gastroenterol* 2004; **99**: 45-51
- 12 **Eloubeidi MA**, Chen VK, Jhala NC, Eltoum IE, Jhala D, Chhieng DC, Syed SA, Vickers SM, Mel Wilcox C. Endoscopic ultrasound-guided fine needle aspiration biopsy of suspected cholangiocarcinoma. *Clin Gastroenterol Hepatol* 2004; **2**: 209-213
- 13 **Shah RJ**, Langer DA, Antillon MR, Chen YK. Cholangioscopy and cholangioscopic forceps biopsy in patients with indeterminate pancreaticobiliary pathology. *Clin Gastroenterol Hepatol* 2006; **4**: 219-225
- 14 **Chen YK**. Preclinical characterization of the Spyglass peroral cholangiopancreatography system for direct access, visualization, and biopsy. *Gastrointest Endosc* 2007; **65**: 303-311
- 15 **Chen YK**, Pleskow DK. SpyGlass single-operator peroral cholangiopancreatography system for the diagnosis and therapy of bile-duct disorders: a clinical feasibility study (with video). *Gastrointest Endosc* 2007; **65**: 832-841
- 16 **Roayaie S**, Guarrera JV, Ye MQ, Thung SN, Emre S, Fishbein TM, Guy SR, Sheiner PA, Miller CM, Schwartz ME. Aggressive surgical treatment of intrahepatic cholangiocarcinoma: predictors of outcomes. *J Am Coll Surg* 1998; **187**: 365-372
- 17 **Weber SM**, Jarnagin WR, Klimstra D, DeMatteo RP, Fong Y, Blumgart LH. Intrahepatic cholangiocarcinoma: resectability, recurrence pattern, and outcomes. *J Am Coll Surg* 2001; **193**: 384-391
- 18 **Nakeeb A**, Pitt HA, Sohn TA, Coleman J, Abrams RA, Piantadosi S, Hruban RH, Lillemoe KD, Yeo CJ, Cameron JL. Cholangiocarcinoma. A spectrum of intrahepatic, perihilar, and distal tumors. *Ann Surg* 1996; **224**: 463-473; discussion 473-475
- 19 **Strasberg SM**. ERCP and surgical intervention in pancreatic and biliary malignancies. *Gastrointest Endosc* 2002; **56**: S213-S217
- 20 **Freeman ML**, Sielaff TD. A modern approach to malignant hilar biliary obstruction. *Rev Gastroenterol Disord* 2003; **3**: 187-201
- 21 **De Palma GD**, Galloro G, Siciliano S, Iovino P, Catanzano C. Unilateral versus bilateral endoscopic hepatic duct drainage in patients with malignant hilar biliary obstruction: results of a prospective, randomized, and controlled study. *Gastrointest Endosc* 2001; **53**: 547-553
- 22 **Soderlund C**, Linder S. Covered metal versus plastic stents for malignant common bile duct stenosis: a prospective, randomized, controlled trial. *Gastrointest Endosc* 2006; **63**: 986-995
- 23 **Prat F**, Chapat O, Ducot B, Ponchon T, Pelletier G, Fritsch J, Choury AD, Buffet C. A randomized trial of endoscopic drainage methods for inoperable malignant strictures of the common bile duct. *Gastrointest Endosc* 1998; **47**: 1-7
- 24 **Kaassis M**, Boyer J, Dumas R, Ponchon T, Coumaros D, Delcenserie R, Canard JM, Fritsch J, Rey JF, Burtin P. Plastic or metal stents for malignant stricture of the common bile duct? Results of a randomized prospective study. *Gastrointest Endosc* 2003; **57**: 178-182

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## TOPIC HIGHLIGHT

Gianfranco D Alpini, PhD, Professor; Sharon DeMorrow, Assistant Professor, Series Editor

# Diagnosis and initial management of cholangiocarcinoma with obstructive jaundice

Takashi Tajiri, Hiroshi Yoshida, Yasuhiro Mamada, Nobuhiko Taniai, Shigeki Yokomuro, Yoshiaki Mizuguchi

Takashi Tajiri, Hiroshi Yoshida, Yasuhiro Mamada, Nobuhiko Taniai, Shigeki Yokomuro, Yoshiaki Mizuguchi, Department of Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan

Author contributions: Tajiri T and Yoshida H wrote the paper. Mamada Y, Taniai N, Yokomuro S, and Mizuguchi Y performed research.

Correspondence to: Takashi Tajiri, Department of Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan. [tajirit@nms.ac.jp](mailto:tajirit@nms.ac.jp)

Telephone: 81-3-58146239 Fax: 81-3-56850989

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## Abstract

Cholangiocarcinoma is the second most common primary hepatic cancer. Despite advances in diagnostic techniques during the past decade, cholangiocarcinoma is usually encountered at an advanced stage. In this review, we describe the classification, diagnosis, and initial management of cholangiocarcinoma with obstructive jaundice.

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**Key words:** Cholangiocarcinoma; Obstructive jaundice; Diagnosis; Treatment; Initial management

**Peer reviewers:** Mitsuo Shimada, Professor, Department of Digestive and Pediatric Surgery, Tokushima University, Kuramoto 3-18-15, Tokushima 770-8503, Japan; Dusan M Jovanovic, Professor, Institute of Oncology, Institutski Put 4, Sremska Kamenica 21204, Serbia

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## INTRODUCTION

Cholangiocarcinoma is the second most common primary hepatic cancer. Despite advances in diagnostic techniques during the past decade, cholangiocarcinoma is usually encountered at an advanced stage. In this review, we

describe the classification, diagnosis, and initial management of cholangiocarcinoma with obstructive jaundice.

## CLASSIFICATION

Cholangiocarcinomas are epithelial neoplasms that originate from cholangiocytes and can occur at any level of the biliary tree. These lesions are broadly classified into intrahepatic cholangiocarcinoma, hilar cholangiocarcinoma, and distal extrahepatic bile duct tumors. Histologically, most cholangiocarcinomas (> 95%) are adenocarcinomas. They are pathologically classified into sclerosing, nodular, and papillary intraductal cancers<sup>[1]</sup>. A recent pathological classification applicable to both intrahepatic and extrahepatic cholangiocarcinomas divides these lesions into mass-forming (nodular), periductal-infiltrating (sclerosing), and intraductal-growing (papillary) cholangiocarcinomas<sup>[2]</sup>.

## DIAGNOSIS

### Laboratory data

Liver test abnormalities reflecting obstruction of the bile duct are usually observed. Strikingly elevated CA19-9 values in symptomatic patients usually signify advanced disease. Carcinoembryonic antigen (CEA) is also elevated in patients with cholangiocarcinoma, but is not diagnostic because of low sensitivity and specificity. Cholangitis and hepatolithiasis commonly lead to increased levels of tumor markers. Cholangiocarcinoma should not be diagnosed on the basis of laboratory data alone.

### Ultrasonography

Ultrasonography is the imaging technique of choice for the diagnosis of cholangiocarcinoma with obstructive jaundice. Visualization allows adequate diagnosis and staging in more than 90% of cases. The presence of dilated ducts without clear communications within a liver lobe indicates the extension of tumor into the segmental bile ducts. Ultrasonography is useful for evaluating the local extent of disease, but is of limited value for staging distant metastases. Intrahepatic cholangiocarcinomas may be identified as mass lesions, sometimes associated with bile duct dilatation proximal to the obstructing lesion. Tumor vascularity is an important characteristic that can be assessed by color Doppler ultrasonography. An abnormal pulsed Doppler signal obtained from the portal venous system due to severe narrowing or occlusion

strongly suggests major involvement and unresectable tumor. However, a normal pulsed Doppler signal does not exclude such involvement, if the tumor is contiguous with vessels showing interruption of the hyperechoic tumor-vessel interface<sup>[3,4]</sup>.

Endoscopic ultrasound (EUS) is useful for assessing the extent of disease and performing fine needle aspiration. Eloubeidi *et al*<sup>[5]</sup> reported that EUS-guided fine needle aspiration biopsy is useful for the diagnosis of suspected cholangiocarcinoma. The sensitivity, specificity, positive predictive value, negative predictive value, and accuracy were 86%, 100%, 100%, 57%, and 88%, respectively. EUS-guided fine needle aspiration of lymph nodes facilitates staging of disease in addition to visualization of the biliary tree<sup>[6]</sup>.

### Computed tomography (CT)

CT permits the identification of bile duct dilatation and assessment of the hepatic parenchyma and lymph nodes. However, the evaluation of horizontal spread by diagnostic imaging via the bile duct remains challenging in patients with cholangiocarcinoma, especially on conventional CT examination. Recently, the development of multidetector row CT scanners has permitted a reduction in the voxel size and facilitated rapid image reconstruction, enhancing the value of CT as an interactive diagnostic tool. Moreover, innovative methods for CT image reconstruction, including multiplanar reconstruction and three-dimensional images, were recently introduced for the visualization of biliary structures<sup>[7]</sup>. CT angiography has been demonstrated to be useful for the detection and assessment of vascular encasement<sup>[8-10]</sup>.

### Magnetic resonance imaging (MRI)

MRI with concurrent magnetic resonance cholangiopancreatography (MRCP) is the radiologic technique of choice for assessing the extent of disease<sup>[11,12]</sup>. The limitations of conventional imaging techniques have led to the increased use of MRCP, which is a noninvasive and highly accurate technique for the evaluation of patients with biliary obstruction. MRCP is optimally suited for the visualization of both intrahepatic and extrahepatic cholangiocarcinomas, which appear as hypointense lesions on T1-weighted images and hyperintense lesions on T2-weighted images. Images can be enhanced with the use of superparamagnetic iron or by delayed gadolinium enhancement<sup>[13,14]</sup>. The overall diagnostic accuracy for assessment of the level and cause of obstruction was 96.3% and 89.7%, respectively<sup>[12]</sup>. MR angiography can be used to evaluate vascular involvement<sup>[15]</sup>.

### Cholangiography

Before the development of MRCP, direct cholangiography was only technique for assessment of the biliary system. Direct cholangiography can be performed by either percutaneous transhepatic cholangiography or endoscopic retrograde cholangiography, and samples of the bile duct can be obtained<sup>[16,17]</sup>. Brushings are analyzed cytologically. In one study, the sensitivity, specificity, positive predictive value, negative predictive value, and accuracy of brush cytology were 75%, 100%, 100%, 12.5%, and 75.9%,

respectively. Biopsy specimens of the bile duct are examined histologically<sup>[18]</sup>. The diagnostic performance of transluminal forceps biopsy for malignant biliary obstructions was as follows: sensitivity, 78.4%; specificity, 100%; and accuracy, 79.2%<sup>[19]</sup>. Savader *et al*<sup>[20]</sup> compared the diagnostic accuracy of three different techniques for percutaneous transhepatic intraductal biopsy: brush cytology, clamshell forceps under choledochoscopic guidance, and clamshell forceps under fluoroscopic guidance. The choledochoscope-directed biopsy technique had the highest sensitivity and specificity among the three techniques, but was not significantly better than either the brush or fluoroscopic clamshell techniques ( $P > 0.10$ ). Multiple biopsies did not increase the overall sensitivity of intraductal biliary biopsy as a diagnostic technique. All three techniques were safe and easy to perform. In patients with malignant biliary obstruction, brush cytology was more sensitive for the diagnosis of cholangiocarcinoma than for the diagnosis of non-cholangiocarcinoma ( $P < 0.05$ ). The site of stenosis was unrelated to sensitivity and technical success ( $P > 0.05$ )<sup>[18,21]</sup>.

Rotational cine cholangiography is used to diagnose bile duct carcinoma. Rotational cine cholangiography is a reliable technique for detecting the confluence of the bile ducts, as well as for diagnosing the longitudinal extent of cancer spread along the bile duct wall<sup>[22]</sup>. Furukawa *et al*<sup>[23]</sup> evaluated the usefulness of three-dimensional cholangiography and rotating cine cholangiography for depicting the anatomy of the hilar bile duct and tumor extension, and for planning surgical procedures for hilar cholangiocarcinoma. Three-dimensional and cine cholangiography allowed accurate assessment of the biliary system in patients with hilar cholangiocarcinoma, facilitating the planning of surgery.

### Angiography

Angiography reveals the anatomy of the hepatic and biliary arteries. Angiography is a superb technique for the detection of vascular encasement. It is also useful for planning surgical procedures.

### Scintigraphy

**Technetium-99m galactosyl human serum albumin scintigraphy:** Technetium-99m-diethylenetriaminepentaacetic acid-galactosyl-human serum albumin (99mTc-GSA) is an analog ligand of asialoglycoprotein that binds specifically to asialoglycoprotein receptors (ASGP-R) residing in mammalian hepatocytes<sup>[24-26]</sup>. The hepatic uptake of 99mTc-GSA at 15 min or later reflects the receptor population or functional hepatocyte mass<sup>[27]</sup>.

Nanashima *et al*<sup>[28]</sup> studied the relation between morphological measurements of hepatic volume on CT and functional volume on 99mTc-GSA scintigraphy. There were no significant differences in the volume measurements between these two volumetric techniques. Volumetric measurement by 99mTc-GSA scintigraphy is useful for detecting changes in the functional volume of individual lobes of the liver and is a more dynamic method than the assessment of morphological changes on CT scanning.

We confirmed hemodynamic changes in the distribution of splenic venous flow in the liver, especially in the cirrhotic liver, and demonstrated the participation



of splenic venous flow in the regeneration or enlargement of the hepatic lobe by means of scintiphotosplenopography after percutaneous intrasplenic injection of  $^{99m}\text{Tc}$ -GSA. We concluded that splenic venous blood flow promotes liver fibrosis in the right lobe of the liver exposed to continuous damage, with gradually increasing flow into the left lobe, showing milder fibrosis<sup>[29]</sup>.

**Positron emission tomography (PET):** PET with  $^{18}\text{F}$ -fluorodeoxyglucose can be used to rule out metastatic disease, although the findings should be interpreted cautiously because of false positive results in inflammatory lesions; moreover a normal PET scan does not exclude cancer<sup>[30]</sup>.

## INITIAL MANAGEMENT

### Biliary drainage

In patients with obstructive jaundice who have cholangiocarcinoma, especially hilar cholangiocarcinoma, preoperative biliary drainage has been recommended to improve liver function before surgery and to reduce postoperative complications. Percutaneous transhepatic biliary drainage (PTBD) with multiple drains was previously the preferred method for the preoperative relief of obstructive jaundice. In patients with hilar cholangiocarcinoma, drainage is currently performed only for liver lobes that will remain after resection and for areas of segmental cholangitis. Endoscopic biliary drainage (EBD) is less invasive than PTBD. However, EBD has to be converted to PTBD in patients with segmental cholangitis, those requiring prolonged drainage, or those in whom the extent of longitudinal tumor extension is poorly defined<sup>[16]</sup>.

Kamiya *et al*<sup>[31]</sup> reported that impaired intestinal barrier function does not respond to external biliary drainage without bile replacement. Bile replacement during external biliary drainage can restore intestinal barrier function in patients with biliary obstruction, primarily by promoting the repair of physical damage to the intestinal mucosa. Koivukangas *et al*<sup>[32]</sup> reported that cell protein synthesis is disturbed earlier than cell dynamics in obstructive jaundice. Decreased baseline skin-collagen synthesis is partly restored by the resolution of jaundice<sup>[33]</sup>.

We previously reported that elevated serum collagen IV is a feature of malignant obstructive jaundice commonly associated with prolonged bilirubin clearance, and a useful indicator of clinical course, postoperative morbidity, and mortality in patients with malignant obstructive jaundice<sup>[34]</sup>.

The procedure of choice for biliary drainage before major hepatectomy in patients with obstructive jaundice remains controversial, i.e. selective biliary drainage of only the future remnant liver or total biliary drainage. Ishizawa *et al*<sup>[35]</sup> reported that selective biliary drainage is superior to total biliary drainage for promoting hypertrophy of the future remnant liver in patients undergoing portal vein embolization and for guaranteeing good liver function before major hepatectomy. Hochwald *et al*<sup>[36]</sup> showed that preoperative biliary stenting in proximal cholangiocarcinoma increases the incidence of contaminated bile and postoperative infectious complications. Cherqui *et al*<sup>[37]</sup> found that major liver resection without preoperative biliary drainage is

a safe procedure in most patients with obstructive jaundice. Recovery of hepatic synthetic function is identical to that of patients without jaundice. Transfusion requirements and the incidence of postoperative complications, especially bile leaks and subphrenic collections, are higher in jaundiced patients. Pitt *et al*<sup>[38]</sup> concluded that preoperative PTBD does not reduce operative risk but does increase hospital costs and, therefore, discouraged routine use. The indication for preoperative biliary stenting in patients with obstructive jaundice remains controversial.

### Portal vein embolization (PVE)

PVE before hepatectomy is designed to induce atrophy of the embolized lobe scheduled to be resected, while inducing compensatory hypertrophy of preserved lobe<sup>[39,40]</sup>. PVE with compensatory contralateral hypertrophy of the future liver remnant has been performed to enable extended hepatectomy (resection of  $\geq 5$  hepatic segments)<sup>[41,42]</sup>. We have reported on combined embolization of the hepatic artery and portal vein<sup>[43]</sup>.

### Biliary ablation

Selective biliary infusion of ethanol can be performed safely without serious complications, inducing lobar ablation with contralateral hypertrophy of the liver<sup>[44,45]</sup>.

### Operation

Surgical resection has been the mainstay of curative treatment for cholangiocarcinoma<sup>[46]</sup>. Major hepatectomy with systematic nodal dissection is associated with a good chance of prolonged survival in patients with carcinoma involving the hepatic hilus, including those with advanced disease<sup>[47,48]</sup>. Extended hemihepatectomy, with or without pancreatoduodenectomy, plus extrahepatic bile duct resection and regional lymphadenectomy has recently been recognized as the standard curative treatment for hilar bile duct cancer. Pancreatoduodenectomy is the choice of treatment for middle and distal bile duct cancer. Major hepatectomy with pancreatoduodenectomy (hepatopancreatoduodenectomy) has been performed in selected patients with widespread disease. Miyazaki *et al*<sup>[49,50]</sup> reported that parenchyma-preserving hepatectomy could result in curative resection and improve the outcomes of patients with hilar cholangiocarcinoma localized to the hepatic duct confluence who do not require vascular resection. Less-extensive procedures were also beneficial for less-advanced disease if the resection margins were free of tumor. Even with carefully selected treatment with curative intent, the 5-year survival of patients with cholangiocarcinoma ranges from 30% to 40%. A tumor-free surgical margin is the best predictor of survival. Several staging schemes have been proposed, but none correlates with resectability. Lymph node involvement is also a predictor of survival<sup>[48,51]</sup>.

### Adjuvant therapy

Neoadjuvant therapy with several types of treatment, including radiation, photodynamic therapy and chemotherapy, provides no clear benefit<sup>[52,53]</sup>.

### Palliative therapy

Previously, plastic endoprostheses were placed for the

palliative treatment of malignant biliary obstruction<sup>[54-57]</sup>. An expandable metal stent (EMS) is used to provide palliation in patients with malignant obstructive jaundice<sup>[58]</sup>. EMSs have been compared with plastic endoprotheses for the palliative treatment of malignant obstructive jaundice<sup>[59,60]</sup>. EMSs are inserted percutaneously<sup>[61-63]</sup> or endoscopically<sup>[59,64]</sup>.

Biliary stent placement combined with local tumor therapy, such as brachytherapy, extra-radiation therapy, or arterial infusion chemotherapy, can prolong the survival time of patients with malignant biliary obstruction<sup>[65-68]</sup>. Mezawa *et al*<sup>[69]</sup> developed a new PTBD tube coated with carboplatin.

Intrahepatic cholangiojejunostomy has been performed in patients with unresectable malignant biliary obstruction<sup>[70-72]</sup>. Endoscopic stenting for the management of this condition costs significantly less than surgical treatment<sup>[73]</sup>. Recently, EUS-guided hepaticogastrostomy has been performed<sup>[74]</sup>.

### Transplantation

Although early survival after transplantation for cholangiocarcinoma is excellent, high recurrence rates have generally discouraged liver replacement. Recent findings, however, have lead to a resurgence in orthotopic liver transplantation for unresectable, albeit locally contained cholangiocarcinoma. Becker *et al*<sup>[75]</sup> reported a series of 280 patients with cholangiocarcinoma who received orthotopic liver transplantation. After a median follow-up of 452 d, the survival rates at 1 and 5 years were 74% and 38%, respectively. Heimbach *et al*<sup>[76]</sup> reported on 56 patients who were treated for unresectable, stage I and II perihilar cholangiocarcinoma. Disease-free survival at 5 years was excellent (82%) in carefully selected patients who underwent neoadjuvant external-beam radiation therapy, transcatheter intrabiliary radiation, chemotherapy, and pretransplant-staging exploratory laparotomy. Neoadjuvant chemoradiotherapy with liver transplantation produces excellent results for selected patients with localized, regional node negative, hilar cholangiocarcinoma<sup>[76,77]</sup>.

### REFERENCES

- Weinbren K, Mutum SS. Pathological aspects of cholangiocarcinoma. *J Pathol* 1983; **139**: 217-238
- Lim JH, Park CK. Pathology of cholangiocarcinoma. *Abdom Imaging* 2004; **29**: 540-547
- Bloom CM, Langer B, Wilson SR. Role of US in the detection, characterization, and staging of cholangiocarcinoma. *Radiographics* 1999; **19**: 1199-1218
- Smits NJ, Reeders JW. Imaging and staging of biliopancreatic malignancy: role of ultrasound. *Ann Oncol* 1999; **10** Suppl 4: 20-24
- Eloubeidi MA, Chen VK, Jhala NC, Eltoum IE, Jhala D, Chhieng DC, Syed SA, Vickers SM, Mel Wilcox C. Endoscopic ultrasound-guided fine needle aspiration biopsy of suspected cholangiocarcinoma. *Clin Gastroenterol Hepatol* 2004; **2**: 209-213
- Fritscher-Ravens A, Broering DC, Sriram PV, Topalidis T, Jaekle S, Thonke F, Soehendra N. EUS-guided fine-needle aspiration cytodiagnosis of hilar cholangiocarcinoma: a case series. *Gastrointest Endosc* 2000; **52**: 534-540
- Unno M, Okumoto T, Katayose Y, Rikiyama T, Sato A, Motoi F, Oikawa M, Egawa S, Ishibashi T. Preoperative assessment of hilar cholangiocarcinoma by multidetector row computed tomography. *J Hepatobiliary Pancreat Surg* 2007; **14**: 434-440
- Teefey SA, Baron RL, Rohrmann CA, Shuman WP, Freeny PC. Sclerosing cholangitis: CT findings. *Radiology* 1988; **169**: 635-639
- Zhang Y, Uchida M, Abe T, Nishimura H, Hayabuchi N, Nakashima Y. Intrahepatic peripheral cholangiocarcinoma: comparison of dynamic CT and dynamic MRI. *J Comput Assist Tomogr* 1999; **23**: 670-677
- Tillich M, Mischinger HJ, Preisegger KH, Rabl H, Szolar DH. Multiphasic helical CT in diagnosis and staging of hilar cholangiocarcinoma. *AJR Am J Roentgenol* 1998; **171**: 651-658
- Craanen ME, van Waesberghe JH, van der Peet DL, Loffeld RJ, Cuesta MA, Mulder CJ. Endoscopic ultrasound in patients with obstructive jaundice and inconclusive ultrasound and computer tomography findings. *Eur J Gastroenterol Hepatol* 2006; **18**: 1289-1292
- Vaishali MD, Agarwal AK, Upadhyaya DN, Chauhan VS, Sharma OP, Shukla VK. Magnetic resonance cholangiopancreatography in obstructive jaundice. *J Clin Gastroenterol* 2004; **38**: 887-890
- Braga HJ, Imam K, Bluemke DA. MR imaging of intrahepatic cholangiocarcinoma: use of ferumoxides for lesion localization and extension. *AJR Am J Roentgenol* 2001; **177**: 111-114
- Peterson MS, Murakami T, Baron RL. MR imaging patterns of gadolinium retention within liver neoplasms. *Abdom Imaging* 1998; **23**: 592-599
- Lee MG, Park KB, Shin YM, Yoon HK, Sung KB, Kim MH, Lee SG, Kang EM. Preoperative evaluation of hilar cholangiocarcinoma with contrast-enhanced three-dimensional fast imaging with steady-state precession magnetic resonance angiography: comparison with intraarterial digital subtraction angiography. *World J Surg* 2003; **27**: 278-283
- Maguchi H, Takahashi K, Katanuma A, Osanai M, Nakahara K, Matuzaki S, Urata T, Iwano H. Preoperative biliary drainage for hilar cholangiocarcinoma. *J Hepatobiliary Pancreat Surg* 2007; **14**: 441-446
- Dillon E, Peel AL, Parkin GJ. The diagnosis of primary bile duct carcinoma (cholangiocarcinoma) in the jaundiced patient. *Clin Radiol* 1981; **32**: 311-317
- Tsai CC, Mo LR, Chou CY, Han SJ, Lin RC, Kuo JY, Chang KK. Percutaneous transhepatic transluminal forceps biopsy in obstructive jaundice. *Hepatogastroenterology* 1997; **44**: 770-773
- Jung GS, Huh JD, Lee SU, Han BH, Chang HK, Cho YD. Bile duct: analysis of percutaneous transluminal forceps biopsy in 130 patients suspected of having malignant biliary obstruction. *Radiology* 2002; **224**: 725-730
- Savader SJ, Prescott CA, Lund GB, Osterman FA. Intraductal biopsy: comparison of three techniques. *J Vasc Interv Radiol* 1996; **7**: 743-750
- Xing GS, Geng JC, Han XW, Dai JH, Wu CY. Endobiliary brush cytology during percutaneous transhepatic cholangiodrainage in patients with obstructive jaundice. *Hepatobiliary Pancreat Dis Int* 2005; **4**: 98-103
- Miura F, Asano T, Okazumi S, Takayama W, Shinohara Y, Makino H, Sugaya M, Ochiai T, Isono K. Rotational cine cholangiography: evaluation for use in diagnosing bile duct carcinoma. *AJR Am J Roentgenol* 1999; **173**: 1043-1048
- Furukawa H, Sano K, Kosuge T, Shimada K, Yamamoto J, Iwata R, Moriyama N. Hilar cholangiocarcinoma evaluated by three-dimensional CT cholangiography and rotating cine cholangiography. *Hepatogastroenterology* 2000; **47**: 615-620
- Ashwell G, Steer CJ. Hepatic recognition and catabolism of serum glycoproteins. *JAMA* 1981; **246**: 2358-2364
- Stockert RJ, Morell AG. Hepatic binding protein: the galactose-specific receptor of mammalian hepatocytes. *Hepatology* 1983; **3**: 750-757
- Chang TM, Chang CL. Hepatic uptake of asialoglycoprotein is different among mammalian species due to different receptor distribution. *Biochim Biophys Acta* 1988; **942**: 57-64
- Matsuzaki S, Onda M, Tajiri T, Kim DY. Hepatic lobar differences in progression of chronic liver disease: correlation of asialoglycoprotein scintigraphy and hepatic functional reserve. *Hepatology* 1997; **25**: 828-832

- 28 **Nanashima A**, Yamaguchi H, Shibasaki S, Morino S, Ide N, Takeshita H, Tsuji T, Sawai T, Nakagoe T, Nagayasu T, Ogawa Y. Relationship between CT volumetry and functional liver volume using technetium-99m galactosyl serum albumin scintigraphy in patients undergoing preoperative portal vein embolization before major hepatectomy: a preliminary study. *Dig Dis Sci* 2006; **51**: 1190-1195
- 29 **Mineta S**, Yoshida H, Mamada Y, Taniai N, Mizuguchi Y, Akimaru K, Kumita S, Kumazaki T, Tajiri T. Changes in distribution of splenic venous flow in the patients with cirrhotic liver. *Hepatogastroenterology* 2005; **52**: 1313-1319
- 30 **Fritscher-Ravens A**, Bohuslavizki KH, Broering DC, Jenicke L, Schafer H, Buchert R, Rogiers X, Clausen M. FDG PET in the diagnosis of hilar cholangiocarcinoma. *Nucl Med Commun* 2001; **22**: 1277-1285
- 31 **Kamiya S**, Nagino M, Kanazawa H, Komatsu S, Mayumi T, Takagi K, Asahara T, Nomoto K, Tanaka R, Nimura Y. The value of bile replacement during external biliary drainage: an analysis of intestinal permeability, integrity, and microflora. *Ann Surg* 2004; **239**: 510-517
- 32 **Koivukangas V**, Oikarinen A, Risteli J, Haukipuro K. Effect of jaundice and its resolution on wound re-epithelization, skin collagen synthesis, and serum collagen propeptide levels in patients with neoplastic pancreaticobiliary obstruction. *J Surg Res* 2005; **124**: 237-243
- 33 **Mann DV**, Lam WW, Magnus Hjelm N, So NM, Yeung DK, Metreweli C, Lau WY. Biliary drainage for obstructive jaundice enhances hepatic energy status in humans: a 31-phosphorus magnetic resonance spectroscopy study. *Gut* 2002; **50**: 118-122
- 34 **Mizuguchi Y**, Yoshida H, Yokomuro S, Arima Y, Mamada Y, Taniai N, Akimaru K, Tajiri T. Collagen IV is a predictor for clinical course in patients with malignant obstructive jaundice. *Hepatogastroenterology* 2005; **52**: 672-677
- 35 **Ishizawa T**, Hasegawa K, Sano K, Imamura H, Kokudo N, Makuuchi M. Selective versus total biliary drainage for obstructive jaundice caused by a hepatobiliary malignancy. *Am J Surg* 2007; **193**: 149-154
- 36 **Hochwald SN**, Burke EC, Jarnagin WR, Fong Y, Blumgart LH. Association of preoperative biliary stenting with increased postoperative infectious complications in proximal cholangiocarcinoma. *Arch Surg* 1999; **134**: 261-266
- 37 **Cherqui D**, Benoist S, Malassagne B, Humeres R, Rodriguez V, Fagniez PL. Major liver resection for carcinoma in jaundiced patients without preoperative biliary drainage. *Arch Surg* 2000; **135**: 302-308
- 38 **Pitt HA**, Gomes AS, Lois JF, Mann LL, Deutsch LS, Longmire WP Jr. Does preoperative percutaneous biliary drainage reduce operative risk or increase hospital cost? *Ann Surg* 1985; **201**: 545-553
- 39 **Takayama T**, Makuuchi M. Preoperative portal vein embolization: is it useful? *J Hepatobiliary Pancreat Surg* 2004; **11**: 17-20
- 40 **Makuuchi M**, Thai BL, Takayasu K, Takayama T, Kosuge T, Gunven P, Yamazaki S, Hasegawa H, Ozaki H. Preoperative portal embolization to increase safety of major hepatectomy for hilar bile duct carcinoma: a preliminary report. *Surgery* 1990; **107**: 521-527
- 41 **Abdalla EK**, Barnett CC, Doherty D, Curley SA, Vauthey JN. Extended hepatectomy in patients with hepatobiliary malignancies with and without preoperative portal vein embolization. *Arch Surg* 2002; **137**: 675-680; discussion 680-681
- 42 **Farges O**, Belghiti J, Kianmanesh R, Regimbeau JM, Santoro R, Vilgrain V, Denys A, Sauvanet A. Portal vein embolization before right hepatectomy: prospective clinical trial. *Ann Surg* 2003; **237**: 208-217
- 43 **Mamada Y**, Tajiri T, Akimaru K, Yoshida H, Taniai N. Long-term prognosis after arterio-portal embolization for hepatocellular carcinoma. *Hepatogastroenterology* 2004; **51**: 234-236
- 44 **Kyokane T**, Nagino M, Oda K, Nimura Y. An experimental study of selective intrahepatic biliary ablation with ethanol. *J Surg Res* 2001; **96**: 188-196
- 45 **Shimizu T**, Yoshida H, Mamada Y, Taniai N, Matsumoto S, Mizuguchi Y, Yokomuro S, Arima Y, Akimaru K, Tajiri T. Postoperative bile leakage managed successfully by intrahepatic biliary ablation with ethanol. *World J Gastroenterol* 2006; **12**: 3450-3452
- 46 **Khan SA**, Davidson BR, Goldin R, Pereira SP, Rosenberg WM, Taylor-Robinson SD, Thillainayagam AV, Thomas HC, Thursz MR, Wasan H. Guidelines for the diagnosis and treatment of cholangiocarcinoma: consensus document. *Gut* 2002; **51** Suppl 6: VI1-VI9
- 47 **Liu CL**, Fan ST, Lo CM, Tso WK, Lam CM, Wong J. Improved operative and survival outcomes of surgical treatment for hilar cholangiocarcinoma. *Br J Surg* 2006; **93**: 1488-1494
- 48 **Kosuge T**, Yamamoto J, Shimada K, Yamasaki S, Makuuchi M. Improved surgical results for hilar cholangiocarcinoma with procedures including major hepatic resection. *Ann Surg* 1999; **230**: 663-671
- 49 **Miyazaki M**, Ito H, Nakagawa K, Ambiru S, Shimizu H, Okaya T, Shinmura K, Nakajima N. Parenchyma-preserving hepatectomy in the surgical treatment of hilar cholangiocarcinoma. *J Am Coll Surg* 1999; **189**: 575-583
- 50 **Miyazaki M**, Ito H, Nakagawa K, Ambiru S, Shimizu H, Shimizu Y, Okuno A, Nozawa S, Nukui Y, Yoshitomi H, Nakajima N. Segments I and IV resection as a new approach for hepatic hilar cholangiocarcinoma. *Am J Surg* 1998; **175**: 229-231
- 51 **Yoshida T**, Matsumoto T, Sasaki A, Morii Y, Aramaki M, Kitano S. Prognostic factors after pancreaticoduodenectomy with extended lymphadenectomy for distal bile duct cancer. *Arch Surg* 2002; **137**: 69-73
- 52 **Heron DE**, Stein DE, Eschelman DJ, Topham AK, Waterman FM, Rosato EL, Alden M, Anne PR. Cholangiocarcinoma: the impact of tumor location and treatment strategy on outcome. *Am J Clin Oncol* 2003; **26**: 422-428
- 53 **Serafini FM**, Sachs D, Bloomston M, Carey LC, Karl RC, Murr MM, Rosemurgy AS. Location, not staging, of cholangiocarcinoma determines the role for adjuvant chemoradiation therapy. *Am Surg* 2001; **67**: 839-843; discussion 843-844
- 54 **Siegel JH**, Pullano W, Kodsi B, Cooperman A, Ramsey W. Optimal palliation of malignant bile duct obstruction: experience with endoscopic 12 French prostheses. *Endoscopy* 1988; **20**: 137-141
- 55 **Siegel JH**, Daniel SJ. Endoscopic and fluoroscopic transpapillary placement of a large caliber biliary endoprosthesis. *Am J Gastroenterol* 1984; **79**: 461-465
- 56 **Coons HG**, Carey PH. Large-bore, long biliary endoprosthesis (biliary stents) for improved drainage. *Radiology* 1983; **148**: 89-94
- 57 **Gouma DJ**, Wesdorp RI, Oostenbroek RJ, Soeters PB, Greep JM. Percutaneous transhepatic drainage and insertion of an endoprosthesis for obstructive jaundice. *Am J Surg* 1983; **145**: 763-768
- 58 **Men S**, Hekimoglu B, Kaderoglu H, Pinar A, Conkbayir I, Soylu SO, Bulut A, Yandakci K, Baran I, Aran Y. Palliation of malignant obstructive jaundice. Use of self-expandable metal stents. *Acta Radiol* 1996; **37**: 259-266
- 59 **Soderlund C**, Linder S. Covered metal versus plastic stents for malignant common bile duct stenosis: a prospective, randomized, controlled trial. *Gastrointest Endosc* 2006; **63**: 986-995
- 60 **Wagner HJ**, Knyrim K. Relief of malignant obstructive jaundice by endoscopic or percutaneous insertion of metal stents. *Bildgebung* 1993; **60**: 76-82
- 61 **Indar AA**, Lobo DN, Gilliam AD, Gregson R, Davidson I, Whittaker S, Doran J, Rowlands BJ, Beckingham IJ. Percutaneous biliary metal wall stenting in malignant obstructive jaundice. *Eur J Gastroenterol Hepatol* 2003; **15**: 915-919
- 62 **Yoshida H**, Mamada Y, Taniai N, Mizuguchi Y, Shimizu T, Yokomuro S, Aimoto T, Nakamura Y, Uchida E, Arima Y, Watanabe M, Uchida E, Tajiri T. One-step palliative treatment method for obstructive jaundice caused by unresectable malignancies by percutaneous transhepatic insertion of an expandable metallic stent. *World J Gastroenterol* 2006; **12**: 2423-2426

- 63 **Tsai CC**, Mo LR, Lin RC, Kuo JY, Chang KK, Yeh YH, Yang SC, Yueh SK, Tsai HM, Yu CY. Self-expandable metallic stents in the management of malignant biliary obstruction. *J Formos Med Assoc* 1996; **95**: 298-302
- 64 **Yoon WJ**, Lee JK, Lee KH, Lee WJ, Ryu JK, Kim YT, Yoon YB. A comparison of covered and uncovered Wallstents for the management of distal malignant biliary obstruction. *Gastrointest Endosc* 2006; **63**: 996-1000
- 65 **Kocak Z**, Ozkan H, Adli M, Garipagaoglu M, Kurtman C, Cakmak A. Intraluminal brachytherapy with metallic stenting in the palliative treatment of malignant obstruction of the bile duct. *Radiat Med* 2005; **23**: 200-207
- 66 **Ishii H**, Furuse J, Nagase M, Kawashima M, Ikeda H, Yoshino M. Relief of jaundice by external beam radiotherapy and intraluminal brachytherapy in patients with extrahepatic cholangiocarcinoma: results without stenting. *Hepatogastroenterology* 2004; **51**: 954-957
- 67 **Bowling TE**, Galbraith SM, Hatfield AR, Solano J, Spittle MF. A retrospective comparison of endoscopic stenting alone with stenting and radiotherapy in non-resectable cholangiocarcinoma. *Gut* 1996; **39**: 852-855
- 68 **Hoevels J**, Lunderquist A, Ihse I. Percutaneous transhepatic intubation of bile ducts for combined internal-external drainage in preoperative and palliative treatment of obstructive jaundice. *Gastrointest Radiol* 1978; **3**: 23-31
- 69 **Mezawa S**, Homma H, Sato T, Doi T, Miyanishi K, Takada K, Kukitsu T, Murase K, Yoshizaki N, Takahashi M, Sakamaki S, Niitsu Y. A study of carboplatin-coated tube for the unresectable cholangiocarcinoma. *Hepatology* 2000; **32**: 916-923
- 70 **Suzuki S**, Kurachi K, Yokoi Y, Tsuchiya Y, Okamoto K, Okumura T, Inaba K, Konno H, Nakamura S. Intrahepatic cholangiojejunostomy for unresectable malignant biliary tumors with obstructive jaundice. *J Hepatobiliary Pancreat Surg* 2001; **8**: 124-129
- 71 **Guthrie CM**, Banting SW, Garden OJ, Carter DC. Segment III cholangiojejunostomy for palliation of malignant hilar obstruction. *Br J Surg* 1994; **81**: 1639-1641
- 72 **Traynor O**, Castaing D, Bismuth H. Left intrahepatic cholangio-enteric anastomosis (round ligament approach): an effective palliative treatment for hilar cancers. *Br J Surg* 1987; **74**: 952-954
- 73 **Martin RC 2nd**, Vitale GC, Reed DN, Larson GM, Edwards MJ, McMasters KM. Cost comparison of endoscopic stenting vs surgical treatment for unresectable cholangiocarcinoma. *Surg Endosc* 2002; **16**: 667-670
- 74 **Bories E**, Pesenti C, Caillol F, Lopes C, Giovannini M. Transgastric endoscopic ultrasonography-guided biliary drainage: results of a pilot study. *Endoscopy* 2007; **39**: 287-291
- 75 **Becker NS**, Rodriguez JA, Barshe NR, O'Mahony CA, Goss JA, Aloia TA. Outcomes Analysis for 280 Patients with Cholangiocarcinoma Treated with Liver Transplantation Over an 18-year Period. *J Gastrointest Surg* 2008; **12**: 117-122
- 76 **Heimbach JK**, Gores GJ, Haddock MG, Alberts SR, Nyberg SL, Ishitani MB, Rosen CB. Liver transplantation for unresectable perihilar cholangiocarcinoma. *Semin Liver Dis* 2004; **24**: 201-207
- 77 **Thelen A**, Neuhaus P. Liver transplantation for hilar cholangiocarcinoma. *J Hepatobiliary Pancreat Surg* 2007; **14**: 469-475

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## GASTRIC CANCER

# Investigation of transcriptional gene silencing and mechanism induced by shRNAs targeted to *RUNX3* *in vitro*

Xue-Zhi Feng, Xiu-Sheng He, Ying-Zhi Zhuang, Qiao Luo, Jun-Hao Jiang, Shuai Yang, Xue-Fang Tang, Ju-Lin Liu, Tao Chen

Xue-Zhi Feng, Xiu-Sheng He, Qiao Luo, Jun-Hao Jiang, Shuai Yang, Xue-Fang Tang, Ju-Lin Liu, Tao Chen, Cancer Research Institute, Nanhua University of South China, Hengyang 421001, Hunan Province, China

Xue-Zhi Feng, Ying-Zhi Zhuang, Department of Oncology, First Affiliated Hospital of Nanhua University of South China, Hengyang 421001, Hunan Province, China

**Author contributions:** Feng XZ designed and performed the research and wrote the paper; He XS contributed the acquisition of funding; He XS and Zhuang YZ guided the research and revised the paper critically for intellectual content; Qiao L contributed technology and materials support; Jiang JH, Yang S, Tang XF, Liu JL and Chen T checked the statistical analysis of data.

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**Correspondence to:** Xiu-Sheng He, MD, Cancer Research Institute, Nanhua University of South China, Hengyang 421001, Hunan Province, China. [hexiusheng@hotmail.com](mailto:hexiusheng@hotmail.com)

Telephone: +86-734-8281725 Fax: +86-734-8281305

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## Abstract

**AIM:** To investigate transcriptional gene silencing induced by short hairpin RNAs (shRNAs) that target gene promoter regions of *RUNX3* gene, and whether shRNAs homologous to DNA sequences may serve as initiators for methylation.

**METHODS:** According to the principle of RNAi design, pSilencer3.1-H1-shRNA/*RUNX3* expression vector was constructed. The recombinant plasmid shRNA was transfected into human stomach carcinoma cell line SGC7901 with Lipofectamine 2000. Then, the positive cell clones were screened by G418. The mRNA and protein expression level of *RUNX3* in the stable transfected cell line SGC7901 were determined by RT-PCR, Western blotting and immunocytochemistry. Characteristics of the cell lines including SGC7901, pSilencer3.1-H1/SGC7901 and pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 were analyzed with growth curves, clone formation rate and cell-cycle distribution. The activated level of *RUNX3* was examined after treatment with the different density of 5'-aza-2'-deoxycytidine (5-Aza-CdR) by using semi-quantitative RT-PCR and Western blotting.

**RESULTS:** In the cell line SGC7901 transfected with pSilencer3.1-H1-shRNA/*RUNX3*, mRNA and protein

expression of the *RUNX3* gene was lost identified by RT-PCR, Western blotting and immunocytochemistry assay. The growth of pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells without expression of *RUNX3* was the fastest ( $P < 0.05$ ), its rate of clone formation was the highest ( $P < 0.01$ ), and the cell distribution in G<sub>0</sub>/G<sub>1</sub> and S/M phases was lowest and highest, respectively ( $P < 0.05$ ), compared with that of the transfected pSilencer3.1-H1 and non-transfected cells. Through RT-PCR and Western blot assay, inactivated *RUNX3* could not be reactivated by 5-Aza-CdR.

**CONCLUSION:** We found that, although shRNAs targeted to gene promoter regions of *RUNX3* could effectively induce transcriptional repression with chromatic changes characteristic of inactivation promoters, this was independent of DNA methylation, and the presence of RNA-dependent transcriptional silencing showed that RNA-directed DNA methylation might be an existing gene regulatory mechanism relative to the methylated in humans.

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**Key words:** RNA interference; Short hairpin RNAs; Promoter; DNA methylation; *RUNX3*; Stomach carcinoma

**Peer reviewer:** Yuan Yuan, Professor, Cancer Institute of China Medical University, 155 North Nanjing Street, Heping District, Shenyang 110001, Liaoning Province, China

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## INTRODUCTION

The human runt-related transcription factor 3 gene (*RUNX3*), a novel tumor suppressor, was originally cloned as *AML2* in human leukocythemia by Levanon in 1994 and is located on human chromosome 1p36.1<sup>[1]</sup>. *RUNX3* contains two promoters, p1 and p2, one at the beginning of exon 1 and the other in front of exon 2. The mRNA expression of *RUNX3* comes from transcription by p2, which has a high GC content due to a large conserved

CpG island around it and contains Sp1 sites and a CCAAT box without a TATA box. Recently, *RUNX3* has been considered to be a vital gene in the occurrence and development of gastric carcinoma<sup>[2]</sup>.

Multiple genes participate in the occurrence and development of gastric carcinoma, which is at the top list of malignant tumors in China. Deactivation of anti-oncogenes is more frequent than activation of proto-oncogenes in the gastric carcinogenesis process. Anti-oncogenes are devitalized by chromosomal absence, gene mutation, and aberrant CpG island hypermethylation of gene promoters. Moreover, the data have suggested that, due to transcription termination of *RUNX3* by aberrant CpG island hypermethylation at the promoter's 5'-terminal CpG island area, the active level of *RUNX3* decreased by 40% and 90%, respectively, in early and advanced gastric cancer. However, the inactivation mechanism of the aberrant CpG island hypermethylation is still unclear<sup>[3-6]</sup>.

The phenomenon by which small interference RNA molecules that target gene promoter regions can induce transcriptional gene silencing in a DNA cytosine methylation-dependent manner (RNA-directed DNA methylation, RdDM) was initially discovered by Wassenegger *et al* in 1994 in plants infected with epiviruses<sup>[7]</sup>. This suggested that RdDM might have an important role in the loss of expression of tumor suppressor genes, and *RUNX3*. We constructed a recombinant plasmid, which expressed short hairpin RNAs (shRNAs) complementary to the CpG island, including the promoter of the human *RUNX3* gene, to investigate transcriptional gene silencing and the mechanism mediated by shRNAs that target gene promoter regions of *RUNX3* in human stomach carcinoma cell lines.

## MATERIALS AND METHODS

### Design and synthesis of shRNAs

shRNAs that target the nucleotide sequence (GCCACTT-GATTCTGGAGGA) in the promoter of human *RUNX3* (NCBI, accession number AL023096) were designed by Ambion using restriction enzyme sites *Bam*H I and *Hind*III, and synthesized by Shanghai Biological Engineering Limited Company (China). The oligonucleotide sequence was as follows: 5'-GATCCGCCACTTGATTCTGGAGGATTCAGAGATCCTCCAGAATCAAGTGGCG GTTTTTTGGAAA-3' and 5'-AGCTTTTCCAAAAAACCGCCACTTGATTCTGGAGGATCTCTTGAATCCTCCAGAATCAAGTGGCG-3'.

### Construction of recombinant plasmid

The eukaryotic expression vector pSilencer3.1-H1 (Ambion), which was kindly provided by Doctor Lei XiaoYong (Institute of Pharmacologic Research, Nanhua University), was digested by *Bam*H I and *Hind*III restriction enzymes (MBI). The digested products, which were reclaimed using Gel-reclamation Kit (Omega), and the oligonucleotide sequence were ligated by T4 DNA ligase (MBI) at 4°C overnight to generate the recombinant pSilencer3.1-H1-shRNA/*RUNX3*. Then, the recombinant plasmid was validated by restriction enzymes digestion and sequencing.

### Cell line and cell culture

Human gastric cancer SGC-7901, MKN-28, SGC7901, MGC803 and BGC823 cell lines, which were first cultured by Academia Sinica (Shanghai, China), were purchased from the Central South University (Hunan, China). All cells were cultured with RPMI 1640 (Gibco) medium and supplemented with 100 mL/L calf serum (Sijiqing, Hangzhou, China) at 37°C in a humidified atmosphere containing 50 mL/L CO<sub>2</sub>.

### Transfection of plasmids

Fifty thousand SGC-7901 cells were seeded into each well in six-well plates and grown overnight. The medium was replaced with complete medium without fetal bovine serum (FBS). The recombinant pSilencer3.1-H1-shRNA/*RUNX3* and the empty pSilencer3.1-H1 were transfected into SGC7901 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The medium was replaced with a fresh medium of calf serum (150 mL/L) after 5 h transfection. Two days later, the transfected cells were selected by G418 (200 µg/mL) (Huamei Biotechnology Company, Beijing, China) until positive clones were discovered after 10 d. The cells were cultured and finally selected by G418 (100 µg/mL) for a further 10 d. Single clones were selected to build a stable transfected cell line.

### Chemical reagents

5'-Aza-2'-deoxycytidine (5-Aza-CdR), which is a DNA methyltransferase inhibitor, was purchased from Sigma (USA). It was diluted with PBS (pH 6.8), and finally prepared as 10 µmol/L mother liquor by filtrated sterilization. The mother liquor was stored at -70°C.

### Total RNA extraction and cDNA preparation

Total RNA was extracted from each sample using the Total RNA Extract Kit (Omega) following the manufacturer's instructions. The concentration of RNA was measured by spectrophotometry. Total RNA was reverse-transcribed to cDNA with reverse transcriptase (RT) reagents (Promega) according to the manufacturer's protocol. Briefly, the RT reaction was carried out in a final volume of 20 µL that contained 4 µL 25 mmol/L MgCl<sub>2</sub>, 2 µL 10 × RT buffer, 2 µL 10 mmol/L dNTP mixed liquor, 1 µL RNase inhibitor, 1 µL Oligo (dT) 15 primers, 15 U AMV retroviralase, 8.37 µL water free from enzyme, and 2 µL total RNA. The mixture was incubated at 70°C for 10 min and 42°C for 60 min, and RT was inactivated by heating at 95°C for 5 min, followed by incubation at 4°C for 5 min.

### Determination of growth curve

SGC-7901, pSilencer3.1-H1/SGC7901 and pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells were suspended in RPMI1640 medium and seeded in a 24-well plate at a density of  $2.0 \times 10^4$  cells/well. Culture medium was changed every 2 d. The number of cells was counted consecutively for 8 d. Each experiment was done in triplicate.

### Colony formation

Single cells were mixed with the semi-solid agar (3 g/L) with growth medium and plated on a basal agar layer

(6 g/L) in three six-well plates. The cells were cultured at 37°C in a humidified atmosphere that contained 50 mL/L CO<sub>2</sub> for 10 d. Then, colonies that contained > 100 cells in soft agar were counted under an inverted microscope and the rate of colony formation was calculated by the mean percentage of colonies.

#### Flow cytometry (FCM) with propidium iodide (PI) staining

The cells were passaged at a density of  $5.0 \times 10^5$ /mL. After 4 h, they were treated with serum-free medium for 12 h, followed by treatment with medium that contained calf serum (100 mL/L) for 24 h. Cells were collected by trypsinization and prepared as a single cell suspension by mechanical blowing with PBS, washed with cold PBS twice, and fixed with 700 mL/L alcohol at 4°C for 24 h. Fixed cells were washed with PBS and stained with PI (50 µg/mL in PBS) for 30 min at room temperature in the dark. DNA content in PI-stained cells was detected by FCM.

#### Primers and real-time RT-PCR

As shown in Table 1, specific primers for *RUNX3* and *β-actin* genes were designed based on sequence data from the GenBank database. The primers were purchased from Shanghai Biological Engineering. Conditions for all PCRs were optimized on Cycler iQ (Bio-Rad, USA) and the optimum annealing temperature was 55°C. The following cycler running protocol was used: denaturation (95°C, 2 min), amplification and quantification repeated 35 times (94°C for 30 s, 55°C for 30 s, and 72°C for 1 min). In addition, a non-template control (ddH<sub>2</sub>O control) was analyzed for each template. All samples were amplified simultaneously in triplicate.

#### Western blot analysis

Cells were collected, washed three times with PBS, lysed in cell lysate that contained 0.1 mol/L NaCl, 0.01 mol/L Tris-HCl (pH 7.6), 0.001 mol/L EDTA (pH 8.0), 1 µg/mL aprotinin and 100 µg/mL PMSF, and then centrifuged at  $13000 \times g$  for 10 min at 4°C. The extracted protein sample (50 µg total protein/lane) was added to the same volume of sample buffer and subjected to denaturation at 100°C for 10 min. The samples were electrophoresed on 100 g/L SDS PAGE at 28 mA for 30 min until they reached the bottom of the spacer gel, separated on the separation gel at 120 V for 1.5 h, and finally transferred onto PVDF membrane at 105 mA for 3.5 h at 4°C. The PVDF membrane was treated with TBST that contained 50 g/L skimmed milk at room temperature for 2 h, followed by incubation with the primary antibody *RUNX3* (1:100 dilution) (Boaosen Biotechnology Company, Beijing China; bs-0378R) at 37°C for 2 h or 4°C overnight. After being washed with TBST for 30 min, the corresponding secondary antibody (1:2000 dilution) (Zhongshan Jinqiao Biotechnology Company, Beijing, China) was added and incubated at room temperature for 1 h. The membrane was then washed three times for 15 min each with TBST. Fluorescence was produced from solution A and B that contained a chemiluminescence generator. Both *RUNX3* and *β-actin* protein expression were quantitatively estimated by densitometric scanning performed with Imaginer 2200. *RUNX3* protein concentration was

Table 1 Sequence of primers and amplified length of genes

Gene	Sequence	Amplified length (bp)
<i>RUNX3</i>	5'-GAGTTTCACCCTGACCA TCACTGTG-3' 5'-GCCCCATCACTGGTCTTGAAGGTTGT-3'	870
<i>β-actin</i>	5'-CTACAATGAGCTGCGTGTGC-3' 5'-AGGAGGACTTCTTCGAT-3'	500

normalized to *β-actin* level and expressed as a densitometric ratio.

#### Immunohistochemistry

Cells were seeded on the axenic cover glass in six-well plates at  $1 \times 10^5$  cells/well, and were grown overnight. The cover glasses with adhered cells were washed three times with PBS, and fixed in 95% alcohol for 10 min. After being rinsed three times in PBS, the endogenous peroxidase activity was suppressed by 30 mg/L hydrogen peroxide for 15 min, followed by rinsing three times in PBS. Antigen repair was performed by immersing the sections in 10 mmol/L sodium citrate buffer (pH 6.0) and heating for 15 min in a microwave oven. Non-specific binding was blocked by incubation with 30 mL/L bovine serum albumin (BSA) for 40 min. The cells were treated for 16 h with goat anti-human polyclonal IgG antibodies of *RUNX3* (Boaosen Biotechnology Company; bs-0378R) at 37°C for 2 h or 4°C overnight, according to the manufacturer's recommended concentration (1:200 dilution). PBS was used as a negative control. After washing three times in PBS, the cover glasses were treated with biotinylated rabbit anti-goat immunoglobulin (Zhongshan Jinqiao Biotechnology Company) for 1 h at room temperature and then by horseradish peroxidase-streptavidin complex (Man Xin Biotechnology Company, Huzhou, China) for 30 min. The cover glasses were then washed three times in PBS and incubated in DAB for 2 min. Next, the cover glasses were rinsed gently with distilled water and counterstained with hematoxylin for 30 s, and dehydrated in alcohol prior to mounting. Images were collected by Olympus DD70 BX51 (Olympus, Japan) and analyzed by IMAGE-PRO plus 4.1 software (Media Cybernetics, USA). Eight visual fields in each section were randomly selected and the mean value of relative OD was measured and calculated by taking the OD of background as 1. The extent of immunohistochemical staining was categorized as positive (1-1.5) and strongly positive (> 1.5).

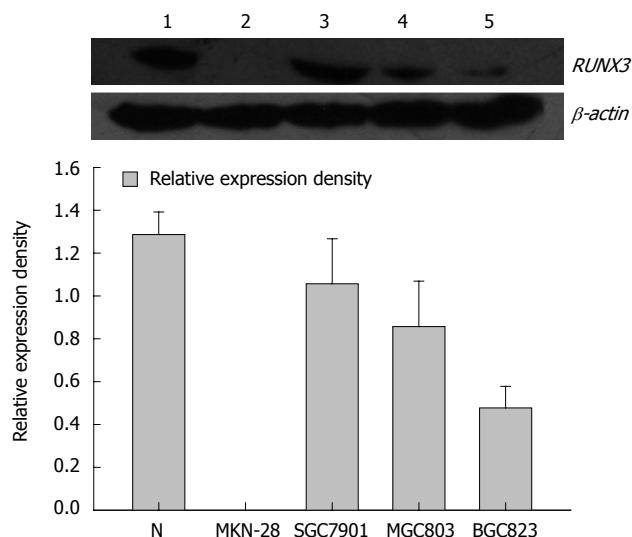
#### Statistical analysis

Experimental data in each group were expressed as mean ± SD. Analysis of variance (ANOVA) was performed with the Statistical Package for the Social Sciences (SPSS 13.0) for Windows by using one way ANOVA and pairwise comparison with Student's *t* test. *P* < 0.05 was considered statistically significantly.

## RESULTS

#### *RUNX3* protein expression in human gastric carcinoma cell lines

Western blot analysis showed that the relative densities



**Figure 1** RUNX3 expression in the gastric cancer cells detected by Western blot. 1: Positive control: Normal gastric tissue; 2: MKN-28 cells; 3: SGC-7901 cells; 4: MGC-803 cells; 5: BGC-823 cells.

of RUNX3 protein bands of normal gastric mucosa, well-differentiated gastric carcinoma cell line MKN-28, moderately differentiated gastric carcinoma cell line SGC7901, poorly differentiated gastric carcinoma cell lines MGC803 and BGC823 were:  $1.279 \pm 0.105$ ,  $0.003 \pm 0.001$ ,  $1.057 \pm 0.610$ ,  $0.857 \pm 0.212$ ,  $0.477 \pm 0.321$  respectively (Figure 1). The moderately differentiated gastric carcinoma cell lines SGC7901 was selected for further experiments.

#### Identification of the recombinant plasmids

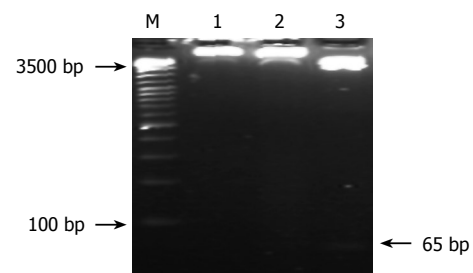
The recombinant eukaryotic expressing vector-pSilencer3.1-H1-shRNA/RUNX3 was cut by the double restriction enzyme *Bam*HI and *Hind*III. Then the enzyme products were electrophoresis using the agar gel (27 g/L). And the objective fragment 65 bp was obtained (Figure 2).

#### mRNA and protein expression of RUNX3 in transfected SGC-7901 cells

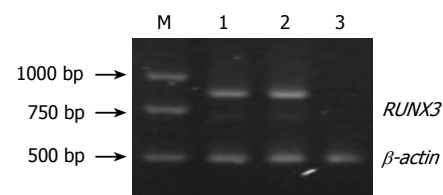
In SGC-7901, pSilencer3.1-H1/SGC7901 and pSilencer3.1-H1-shRNA/RUNX3/SGC790 cells, RT-PCR showed that the relative densities of RUNX3 mRNA bands were  $1.116 \pm 0.217$ ,  $1.057 \pm 0.187$  and  $0.002 \pm 0.001$ , respectively (Figure 3), and Western blot analysis indicated that the relative densities of RUNX3 protein bands were  $0.812 \pm 0.091$ ,  $0.786 \pm 0.103$  and  $0.021 \pm 0.002$ , respectively (Figure 4). Immunocytochemistry disclosed that RUNX3 protein was located in the endochylema and the nucleus, and loss of expression of RUNX3 in SGC790 cells transfected with recombinant plasmid-pSilencer3.1-H1-shRNA/RUNX3 (Figure 5).

#### Effect of RUNX3 on proliferation of SGC-7901 cell line

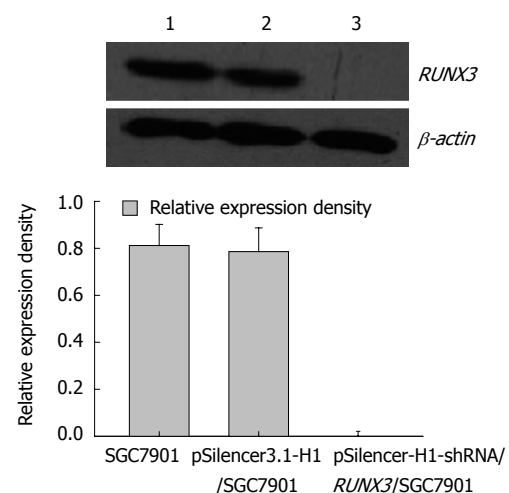
The growth curves showed that the pSilencer3.1-H1-shRNA/RUNX3/SGC7901 cells grew more quickly than the control cells, SGC7901 and pSilencer3.1-H1/SGC7901 cells ( $P < 0.05$ , Figure 6), but that the rate of cell growth



**Figure 2** Identification of recombinant by restrict endonucleases digestion. M: DNA Ladder; 1: pSilencer3.1-H1/SGC7901; 2: pSilencer3.1-H1-shRNA/RUNX3/SGC-7901; 3: pSilencer3.1-H1-shRNA/RUNX3/SGC7901 cut with *Bam*HI/*Hind*III.



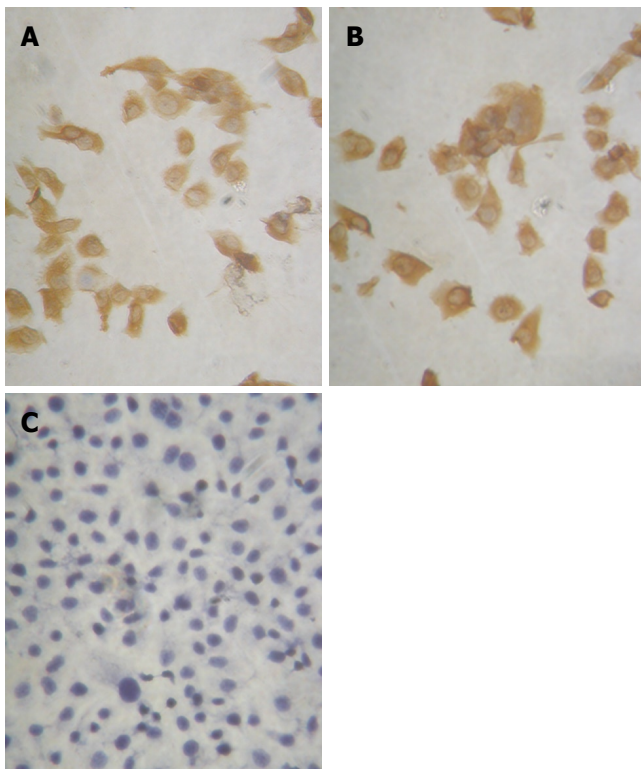
**Figure 3** RUNX3 expression in the three groups of cells detected by RT-PCR. M: DL2000 marker; 1: SGC7901 cells; 2: pSilencer3.1-H1/SGC7901 cells; 3: pSilencer3.1-H1-shRNA/RUNX3/SGC7901 cells.



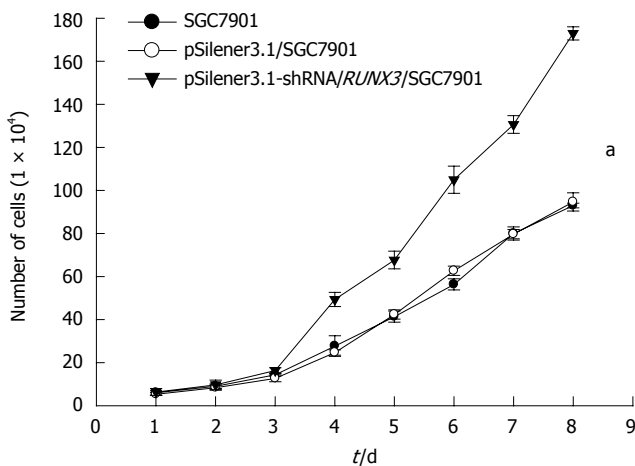
**Figure 4** RUNX3 expression detected by Western blot. 1: SGC7901 cells; 2: pSilencer3.1-H1/c SGC7901 cells; 3: pSilencer3.1-H1-shRNA/RUNX3/SGC7901 cells.

was similar in the two control groups. The results of the soft-agar colony-formation experiment indicated that a significant increase in the colony-formation rate in the pSilencer3.1-H1-shRNA/RUNX3/SGC790 cells ( $17.4 \pm 0.31\%$ ) was found compared with that in the control SGC-79011 ( $9.9 \pm 0.3\%$ ) and pSilencer3.1-H1/SGC7901 ( $9.7 \pm 0.6\%$ ) cells ( $P < 0.01$ , Figure 7). The colony-formation rates of the two control cells were similar. FCM with PI staining suggested that the proportion of cells in  $G_0/G_1$  and S phases in pSilencer3.1-H1-shRNA/RUNX3/SGC7901 cells significantly decreased and increased, respectively ( $P < 0.05$ , Figure 8 and Table 2), compared with the control cells.





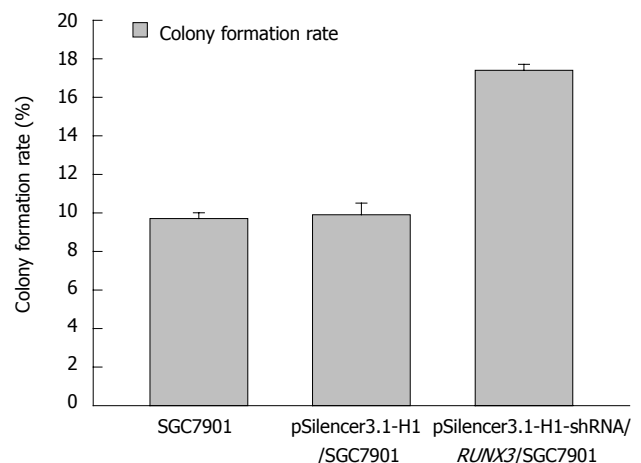
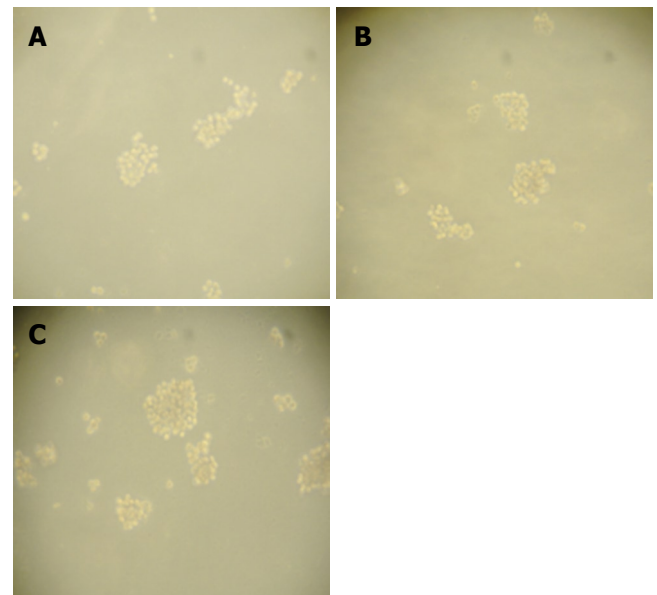
**Figure 5** RUNX3 expression in SGC7901 cells by immunohistochemistry ( $\times 400$ ). **A:** SGC7901 cells, the protein of RUNX3 expressed and located in the endochylema and the nucleus in the SGC7901 cell without transfection; **B:** pSilencer3.1-H1/SGC7901 cells, the protein of RUNX3 expressed and located in the endochylema and the nucleus in the SGC7901 cell with the plasmid DNA of pSilencer3.1-H1; **C:** pSilencer3.1-H1-shRNA/RUNX3/SGC7901 cells, the protein of RUNX3 lost expression in the SGC7901 cells transfected with the recombinant plasmid-pSilencer 3.1-H1-shRNA/RUNX3.



**Figure 6** The growth effect of SGC7901 cell by silencing *RUNX3* ( $^aP < 0.05$ ).

### Expression of *RUNX3* in transfected cells treated with 5-Aza-CdR

The pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells ( $1 \times 10^6/100$  mL) were treated with  $5 \times 10^{-6}$  mol/L and  $1 \times 10^{-5}$  mol/L 5-Aza-CdR. Cells were collected after 3 d treatment. The cells treated with  $5 \times 10^{-6}$  mol/L 5-Aza-CdR were named experimental group 1, and those treated with  $1 \times 10^{-5}$  mol/L 5-Aza-CdR were experimental group 2.

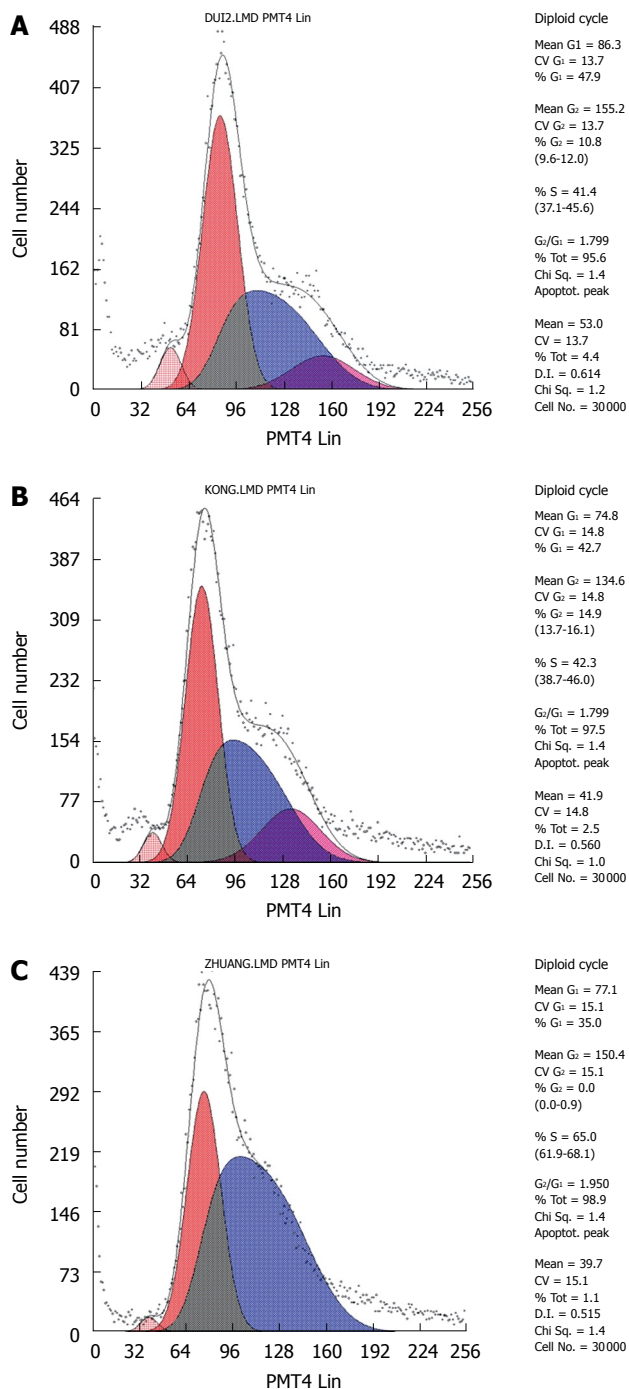


**Figure 7** The colony formation assay of SGC7901 in the soft agar ( $\times 100$ ). **A:** SGC7901 cells, the cloning efficiency was  $9.9\% \pm 0.3\%$  in the SGC7901 cell without transfection; **B:** pSilencer3.1-H1/SGC7901 cells, the cloning efficiency was  $9.7\% \pm 0.6\%$  in the SGC7901 cell with the plasmid DNA of pSilencer3.1-H1; **C:** pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells, the cloning efficiency was  $17.4\% \pm 0.31\%$  in the SGC7901 cells transfected with the recombinant plasmid-pSilencer3.1-H1-shRNA/*RUNX3*. A significant increase of the colony formation rate in the pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells was discovered compared with the controls-the SGC7901 cells and pSilencer3.1-H1/SGC7901 cells ( $P < 0.01$ ).

**Table 2** Cell cycle distribution of three groups by FCM ( $n = 3$ , mean  $\pm$  SD, %)

Groups	G <sub>0</sub> /G <sub>1</sub>	S	G <sub>2</sub> /M
SGC7901	43.2 $\pm$ 1.2	47.7 $\pm$ 1.1	9.0 $\pm$ 1.5
pSilencer3.1-H1-shRNA/SGC7901	40.3 $\pm$ 2.0	49.3 $\pm$ 0.9	8.1 $\pm$ 0.3
pSilencer3.1-H1-shRNA/ <i>RUNX3</i> /SGC7901	37.2 $\pm$ 1.9	60.5 $\pm$ 0.8	7.3 $\pm$ 0.9

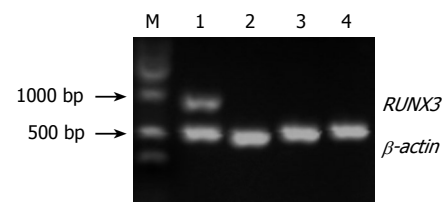
RT-PCR showed that the relative densities of *RUNX3* mRNA bands were  $0.861 \pm 0.167$ ,  $0.004 \pm 0.001$ ,  $0.002 \pm 0.001$  and  $0.002 \pm 0.001$ , respectively (Figure 9), and Western blot analysis that the band densities of *RUNX3* were  $1.013 \pm 0.138$ ,  $0.003 \pm 0.001$ ,  $0.002 \pm 0.001$  and  $0.005 \pm 0.001$ , respectively (Figure 10).



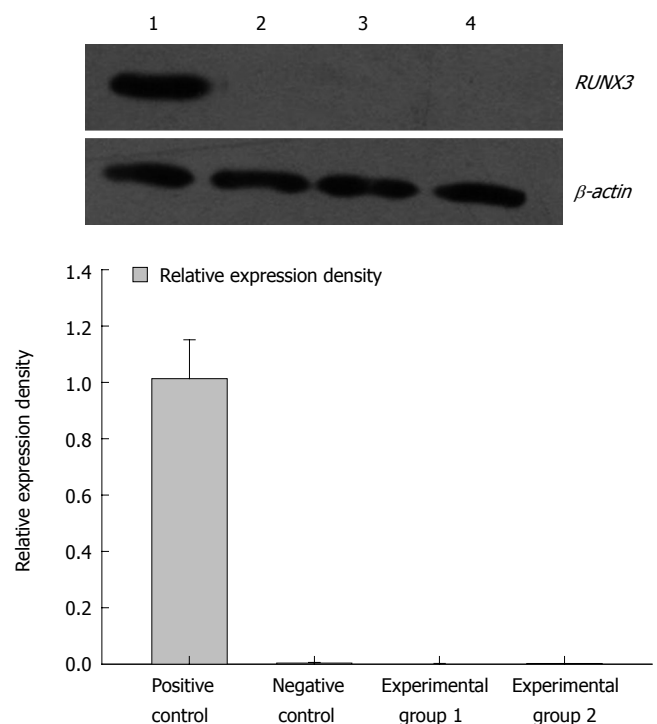
**Figure 8** The cell cycle analysis by FCM. **A:** SGC790 cells, the cell proportions of G<sub>0</sub>/G<sub>1</sub> and S stages were  $43.2\% \pm 1.2\%$  and  $47.7\% \pm 1.1\%$  in the SGC7901 cell without transfection, respectively; **B:** pSilencer3.1-H1/SGC7901 cells, the cell proportions of G<sub>0</sub>/G<sub>1</sub> and S stages were  $40.3\% \pm 2.0\%$  and  $49.3\% \pm 0.9\%$  in the SGC7901 cell with the plasmid DNA of pSilencer3.1-H1 respectively; **C:** pSilencer3.1-shRNA/RUNX3/SGC7901 cells, the cell proportions of G<sub>0</sub>/G<sub>1</sub> and S stages were  $37.2\% \pm 1.9\%$  and  $60.5\% \pm 0.8\%$  in the SGC790 cells transfected with the recombinant plasmid-pSilencer3.1-H1-shRNA/RUNX3 respectively. Compared with that of G<sub>0</sub>/G<sub>1</sub> and S stages of two controls, the cell proportions of G<sub>0</sub>/G<sub>1</sub> and S stages in the pSilencer3.1-H1-shRNA/RUNX3/SGC7901 cells decreased and increased obviously, respectively ( $P < 0.05$ ).

## DISCUSSION

In the occurrence and development of gastric carcinoma, the mechanism of abnormal silencing of anti-oncogenes mediated by epigenomics in the un-first-class structure



**Figure 9** *RUNX3* expression detected by RT-PCR in the four groups of SGC7901 cells. M: Marker; 1: Positive control, SGC7901 cells untreated with 5-Aza-CdR; 2: Negative control, pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells untreated with 5-Aza-CdR; 3: Experimental group 1, pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells treated with  $5 \times 10^{-6}$  mol/L 5-Aza-CdR; 4: Experimental group 2, pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells treated with  $1 \times 10^{-5}$  mol/L 5-Aza-CdR.



**Figure 10** *RUNX3* protein detected by Western blotting in the four groups of SGC7901 cells. 1: Positive control, SGC7901 cells untreated with 5-Aza-CdR; 2: Negative control, pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells untreated with 5-Aza-CdR; 3: Experimental group 1, pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells treated with  $5 \times 10^{-6}$  mol/L 5-Aza-CdR; 4: Experimental group 2, pSilencer3.1-H1-shRNA/*RUNX3*/SGC7901 cells treated with  $1 \times 10^{-5}$  mol/L 5-Aza-CdR.

levels of nucleotide sequence such as DNA methylation and histone modification, is still unclear. Many researchers have assumed that aberrant CpG island hypermethylation of the promoters of anti-oncogenes exists frequently in tumors. However, it is still not known how the aberrant CpG island hypermethylation takes place. Recently, the phenomenon by which siRNA molecules that target gene promoter regions can induce transcriptional gene silencing in a DNA cytosine methylation-dependent manner has attracted much attention.

siRNA can induce post-transcriptional gene silencing through RNA-RNA binding and transcriptional gene silencing through RNA-DNA binding. Transcriptional gene silencing refers to siRNA molecules that hinder production of mRNA from DNA before gene transcription, by

modifying chromosomal DNA and histones. It include three patterns; RNA-directed DNA methylation, RNA-directed heterochromatinization and RNA-directed DNA ablation<sup>[8-10]</sup>. The idea of RNA-directed DNA methylation was obtained from a propagation study in *Arabidopsis thaliana*<sup>[11]</sup>. It is not known whether a similar mechanism exists in mammalian systems.

Some elements, such as the required constituents of RNA-directed DNA methylation in plants, have been discovered in mammals. They contain three DNA methyltransferases (DNMT) 1, which play a role in maintaining methyltransferases. DNMT3A and DNMT3B can be let in the new methylated sites. The two processes including the methylated maintenance and repeated methylation might exist to affect genome DNA in mammals as in plants<sup>[12]</sup>. DNMT3A and DNMT3B have been observed to participate in the orientation of siRNA that target gene promoters<sup>[13]</sup>. As to human HeLa cells, the percentage of the mature-type miR-21 located in the cytoplasm and the nucleus were 80% and 20%, respectively. After transfection with fluorescently-labeled siRNA/miR-21, the fluorescence which siRNA/miR-21 binds the complementary sequences was observed in the cytoplasm and the nucleus. A identical consequence has appeared in cells transfected with fluorescently-labeled siRNA/let-7a<sup>[14]</sup>.

Some previous investigations have reported that siRNA molecules that target gene promoter regions can induce transcriptional gene silencing and DNA cytosine methylation around the promoter region. In the study by Morris *et al*, elongation factor 1  $\alpha$  (EF1 $\alpha$ ) was knocked down by the promoter-directed siRNA in HEK293 cells by transfection. After treatment with 5-Aza-CdR (4  $\mu$ mol/L) and trichostatin A (TSA, 0.05 mmol/L), deactivation of EF1 $\alpha$  was reversed and detected through nuclear run-on assays and RT-PCR. According to the above data, the transcriptional silencing of EF1 $\alpha$  might be associated with DNA methylation of the targeted sequence<sup>[15]</sup>. According to research by Castanotto *et al*, shRNAs, molecules homologous to DNA sequences in the promoter and early transcribed regions of RASSF1, can direct the partial gene silencing and low levels of *de novo* DNA methylation. They used a methylation-specific polymerase chain reaction (MSP) and bisulfite sequencing in HeLa cells<sup>[16]</sup>. Moreover, Weinberg's investigation showed that transcriptional silencing and DNA methylation of EF1 $\alpha$  can be directed by antisense interference alone in human 293T cells transfected with EF1 $\alpha$  siRNA. This targets the promoter of it using the peptide MPG, which transports siRNAs to the nucleus. This silencing is accompanied by increased methylation level in histone 3 lysine 9 (H3K9) and histone 3 lysine 27 (H3K27). Furthermore, siRNAs EF52 is associated with the transient expression of Flag-tagged DNMT3A, the targeted EF1 $\alpha$  promoter, and trimethylated H3K27<sup>[17]</sup>. Previous studies have indicated that, in mammalian cells, the unique RNA endonuclease Dicer, which cuts long dsRNA to form siRNA, is situated in the cytoplasm<sup>[18-20]</sup>. RNA-induced silencing complex (RISC), which binds to siRNAs in the cell-substance, may gain access to the nucleus when caryotheca vanishes during cell division, or it may be transported into the nucleus, as in

plants. Furthermore, siRNA can integrate the homologous DNA sequences (promoters) and induce transcriptional gene silencing in a DNA cytosine methylation-dependent manner<sup>[11,21]</sup>.

We confirmed that aberrant CpG island hypermethylation of the promoter of RUNX3 is a critical pathway to cause down-regulation or loss of expression of the gene. How may the pathway be connected with the mechanism of RNA-directed DNA methylation? In our experiment, on the basis of the principle of RNAi design, pSilencer3.1-H1-shRNA/RUNX3 expression vector was constructed, and transfected in SGC7901 cells by liposomes. RT-PCR, western blot and immunocytochemistry showed that mRNA and protein expression of RUNX3 were absent in the stable cell line SGC7901 transfected with the recombinant plasmid. Moreover, compared with those transfected with pSilencer3.1-H1 and non-transfected cells, cells transfected with pSilencer3.1-H1-shRNA/RUNX3 grew most quickly. Both the clone number and size were largest following soft-agar colony-formation assay ( $P < 0.01$ ) and the number of cells in G<sub>0</sub>/G<sub>1</sub> and S/M phases was lowest and highest following FCM ( $P < 0.05$ ). The above results indicated that, through RNA-dependent transcriptional silencing (RdTS), knockdown of the gene at the transcriptional stage was feasible and effective. After loss of expression of RUNX3 protein, the proliferation rate of SGC7901 cells increased. These results, like the previous description<sup>[3-6]</sup>, suggested that RUNX3, a putative tumor suppressor, might have an important role in stomach tumorigenesis. However, after the deal with the different density of 5-Aza-CdR, which may reactivate anti-oncogenes silenced by *de novo* methylation, inactivated RUNX3 was not reactivated, as shown by RT-PCR and the silenced. The phenomenon was different from the RdDM described in plants. At the same time, similar results have been reported. In two human glioblastoma cell lines, U-87 and U-118, siRNA homologous to the promoter region of *huntingtin* gene can repress transcriptional expression of the target gene. However, no CpG methylation has been observed on the target sequences by bisulfite-mediated genomic sequencing<sup>[21,22]</sup>. In the research of Ting *et al*, the human colorectal cancer cells, HCT116, were transfected with two 21-nucleotide-long dsRNAs (dsCDH1-1 and dsCDH1-2). The two sequences were homologous to the CpG island of the endogenous gene promoter of *CDH1* but did not overlap any known transcribed sequences. The findings suggested that promoter-targeting dsRNAs could effectively silence *CDH1* transcription, which results in a net decrease in mRNA and protein production, as demonstrated by RT-PCR and Western blot. Absence of DNA methylation at the targeted sequences was reviewed by MSP and bisulfite sequencing. Transfection of the human breast cancer cells, MCF-7, used a silencing strategy virtually identical to that above for *CDH1*. The transcriptional silencing without DNA methylation was reviewed and the results were identical<sup>[23]</sup>.

What accounts for these opposing results? We believe the answer may lie in the types of DNA methylation assays performed. In the work by Morris *et al*, the DNA methylation data remain to be verified because DNA

methylation was not as extensive, and there was indirect evidence for 5-Aza-CdR and TSA, a histone-deacetylase inhibitor. Co-administration alleviating the silencing was difficult to interpret as the reason of the *EF1 $\alpha$*  silencing. In other investigations, MSP distinguishes between non-methylated and methylated alleles by using two sets of primers to amplify either non-methylated or methylated sequences after bisulfite treatment, which specifically converts non-methylated cytosines to uracils. The shortcoming of MSP analysis is that it examines a few CpG sites in the sequences that are recognized by the primers, and the design of primers is very difficult. Mismatch sequencing was used to verify the overall target region by unbiased primers that did not contain CpG dinucleotides to amplify the bisulfite-converted promoter region. Due to the existence of incomplete mismatch conversions, unconverted cytosine residues in both CpG and CpG contexts remained as cytosine and created a false negative result. Furthermore, the PCR primers that were used previously to obtain the initial mismatch sequencing template that contains CpG sites may bias the amplification step and produce problematic mismatch sequencing results<sup>[5]</sup>. Such non-conversions may partly explain the different results in the mismatch sequencing. In the current study, the level of DNA methylation was testified preliminary and indirect. The finding that inactivation of *RUNX3* was not reactivated with 5-Aza-CdR could not confirm sufficiently that the transcriptional silencing of *RUNX3* was independent of DNA methylation. Therefore, further study is needed.

Taken together, the presence of RdTS indicated that RdDM might be an existing gene regulatory mechanism relevant to methylation in humans, even though we are currently unable to verify this. A recent study has shown that, in *A. thaliana*, RdDM is studied in the progeny of plants being silenced, and DNA methylation may still be involved in a prolonged silencing event in human cells<sup>[24]</sup>. Therefore, further experiments, which include a thorough examination of the long-term and full-scale outcomes of RdTS, are needed to research RNA-directed DNA methylation in mammalian systems.

## COMMENTS

### Background

Aberrant CpG island hypermethylation of the promoter of *RUNX3*, a tumor suppressor, is associated with its transcriptional silencing and the loss of gene functions in stomach cancer. However, the mechanism of the aberrant CpG island hypermethylation is still unclear.

### Research frontiers

In plants, siRNA molecules that target gene promoter regions can induce transcriptional gene silencing in a DNA cytosine methylation-dependent manner (RdDM). Whether a similar mechanism exists in mammalian systems is a vital and controversial issue. Recently, the RdDM theory on gene regulation has attracted close attention from researchers and has become a highlight in tumor studies.

### Innovations and breakthroughs

This article focuses on the relationship between siRNA molecules and aberrant CpG island hypermethylation in the promoter domain of *RUNX3*, through the RNA interference principle and gene transfection technology in human gastric cancer cell lines.

### Applications

*RUNX3* is an important anti-oncogene. Its mechanism of methylation may contribute to the study of the etiology of gastric cancer, and offer a theoretical basis for gene therapy of gastric cancer

### Terminology

RNA interference is an evolutionarily conserved mechanism of gene silencing. It can affect transcriptional gene silencing induced by siRNA that target gene promoter regions in a DNA cytosine methylation-dependent manner.

### Peer review

This is a good study in which the authors demonstrated that, shRNAs that targeted gene promoter regions of *RUNX3* could effectively induce transcriptional repression with chromatic changes characteristic of inaction promoters, but it was independent of DNA methylation. The study was well designed and the results are convincing.

## REFERENCES

- 1 **Levanon D**, Negreanu V, Bernstein Y, Bar-Am I, Avivi L, Groner Y. AML1, AML2, and AML3, the human members of the runt domain gene-family: cDNA structure, expression, and chromosomal localization. *Genomics* 1994; **23**: 425-432
- 2 **Li QL**, Ito K, Sakakura C, Fukamachi H, Inoue K, Chi XZ, Lee KY, Nomura S, Lee CW, Han SB, Kim HM, Kim WJ, Yamamoto H, Yamashita N, Yano T, Ikeda T, Itohara S, Inazawa J, Abe T, Hagiwara A, Yamagishi H, Ooe A, Kaneda A, Sugimura T, Ushijima T, Bae SC, Ito Y. Causal relationship between the loss of *RUNX3* expression and gastric cancer. *Cell* 2002; **109**: 113-124
- 3 **Wei D**, Gong W, Oh SC, Li Q, Kim WD, Wang L, Le X, Yao J, Wu TT, Huang S, Xie K. Loss of *RUNX3* expression significantly affects the clinical outcome of gastric cancer patients and its restoration causes drastic suppression of tumor growth and metastasis. *Cancer Res* 2005; **65**: 4809-4816
- 4 **Homma N**, Tamura G, Honda T, Matsumoto Y, Nishizuka S, Kawata S, Motoyama T. Spreading of methylation within *RUNX3* CpG island in gastric cancer. *Cancer Sci* 2006; **97**: 51-56
- 5 **So K**, Tamura G, Honda T, Homma N, Endoh M, Togawa N, Nishizuka S, Motoyama T. Quantitative assessment of *RUNX3* methylation in neoplastic and non-neoplastic gastric epithelia using a DNA microarray. *Pathol Int* 2006; **56**: 571-575
- 6 **Zeng C**, He XS, Luo Q, Zhao S, Deng M, Li YN. The expression and the mechanism of the down regulation of *RUNX3* in the gastric carcinoma. *Shijie Huaren Xiaohua Zazhi* 2006; **14**: 250-255
- 7 **Wassenegger M**, Heimes S, Riedel L, Sunger HL. RNA-directed de novo methylation of genomic sequences in plants. *Cell* 1994; **76**: 567-576
- 8 **Kawasaki H**, Taira K, Morris KV. siRNA induced transcriptional gene silencing in mammalian cells. *Cell Cycle* 2005; **4**: 442-448
- 9 **Kawasaki H**, Taira K. Transcriptional gene silencing by short interfering RNAs. *Curr Opin Mol Ther* 2005; **7**: 125-131
- 10 **Gaur RK**, Rossi JJ. The diversity of RNAi and its applications. *Biotechniques* 2006; Suppl: 4-5
- 11 **Matzke MA**, Birchler JA. RNAi-mediated pathways in the nucleus. *Nat Rev Genet* 2005; **6**: 24-35
- 12 **Szyf M**. DNA methylation and demethylation as targets for anticancer therapy. *Biochemistry (Mosc)* 2005; **70**: 533-549
- 13 **Morris KV**. siRNA-mediated transcriptional gene silencing: the potential mechanism and a possible role in the histone code. *Cell Mol Life Sci* 2005; **62**: 3057-3066
- 14 **Meister G**, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell* 2004; **15**: 185-197
- 15 **Morris KV**, Chan SW, Jacobsen SE, Looney DJ. Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* 2004; **305**: 1289-1292
- 16 **Castanotto D**, Tommasi S, Li M, Li H, Yanow S, Pfeifer



- GP, Rossi JJ. Short hairpin RNA-directed cytosine (CpG) methylation of the RASSF1A gene promoter in HeLa cells. *Mol Ther* 2005; **12**: 179-183
- 17 **Weinberg MS**, Villeneuve LM, Ehsani A, Amarzguioui M, Aagaard L, Chen ZX, Riggs AD, Rossi JJ, Morris KV. The antisense strand of small interfering RNAs directs histone methylation and transcriptional gene silencing in human cells. *RNA* 2006; **12**: 256-262
- 18 **Hutvagner G**, McLachlan J, Pasquinelli AE, Balint E, Tuschl T, Zamore PD. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* 2001; **293**: 834-838
- 19 **Rossi JJ**. Mammalian Dicer finds a partner. *EMBO Rep* 2005; **6**: 927-929
- 20 **Schmitter D**, Filkowski J, Sewer A, Pillai RS, Oakeley EJ, Zavolan M, Svoboda P, Filipowicz W. Effects of Dicer and Argonaute down-regulation on mRNA levels in human HEK293 cells. *Nucleic Acids Res* 2006; **34**: 4801-4815
- 21 **Svoboda P**, Stein P, Filipowicz W, Schultz RM. Lack of homologous sequence-specific DNA methylation in response to stable dsRNA expression in mouse oocytes. *Nucleic Acids Res* 2004; **32**: 3601-3606
- 22 **Park CW**, Chen Z, Kren BT, Steer CJ. Double-stranded siRNA targeted to the huntingtin gene does not induce DNA methylation. *Biochem Biophys Res Commun* 2004; **323**: 275-280
- 23 **Ting AH**, Schuebel KE, Herman JG, Baylin SB. Short double-stranded RNA induces transcriptional gene silencing in human cancer cells in the absence of DNA methylation. *Nat Genet* 2005; **37**: 906-910
- 24 **Aufsatz W**, Mette MF, Matzke AJ, Matzke M. The role of MET1 in RNA-directed de novo and maintenance methylation of CG dinucleotides. *Plant Mol Biol* 2004; **54**: 793-804

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# Transplanted bone marrow stromal cells are not cellular origin of hepatocellular carcinomas in a mouse model of carcinogenesis

Jin-Fang Zheng, Li-Jian Liang

Jin-Fang Zheng, Department of Hepatobiliary Surgery, the People's Hospital of Hainan Province, Haikou 570311, Hainan Province, China

Li-Jian Liang, Department of Hepatobiliary Surgery, the First Affiliated Hospital, Sun Yat-Sen University, Guangzhou 510080, Guangdong Province, China

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Author contributions: Zheng JF and Liang LJ contributed equally to this work; Zheng JF and Liang LJ designed the research; Zheng JF performed the research; Liang LJ provided new reagents/analytic tools; Liang LJ analyzed data; and Zheng JF wrote the paper.

Correspondence to: Dr. Jin-Fang Zheng, Department of Hepatobiliary Surgery, the People's Hospital of Hainan Province, 19# Xiuhua Road, Haikou 570311, Hainan Province, China. [zhengjf2000@hotmail.com](mailto:zhengjf2000@hotmail.com)

Telephone: +86-898-68642216 Fax: +86-898-68661664

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**Key words:** Bone marrow stromal cell; Stem cell; Hepatocarcinogenesis; Animal study

**Peer reviewers:** Toru Hiyama, MD, Department of Health Service Center, Hiroshima University, 1-7-1 Kagamiyama, Hiagshihiroshima 7398521, Japan; Jean Rosenbaum, MD, Inserm E362, Universite Victor Segalen Bordeaux 2, Bordeaux 33076, France

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## Abstract

**AIM:** To investigate the malignant potential of hepatic stem cells derived from the bone marrow stromal cells (BMSCs) in a mouse model of chemical hepatocarcinogenesis.

**METHODS:** BMSCs from male BALB/c mice were harvested and cultured, then transplanted into female syngenic BALB/c mice *via* portal vein. Hepato-carcinogenesis was induced by 6 mo of treatment with diethylnitrosamine (DEN). Six months later, the liver was removed from each treated mouse and evaluated by immunohistochemistry and fluorescence *in situ* hybridization (FISH).

**RESULTS:** Twenty-six percent of recipient mice survived and developed multiple hepatocellular carcinomas (HCCs). Immunohistochemically, HCC expressed placental form of glutathione-S-transferase (GST-P) and  $\alpha$ -fetoprotein, but did not express cytokeratin 19. Y chromosome positive hepatocytes were detected by fluorescent *in situ* hybridization (FISH) in the liver of mice treated with DEN after BMSCs transplantation while no such hepatocytes were identified in the liver of mice not treated with DEN. No HCC was positive for the Y chromosome by FISH.

**CONCLUSION:** Hepatic stem cells derived from the bone marrow stromal cells have a low malignant potential in our mouse model of chemical hepatocarcinogenesis.

## INTRODUCTION

Hepatocellular carcinoma (HCC) is the fifth most common cancer in the world<sup>[1]</sup>. Hepatitis B or C virus can induce chronic hepatitis and potentially result in liver cirrhosis and HCC, and these viral infections are frequently seen among HCC patients<sup>[2]</sup>. However, there is no clear evidence as to which cell is directly involved in the development of HCCs<sup>[3-5]</sup>. Two cell lineages have been considered as candidates: the first is hepatic stem cell, and the second is mature hepatocyte.

Oval cells are small, oval shaped epithelial cells identified as hepatic stem cells in the adult liver only following severe, repetitive liver injury<sup>[6]</sup>. There are increasing evidences that oval cells are the cellular targets for transformation in the development of HCC<sup>[7-8]</sup>. Oval cells might give rise to HCC as a result of the arrest of stem cell maturation<sup>[9]</sup>. Previous studies indicated that bone marrow cells can differentiate into oval cells in rodents and that a similar process could possibly take place in humans<sup>[10,11]</sup>. The incidence of plasticity has been shown to be very variable, from extremely rare to a range from 20% to 40%<sup>[12,13]</sup>. Although there is still controversy about which part of bone marrow cells can differentiate into hepatocytes, the present study clearly shows that transplanted bone marrow cells may help restore the hepatic degenerative diseases and reduce CCl<sub>4</sub>-induced liver fibrosis<sup>[14]</sup>. Some studies readily demonstrated bone marrow stromal cells (BMSCs) differentiated into hepatocyte-like cells in culture

after HGF treatment *in vitro*<sup>[15]</sup>. Therefore, BMSCs could be a valuable strategy for future replacement therapy of damaged or malfunctioned hepatocytes, because getting autologous BMSCs is easier than obtaining other tissue-specific stem cells. However, the safety and efficacy of hepatic stem cells derived from bone marrow cells should be adequately confirmed before any such therapies are tested in humans.

Our aim was to study the malignant potential of hepatic stem cells derived from BMSCs *in vivo*. To identify hepatic stem cells, BMSCs of male mice were transplanted into recipient female mice. After BMSCs transplantation, HCC was induced in the recipients by chemical hepatocarcinogenic compounds and the presence of the Y chromosome was evaluated in HCC.

## MATERIALS AND METHODS

### Animals

Six to eight week old BALB/c mice were purchased from the Animal Breeding Center of Sun Yat-Sen University (Guangzhou, China). Mice were bred and maintained in an air-conditioned animal house with specific pathogen-free conditions, using an alternate 12 h cycle of daylight and darkness, and unlimited access to chow and water. All animal experiments were performed in accordance with the guidelines of the Animal Care and Use Committee of Sun Yat-Sen University.

### Isolation and culture of bone marrow stromal cells (BMSCs)

BMSCs were harvested from bone marrow of the femurs and tibias of male mice by inserting a 21-gauge needle into the shaft of the bone and flushing it with DMEM medium supplemented with heparin<sup>[16]</sup>. The cell suspension was centrifuged over a Ficoll step gradient (density 1.077 g/mL) (Sigma, St. Louis, MO) at 1500 r/min for 10 min. The interface fraction was then collected and cultured in DMEM medium, supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin. Isolated cells were grown at 37°C and 5% CO<sub>2</sub> for 3 d. After removing the suspended cells, the adherent BMSCs were grown to 90% confluence and used between passages 3 and 4. After serum starvation for 4 h, BMSCs were treated with human recombinant HGF (Sigma-Aldrich, USA) at a concentration of 50 µg/L. Cultures were maintained by media exchange every 3 d. On d 21, all cells were detached for the next experiment.

The above detached cells were coated on the glass slides and fixed with 4% paraformaldehyde for 10 min at room temperature, followed by methanol for 2 min at -20°C, and permeabilized with 0.1% Triton X-100 for 10 min. Slides were blocked for 30 min using blocking and diluent solution, then incubated with rabbit anti- $\alpha$ -fetoprotein antibody or goat anti-albumin antibody at 4°C overnight. The cells were reincubated sequentially for 30 min with FITC-conjugated secondary antibody or PE-conjugated secondary antibody. The slides were counterstained with 4',6-diamidino-2-phenylindole (DAPI, Sigma) before mounting and observed under fluorescence microscope.

### Transplantation of BMSCs

BMSCs were harvested after cultured for passages 3 or 4 and suspended in DMEM medium supplemented with penicillin/streptomycin. BMSCs were washed twice in DMEM medium before intraportal injection. Cell viability (> 95%) was measured by trypan blue dye exclusion.

Anesthesia was performed with ether and partial hepatectomy used the standard method for two-thirds resection<sup>[17]</sup>. Briefly, after ligation of the pedicle and resection of the two largest lobes (median and left), the remaining liver was composed of the caudate and epiploic lobes. BMSCs were injected into the female liver *via* the superior mesenteric vein using insulin syringes after hepatectomy<sup>[18]</sup>. A total of 10<sup>6</sup> cells were injected per mouse.

### Diethylnitrosamine(DEN)-induced hepatocarcinogenesis

After partial hepatectomy and BMSCs injection, mice were allowed to recover for one week. Thereafter, DEN (Sigma) was continuously administered for 12 wk through drinking water at a final concentration of 100 µg/L to induce hepatocarcinogenesis<sup>[3]</sup>.

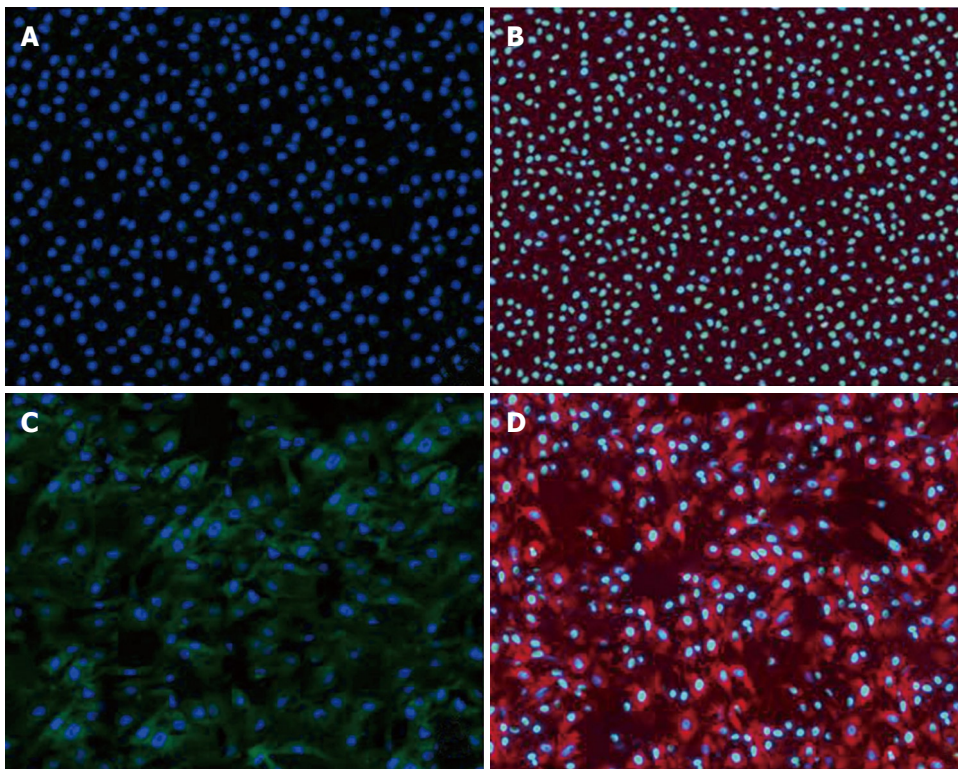
Sixty female BALB/c mice were randomly assigned to three groups. Ten mice in the normal control group were given BMSCs and non-supplemented drinking water. Twenty-five mice in the model group were continuously administered DEN in the drinking water. Twenty-five mice in the experimental group received BMSCs and DEN. The animals were sacrificed at 6 mo after the carcinogen regimen and the livers were fixed in 10% formalin for 24 h and embedded with paraffin. Routine histology was performed with haematoxylin-eosin staining. Serial sections were cut from liver samples with the macroscopically visible liver tumors and the right lobe for pathologic examination.

### Liver histopathology

To identify characteristics of tumors in the liver after BMSCs transplantation and DNE administration, placental form of glutathione-S-transferase (GST-P),  $\alpha$ -fetoprotein, and cytokeratin 19 were assayed immunohistochemically for these tumor nodules as previously described<sup>[19]</sup>. Briefly, after being deparaffinized with xylene, quenched with hydrogen peroxide and blocked with normal serum, the liver tissue sections were incubated for 1 h with rabbit anti- $\alpha$ -fetoprotein polyclonal antibody (dilution 1:100; Santa Cruz, USA), goat anti-cytokeratin 19 monoclonal antibody (dilution 1:100; Santa Cruz), or goat anti-GST-P polyclonal antibody (dilution 1:1000; Stressgen, Canada). FITC-conjugated secondary antibody or PE-conjugated secondary antibody was added. Counterstaining of nuclei was performed with DAPI for fluorescence staining.

### Fluorescent in situ hybridization (FISH)

Because BMSCs transplantation was performed from male donor mice to female recipient mice, the transplanted bone marrow derived cells could be recognized in the recipient by the presence of the Y chromosome in the nucleus. Therefore, FISH for the mouse Y chromosome was conducted to detect the transplanted bone marrow derived cells according to the Cambio protocol (<http://www.cambio.com>).



**Figure 1** Immunofluorescence of  $\alpha$ -fetoprotein and albumin in bone marrow stromal cells, with or without treatment of HGF in culture ( $\times 400$ ). **A:**  $\alpha$ -fetoprotein expression was negative in the absence of HGF; **B:** Albumin expression was negative in the absence of HGF; **C:**  $\alpha$ -fetoprotein expression localized at the cytoplasm in hepatocyte-like BMSCs; **D:** Albumin expression localized at the cytoplasm in hepatocyte-like BMSCs.

cambio.co.uk/). Paraffin-embedded slides were deparaffinized by baking in an oven overnight at  $37^{\circ}\text{C}$  and cleared in xylene three times for 10 min each; and they were then dehydrated and air-dried. Sections were incubated in 1 mol/L sodium thiocyanate for 10 min at  $80^{\circ}\text{C}$ , washed in PBS, and digested in pepsin (0.4% w/v) in 0.1 mol/L HCl at  $37^{\circ}\text{C}$  for 10 min. The protease was quenched in glycine (0.2% v/w) in  $2 \times$  PBS, post-fixed in paraformaldehyde (4% w/v) in PBS, dehydrated through graded alcohols and air-dried. A fluorescein isothiocyanate (FITC)-labeled Y-chromosome paint (Cambio, Cambridge, UK) was added to the sections, sealed under glass with rubber cement, heated to  $80^{\circ}\text{C}$  for 10 min, and incubated overnight at  $37^{\circ}\text{C}$ . The slides were washed in formamide (50% w/v)/ $2 \times$  saline sodium citrate (SSC) at  $37^{\circ}\text{C}$ , washed with  $2 \times$  SSC and  $4 \times$  SSC/Tween-20 (0.05% w/v) at  $37^{\circ}\text{C}$ . The slides were rinsed in  $0.5 \times$  SSC at  $37^{\circ}\text{C}$ . FITC amplification kit (Cambio) was used to amplify fluorescence signal. The slides were counterstained with DAPI before mounting and observed under confocal microscope (Zeiss, German).

### Statistical analysis

Data were presented as mean  $\pm$  SD. Significant differences were determined using ANOVA in SPSS10.0.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Differentiation of BMSCs into hepatocytes in vitro

To confirm the differentiation of BMSCs into hepatocytes, we selected cultural BMSCs with or without treatment of HGF for 21 d in culture and examined the expression of  $\alpha$ -fetoprotein and albumin by immunofluorescence. We found that

cultural BMSCs without treatment of HGF could not express  $\alpha$ -fetoprotein and albumin (Figure 1A and B), while differentiated hepatocyte-like BMSCs with treatment of HGF expressed  $\alpha$ -fetoprotein and albumin (Figure 1C and D).

### Survival rate

We evaluated the survival rate of mice that underwent BMSCs transplantation and/or DEN treatment. All mice that underwent BMSCs transplantation were still alive at the end of the study. Thirteen (26.0%) of 50 mice induced with DEN survived at the end of the 6-month study period, including six mice in the model group and seven in the experimental group. The survival rates were similar between the model group and the experimental group ( $P > 0.05$ ).

### Tumor development in the livers of recipient mice

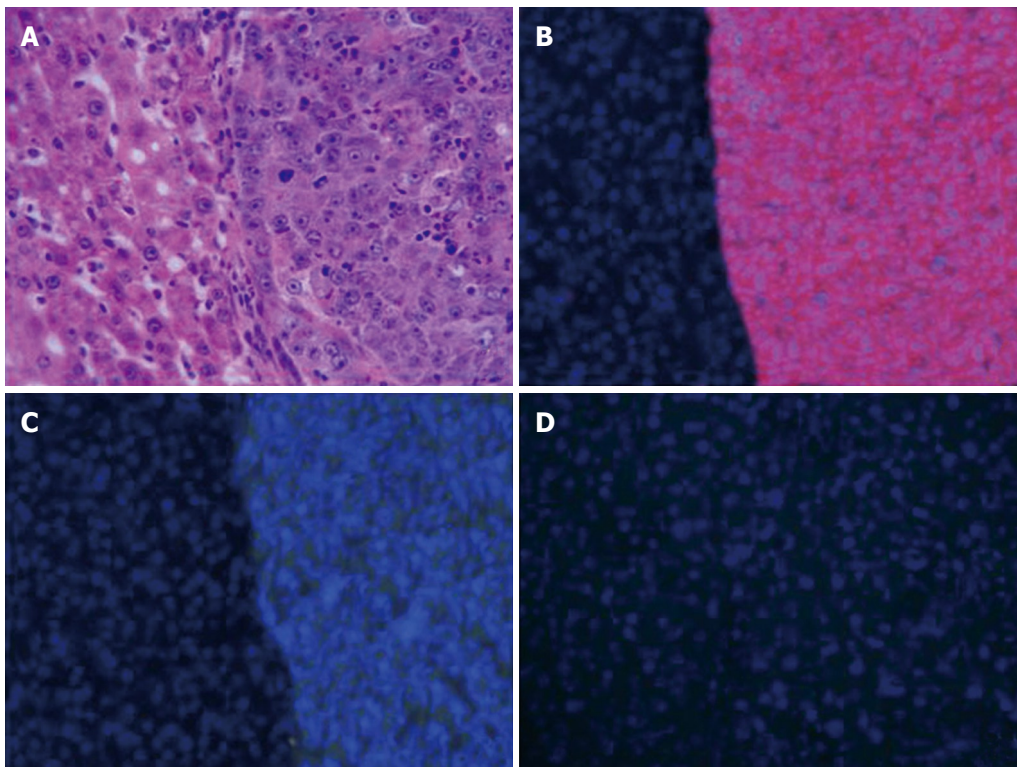
All of the survived recipient mice developed multiple HCCs. Thirteen mice developed HCCs including six mice in the model group and seven mice in the experiment group. These tumors were evenly distributed among the liver lobes of mice. The average sizes of hepatic tumors were not different between the two groups ( $4.8 \pm 1.5$  mm vs  $4.4 \pm 1.1$  mm;  $P > 0.05$ ).

HE stained sections of these tumors confirmed to be HCCs (Figure 2A) expressed GST-P and  $\alpha$ -fetoprotein (Figure 2B and C), but not Cytokeratin 19 (Figure 2D). No other types of liver tumors, such as hepatoblastoma or cholangiocellular carcinoma, were noted in our experiment.

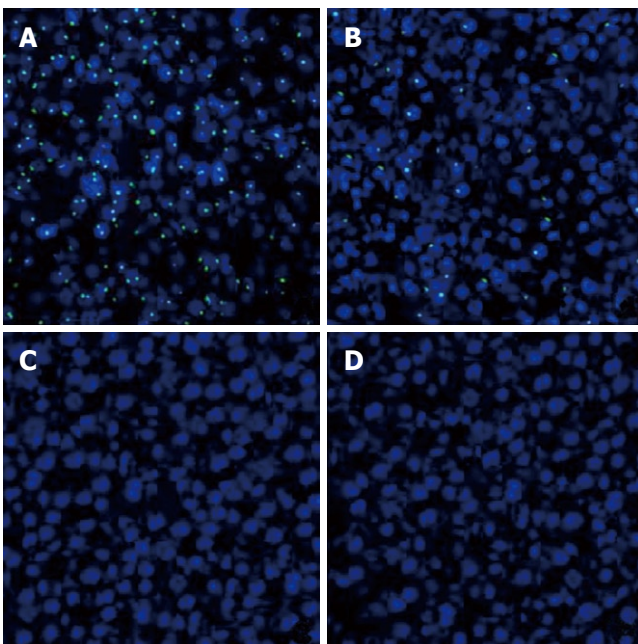
### Repopulation and carcinogenesis of transplanted BMSCs

To follow the repopulation and differentiation of BMSCs, we transplanted male BMSCs into the liver of normal and





**Figure 2** Histopathological analysis of the tumors in the liver serial sections of recipient mice after 6 mo of DEN treatment ( $\times 400$ ). **A:** HCC nodules development at 6 mo stained with haematoxylin-eosin; **B:** HCC expressing GST-P appeared as red fluorescence by immunofluorescence; **C:** HCC expressing  $\alpha$ -fetoprotein appeared as green fluorescence by immunofluorescence; **D:** HCC was negative for cytokeratin 19 by immunofluorescence.



**Figure 3** Repopulation and carcinogenesis of male bone marrow-derived cells in female recipient liver tissues by FISH for Y chromosome ( $\times 400$ ). **A:** normal male liver, positive Y-chromosome signals appeared as green dots in the nuclei stained with DAPI, a chromosomal marker that appears as blue fluorescence; **B:** Six months after BMSCs transplantation and DEN treatment, some of hepatocyte nuclei were positive for the Y chromosome in the liver of female recipients; **C:** Six months after BMSCs transplantation without DEN treatment, none of hepatocyte nuclei was positive for Y chromosome in the liver of female recipients; **D:** HCC was negative for Y chromosome in nucleus.

DEN-treated female mice. FISH was performed to detect Y chromosome in the female recipients. A positive FISH signal was detected in the nucleus which was confirmed

by counterstaining with DAPI. In male mouse liver, which served as positive control, most of the cells stained positive for Y-chromosome with fluorescein signal in the nuclei (Figure 3A).

We found that male BMSCs infused *via* portal vein into female syngeneic mouse liver could engraft and differentiate into hepatocytes after induction with DEN using FISH for Y chromosome. Six months after BMSCs transplantation and DEN challenge, FISH revealed that 15% of hepatocyte nuclei were positive for the Y chromosome in the liver of female recipients (Figure 3B). In addition, no nucleus showed two or more signals. However, donor-derived cells were not detected when BMSCs were transplanted to normal recipients without DEN treatment (Figure 3C). Moreover, no HCC was positive for the Y chromosome by FISH (Figure 3D).

## DISCUSSION

The liver is classified as a conditionally renewing tissue and hepatocytes proliferate quiescently and hepatic stem cells are not needed under normal circumstances. Oval cells reside within or adjacent to the canals of Hering and comprise a quiescent compartment of dormant stem cells in adult livers<sup>[6]</sup>. They can be activated to proliferate and differentiate into hepatocytes or bile duct epithelial cells when there is severe hepatic liver damage and coexistent impaired hepatocyte regeneration.

Accumulated evidence indicates that bone marrow cells can differentiate into specific cell types<sup>[20]</sup>. It has been reported that 30%-50% liver regeneration with bone marrow-derived cells in the FAH mouse model offers a selective proliferative advantage in the transplanted cells<sup>[21]</sup>. Bone marrow-derived hepatocytes may originate from the

mesenchymal compartment, rather than the hematopoietic compartment<sup>[22]</sup>. However, other data demonstrate that bone marrow-derived hepatocyte is only a possible but rare event, even in the presence of very strong selection pressure<sup>[23]</sup>. Several reports have demonstrated that cell fusion is the principal source of bone marrow-derived hepatocytes<sup>[24]</sup>, and bone marrow-derived hepatocytes are primarily of mature myelomonocytic cells which fuse spontaneously with host hepatocytes producing functional liver repopulation<sup>[14]</sup>.

The identity of the specific cell types that differentiate to express hepatocyte characteristics remains undetermined. BMSCs comprise marrow stromal stem cells, sharing characteristics with other multi-potent stem cells such as neural stem cells and hematopoietic stem cells, because they possess the capability of self-renewal and progeny differentiation potentials<sup>[25]</sup>. Our study demonstrates that cultured BMSCs differentiated hepatocyte-like cells which expressed  $\alpha$ -fetoprotein and albumin with the treatment of HGF *in vitro*. After BMSCs transplantation, Y chromosome positive cells appeared only in mice treated with DEN and not in mice who did not receive DEN. These results suggest that in our model, BMSCs can differentiate into hepatocytes under limited conditions. Bone marrow-derived hepatic stem cells seem not to be required for normal hepatocyte substitution. Indeed, in the present study, we found that in positive hepatocytes, no nucleus had two or more Y chromosomes by FISH. This finding indicates that transdifferentiation, rather than cell fusion, was the main process in our model.

DEN is a DNA alkylating agent that is rapidly metabolized to reactive metabolites. These metabolites interact with DNA to form various DNA adducts, leading to genetic alterations<sup>[26]</sup>. GST-P is a highly expressed cytoplasmic protein during early and late steps of carcinogenesis and GST-P sensitivity is higher than that of other enzymes for the detection of malignant transformation<sup>[27]</sup>. Seventy percent of HCCs were stained positively for  $\alpha$ -fetoprotein in clinical cases. In our study, chronic exposure to DEN caused multiple HCCs. These HCCs express GST-P and  $\alpha$ -fetoprotein, but not cytokeratin 19 which was expressed in cholangiocellular carcinoma.

In this study, we focused our interest on the original cell lineage of carcinogenesis. There are two major nonexclusive hypotheses of the cellular origin of cancer: from stem cells due to maturation arrest or from dedifferentiation of mature cells. Debate has centered on whether hepatocytes are responsible for HCCs through dedifferentiation, or whether oval cells are the prime target for malignant changes after a differential "block"<sup>[3,5]</sup>. Oval cells are possibly involved in hepatocarcinogenesis based on the followings: (1) massive existence of oval cells in an animal rodent hepatocarcinogenic model<sup>[28]</sup>; (2) development of HCC after transformation of oval cells<sup>[8]</sup>; and (3) occurrence of mixed hepatocellular and cholangiocarcinomatous tumors (oval cell exhibits bipotential developmental ability)<sup>[29]</sup>. However, the relationship between oval cells and cancer is only circumstantial. In this study, no HCC was positive for Y chromosome after long-term carcinogenic induction. However, as all hepatic stem cells might not be labeled by our method as mentioned above, we cannot completely exclude the stem cell theory. Although

our results may be limited to BMSCs transplanted mice treated with DEN, we can state that the malignant potential of the hepatic stem cell derived from bone marrow seems to be low. Further studies are needed to clarify the precise interaction of bone marrow cells with hepatic regeneration and carcinogenesis using other animal models or human studies.

In conclusion, our study demonstrates that cultured BMSCs could differentiate hepatocyte-like cells with HGF treatment *in vitro*. BMSCs can differentiate into hepatocytes in our model. Hepatic stem cells derived from bone marrow stromal cells are not cellular origin of hepatocellular carcinomas in the DEN model of carcinogenesis. Bone marrow cells may potentially be used in cell based replacement therapy or gene delivery systems. Under these circumstances, our results indicate that hepatic stem cell therapy derived from bone marrow is safe.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Bone marrow stromal cells can differentiate into hepatic stem cells in rodents and in humans. Bone marrow stromal cells could be a valuable strategy for future replacement therapy of damaged or malfunctioned hepatocytes, because getting autologous bone marrow cells is easier than obtaining other tissue-specific stem cells. However, the safety and efficacy of hepatic stem cells derived from bone marrow stromal cells should be adequately confirmed before any such therapies are tested in humans.

### Research frontiers

There are two major nonexclusive hypotheses of the cellular origin of cancer: from stem cells due to maturation arrest or from dedifferentiation of mature cells. Debate has centered on whether hepatocytes are responsible for hepatocellular carcinoma (HCC) through a process of dedifferentiation, or whether oval cells are the prime target for malignant changes after a differential "block". There are increasing evidences that oval cells are the cellular targets for transformation in the development of HCC. Accumulating evidences indicate that bone marrow cells can differentiate into specific cell types. It has been reported that 30%-50% liver regeneration with bone marrow-derived cells in the FAH mouse model offers a selective proliferative advantage in the transplanted cells. Bone marrow-derived hepatocytes may originate from the mesenchymal compartment, rather than the hematopoietic compartment.

### Innovations and breakthroughs

This study demonstrates that cultured bone marrow stromal cells could differentiate hepatocyte-like cells *in vitro*. Bone marrow stromal cells can differentiate into hepatocytes. Hepatic stem cells derived from bone marrow stromal cells are not cellular origin of hepatocellular carcinomas in a mouse model of carcinogenesis. Bone marrow cells may potentially be used in cell based replacement therapy or gene delivery systems. The results in this study indicate that hepatic stem cell therapy derived from bone marrow is safe.

### Applications

Bone marrow stromal cells might be applicable for future replacement therapy of damaged or malfunctioned hepatocytes.

### Peer review

This paper is interesting and the study appears well conducted. The conclusions, although of a limited scope given the design of the study, are in agreement with the results.



## REFERENCES

- 1 **Kao JH**, Chen DS. Changing disease burden of hepatocellular carcinoma in the Far East and Southeast Asia. *Liver Int* 2005; **25**: 696-703
- 2 **Perz JF**, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contributions of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol* 2006; **45**: 529-538
- 3 **Bralet MP**, Pichard V, Ferry N. Demonstration of direct lineage between hepatocytes and hepatocellular carcinoma in diethylnitrosamine-treated rats. *Hepatology* 2002; **36**: 623-630
- 4 **Gournay J**, Auvigne I, Pichard V, Ligeza C, Bralet MP, Ferry N. In vivo cell lineage analysis during chemical hepatocarcinogenesis in rats using retroviral-mediated gene transfer: evidence for dedifferentiation of mature hepatocytes. *Lab Invest* 2002; **82**: 781-788
- 5 **Lee JS**, Heo J, Libbrecht L, Chu IS, Kaposi-Novak P, Calvisi DF, Mikaelyan A, Roberts LR, Demetris AJ, Sun Z, Nevens F, Roskams T, Thorgeirsson SS. A novel prognostic subtype of human hepatocellular carcinoma derived from hepatic progenitor cells. *Nat Med* 2006; **12**: 410-416
- 6 **Kofman AV**, Morgan G, Kirschenbaum A, Osbeck J, Hussain M, Swenson S, Theise ND. Dose- and time-dependent oval cell reaction in acetaminophen-induced murine liver injury. *Hepatology* 2005; **41**: 1252-1261
- 7 **Yamamoto T**, Uenishi T, Ogawa M, Ichikawa T, Hai S, Sakabe K, Tanaka S, Kato H, Mikami S, Ikebe T, Tanaka H, Ito S, Kaneda K, Hirohashi K, Kubo S. Immunohistologic attempt to find carcinogenesis from hepatic progenitor cell in hepatocellular carcinoma. *Dig Surg* 2005; **22**: 364-370
- 8 **Dumble ML**, Croager EJ, Yeoh GC, Quail EA. Generation and characterization of p53 null transformed hepatic progenitor cells: oval cells give rise to hepatocellular carcinoma. *Carcinogenesis* 2002; **23**: 435-445
- 9 **Dumble ML**, Knight B, Quail EA, Yeoh GC. Hepatoblast-like cells populate the adult p53 knockout mouse liver: evidence for a hyperproliferative maturation-arrested stem cell compartment. *Cell Growth Differ* 2001; **12**: 223-231
- 10 **Lagasse E**, Connors H, Al-Dhalimy M, Reitsma M, Dohse M, Osborne L, Wang X, Finegold M, Weissman IL, Grompe M. Purified hematopoietic stem cells can differentiate into hepatocytes in vivo. *Nat Med* 2000; **6**: 1229-1234
- 11 **Petersen BE**, Bowen WC, Patrene KD, Mars WM, Sullivan AK, Murase N, Boggs SS, Greenberger JS, Goff JP. Bone marrow as a potential source of hepatic oval cells. *Science* 1999; **284**: 1168-1170
- 12 **Wagers AJ**, Sherwood RI, Christensen JL, Weissman IL. Little evidence for developmental plasticity of adult hematopoietic stem cells. *Science* 2002; **297**: 2256-2259
- 13 **Theise ND**, Wilmut I. Cell plasticity: flexible arrangement. *Nature* 2003; **425**: 21
- 14 **Sakaida I**, Terai S, Yamamoto N, Aoyama K, Ishikawa T, Nishina H, Okita K. Transplantation of bone marrow cells reduces CCl4-induced liver fibrosis in mice. *Hepatology* 2004; **40**: 1304-1311
- 15 **Wang PP**, Wang JH, Yan ZP, Hu MY, Lau GK, Fan ST, Luk JM. Expression of hepatocyte-like phenotypes in bone marrow stromal cells after HGF induction. *Biochem Biophys Res Commun* 2004; **320**: 712-716
- 16 **Luk JM**, Wang PP, Lee CK, Wang JH, Fan ST. Hepatic potential of bone marrow stromal cells: development of in vitro co-culture and intra-portal transplantation models. *J Immunol Methods* 2005; **305**: 39-47
- 17 **Oertel M**, Rosencrantz R, Chen YQ, Thota PN, Sandhu JS, Dabeva MD, Pacchia AL, Adelson ME, Dougherty JP, Shafritz DA. Repopulation of rat liver by fetal hepatoblasts and adult hepatocytes transduced ex vivo with lentiviral vectors. *Hepatology* 2003; **37**: 994-1005
- 18 **Kushida T**, Inaba M, Hisha H, Ichioka N, Esumi T, Ogawa R, Iida H, Ikehara S. Crucial role of donor-derived stromal cells in successful treatment for intractable autoimmune diseases in mrl/lpr mice by bmt via portal vein. *Stem Cells* 2001; **19**: 226-235
- 19 **Vig P**, Russo FP, Edwards RJ, Tadrous PJ, Wright NA, Thomas HC, Alison MR, Forbes SJ. The sources of parenchymal regeneration after chronic hepatocellular liver injury in mice. *Hepatology* 2006; **43**: 316-324
- 20 **Alison MR**, Poulosom R, Jeffery R, Dhillon AP, Quaglia A, Jacob J, Novelli M, Prentice G, Williamson J, Wright NA. Hepatocytes from non-hepatic adult stem cells. *Nature* 2000; **406**: 257
- 21 **Jang YY**, Collector MI, Baylin SB, Diehl AM, Sharkis SJ. Hematopoietic stem cells convert into liver cells within days without fusion. *Nat Cell Biol* 2004; **6**: 532-539
- 22 **Jiang Y**, Jahagirdar BN, Reinhardt RL, Schwartz RE, Keene CD, Ortiz-Gonzalez XR, Reyes M, Lenvik T, Lund T, Blackstad M, Du J, Aldrich S, Lisberg A, Low WC, Largaespada DA, Verfaillie CM. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* 2002; **418**: 41-49
- 23 **Kanazawa Y**, Verma IM. Little evidence of bone marrow-derived hepatocytes in the replacement of injured liver. *Proc Natl Acad Sci USA* 2003; **100** Suppl 1: 11850-11853
- 24 **Wang X**, Willenbring H, Akkari Y, Torimaru Y, Foster M, Al-Dhalimy M, Lagasse E, Finegold M, Olson S, Grompe M. Cell fusion is the principal source of bone-marrow-derived hepatocytes. *Nature* 2003; **422**: 897-901
- 25 **Mangi AA**, Noiseux N, Kong D, He H, Rezvani M, Ingwall JS, Dzau VJ. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med* 2003; **9**: 1195-1201
- 26 **Kagawa M**, Sano T, Ishibashi N, Hashimoto M, Okuno M, Moriawaki H, Suzuki R, Kohno H, Tanaka T. An acyclic retinoid, NIK-333, inhibits N-diethylnitrosamine-induced rat hepatocarcinogenesis through suppression of TGF- $\alpha$  expression and cell proliferation. *Carcinogenesis* 2004; **25**: 979-985
- 27 **Sakata K**, Hara A, Hirose Y, Yamada Y, Kuno T, Katayama M, Yoshida K, Zheng Q, Murakami A, Ohgashi H, Ikemoto K, Koshimizu K, Tanaka T, Mori H. Dietary supplementation of the citrus antioxidant auranthene inhibits N,N-diethylnitrosamine-induced rat hepatocarcinogenesis. *Oncology* 2004; **66**: 244-252
- 28 **Choudhury S**, Zhang R, Frenkel K, Kawamori T, Chung FL, Roy R. Evidence of alterations in base excision repair of oxidative DNA damage during spontaneous hepatocarcinogenesis in Long Evans Cinnamon rats. *Cancer Res* 2003; **63**: 7704-7707
- 29 **Wakasa T**, Wakasa K, Shutou T, Hai S, Kubo S, Hirohashi K, Umeshita K, Monden M. A histopathological study on combined hepatocellular and cholangiocarcinoma: cholangiocarcinoma component is originated from hepatocellular carcinoma. *Hepatogastroenterology* 2007; **54**: 508-513

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# Cost effectiveness analysis of population-based serology screening and $^{13}\text{C}$ -Urea breath test for *Helicobacter pylori* to prevent gastric cancer: A markov model

Feng Xie, Nan Luo, Hin-Peng Lee

Feng Xie, Department of Clinical Epidemiology and Biostatistics, McMaster University, Ontario L8P1H1, Canada

Nan Luo, Department of Community, Occupational, and Family Medicine, and Research Fellow, Centre for Health Services Research, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore

Hin-Peng Lee, Department of Community, Occupational, and Family Medicine, Yong Loo Lin School of Medicine, National University of Singapore, Singapore 117597, Singapore

Author contributions: Xie F designed the study, analyzed the data and wrote the manuscript; Luo N designed the study and revised the manuscript; and Lee HP revised the manuscript.

Correspondence to: Feng Xie, Dr, Programs for Assessment of Technology in Health (PATH), Department of Clinical Epidemiology and Biostatistics, McMaster University, 25 Main Street West Suite 2000, Hamilton, Ontario L8P 1H1, Canada. [fengxie@mamaster.ca](mailto:fengxie@mamaster.ca)

Telephone: +1-905-5237284 Fax: +1-905-5220568

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screening for *H. pylori* was more cost-effective than the UBT in prevention of gastric cancer in Singapore Chinese males.

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**Key words:** Cost-effectiveness analysis; Gastric cancer; *Helicobacter pylori*;  $^{13}\text{C}$ -Urea breath test; Serology

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Xie F, Luo N, Lee HP. Cost effectiveness analysis of population-based serology screening and  $^{13}\text{C}$ -Urea breath test for *H. pylori* to prevent gastric cancer: A markov model. *World J Gastroenterol* 2008; 14(19): 3021-3027 Available from: URL: <http://www.wjg-net.com/1007-9327/14/3021.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3021>

## Abstract

**AIM:** To compare the costs and effectiveness of no screening and no eradication therapy, the population-based *Helicobacter pylori* (*H. pylori*) serology screening with eradication therapy and  $^{13}\text{C}$ -Urea breath test (UBT) with eradication therapy.

**METHODS:** A Markov model simulation was carried out in all 237900 Chinese males with age between 35 and 44 from the perspective of the public healthcare provider in Singapore. The main outcome measures were the costs, number of gastric cancer cases prevented, life years saved, and quality-adjusted life years (QALYs) gained from screening age to death. The uncertainty surrounding the cost-effectiveness ratio was addressed by one-way sensitivity analyses.

**RESULTS:** Compared to no screening, the incremental cost-effectiveness ratio (ICER) was \$16166 per life year saved or \$13571 per QALY gained for the serology screening, and \$38792 per life year saved and \$32525 per QALY gained for the UBT. The ICER was \$477079 per life year saved or \$390337 per QALY gained for the UBT compared to the serology screening. The cost-effectiveness of serology screening over the UBT was robust to most parameters in the model.

**CONCLUSION:** The population-based serology

## INTRODUCTION

Gastric cancer is the second leading cause of cancer death worldwide, which leads to a substantial burden of morbidity, mortality, and health care costs<sup>[1,2]</sup>. *H. pylori* infection has been recognized as an important risk factor for cancer of gastric body and antrum (distal cancers)<sup>[3,4]</sup>. Approximately 50% of the world population has been affected by *H. pylori*<sup>[5]</sup>. Although less than 1% of the infected will develop cancer, population-based *H. pylori* screening in high-risk population has been proposed as a cost-effective strategy in the long term in Western countries<sup>[6-8]</sup>.

The East Asian countries such as China and Japan have the highest incidence of distal gastric cancer, which is twice as common in males as in females<sup>[1]</sup>. *H. pylori* infection was also found to be strongly linked to increased risk of gastric cancer in ethnic Chinese and Japanese<sup>[9]</sup>. Early detection and eradication of *H. pylori* infection might be a useful way to reduce the risk of gastric cancer in Asian populations where prevalence of *H. pylori* infection and gastric cancer are significantly higher than in the West<sup>[1]</sup>. However, it is unknown whether it is cost-effective to implement population-based *H. pylori* screening



in high-risk Asian populations. Moreover, two widely used screening programs demonstrated good sensitivity and specificity in detection of *H pylori* infection in Chinese<sup>[10,11]</sup>, therefore the question arises which screening program is more cost effective?

This study was aimed to evaluate the clinical and economic effects associated with no screening, population-based *H pylori* serology screening, and population-based <sup>13</sup>C-Urea breath test (UBT) in Singapore Chinese males using a Markov model.

## MATERIALS AND METHODS

### Model structure

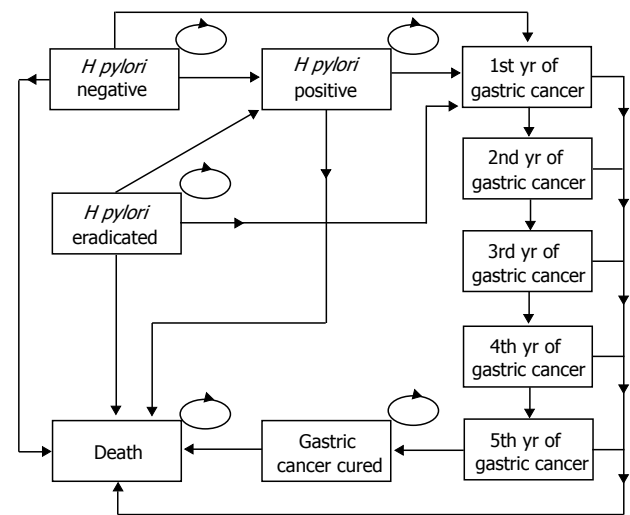
The decision analytical model compared three strategies: strategy 1, no screening and no eradication therapy; strategy 2, single serology screening for *H pylori* and treating those tested positive with eradication therapy; and strategy 3, single screening for *H pylori* using the UBT and treating those tested positive with the same eradication therapy as used in strategy 2. After the screening and treatment, both costs and outcomes of the strategies were evaluated using a Markov model (Figure 1)<sup>[12,13]</sup>, which, from the public healthcare provider's perspective, estimated the costs, number of gastric cancer cases prevented, life years saved, and quality-adjusted life years (QALYs) gained from screening age to death (either died of gastric cancer or other causes, or achieved full life expectancy<sup>[14]</sup>). The distribution of people in the Markov states before the simulation started (i.e. cycle 0) was determined by the sensitivity and specificity of the screening strategies and prevalence of *H pylori* infection. The transition probabilities and corresponding plausible ranges in the model were obtained from a critical review of the published literature on target population where available (Table 1). Probabilities were converted from available rates using the formula recommended<sup>[13]</sup>.

### Sensitivity analyses

One-way sensitivity analyses were conducted by altering individual variables within the aforementioned ranges. Based on the one-way sensitivity analyses, we additionally performed the best-case and the worst-case analyses, which included the most optimistic and pessimistic values for selected key variables.

### Incidence and prevalence rates

We evaluated all Singapore Chinese males aged from 35 to 44 as the prevalence of *H pylori* infection at this age group increased substantially compared to the younger age<sup>[10,15]</sup>. Age-specific *H pylori* infection rate, gastric cancer incidence, and mortality were applied when the cohort aged in the model<sup>[10,16,17]</sup>. The relative risk in developing gastric cancer in *H pylori* infected persons compared to the uninfected was obtained from published literature<sup>[3,18]</sup>. Proportion of gastric cancer death among deaths from all causes was derived from local reports<sup>[17]</sup>. The 1- to 5-year survival rates were estimated from a large prospective cohort study in Chinese<sup>[19]</sup>. Persons who survived for more



**Figure 1** Markov model schematic. *H pylori* eradicated referred to the state of persons with positive screening test and the infection was successfully eradicated by the triple therapy.

than 5 years after diagnosis of gastric cancer were assumed to be cured and therefore achieved full life expectancy as the 5-year survival rate adequately reflected the curative success of gastric cancer treatment<sup>[7,20]</sup>.

### Screening and eradication therapy

The screening strategies included 1 single serology screening by using enzyme-linked immunosorbent assay (ELISA) with a sensitivity and specificity of 93% and 79%, respectively (strategy 2)<sup>[10]</sup> and 1 single UBT using simple gas chromatograph-mass selective detector with a sensitivity and specificity of 97.9% and 95.8%, respectively (strategy 3)<sup>[11]</sup>. In both strategies, persons with positive test for *H pylori* (including both true and false positive) were treated with a triple therapy (i.e. rabeprazole 20 mg, amoxicillin 1000 mg, clarithromycin 500 mg, all twice a day for 4 d) with an eradication rate of 91%<sup>[21,22]</sup>. This regimen was specifically chosen because it is safe and effective with less resistance rate in patients and is recommended by the Asia-Pacific consensus conference<sup>[23-25]</sup>. Persons who stopped the triple therapy due to side effects or did not comply with the regimen were considered as treatment failure and thus remained infected. Persons who remained infected despite attempts at eradication had life expectancies and other outcomes identical to the infected who did not undergo treatment. The reinfection rate of the persons whose infection had been successfully eradicated was assumed to be identical to the persons who had never been infected (i.e. 1% annually in the base-case analysis)<sup>[6,26]</sup>. Once the reinfection occurred, an individual's gastric cancer risk was considered the same as that of an untreated, infected person of the same age.

An underlying assumption of the present study is that eradication of *H pylori* infection can reduce only the certain level of excess risk of distal gastric cancer (60% of all gastric cancers)<sup>[4,27]</sup>. We conservatively assumed that persons cured of *H pylori* infection will

Table 1 Parameter estimates in the base-case analysis

Input variable	Base-case analysis	Range	Ref.
Incidence and prevalence rates			
Age-specific prevalence of <i>H pylori</i> (%)	20.0-43.3	-	[10]
Age-specific prevalence of gastric cancer per 100 000	3-342	-	[17]
Gastric cancer in distal stomach (%)	60	50-80	[7]
Relative risk of gastric cancer in persons with <i>H pylori</i> infection	3.6	2-12	[7]
Age-specific mortality from age of 25, per 1000	0.5-50.6	-	[16]
Gastric cancer death in deaths from all causes (%)	2.27	2.20-2.33	[14,17]
Survival rate of gastric cancer after treatment (%)			[19]
1-yr	54.2	51-58	
2-yr	41.8	38-45	
3-yr	37.9	34-42	
4-yr	34.0	30-38	
5-yr	30.5	27-35	
Screening and treatment variables (%)			
<i>H pylori</i> serology screening sensitivity	93	82-95	[10]
<i>H pylori</i> serology screening specificity	79	70-92	[10]
<i>H pylori</i> <sup>13</sup> C-Urea breath test sensitivity	97.9	90-100	[11]
<i>H pylori</i> <sup>13</sup> C-Urea breath test specificity	95.8	90-100	[11]
Effectiveness of <i>H pylori</i> eradication	92.0	87-98	[21]
Probability of adverse effects related to eradication therapy necessitating medical intervention	2.5	2-5	[6]
Annual <i>H pylori</i> infection rate	1.0	1-3	[6,26]
Excess gastric cancer risk reduction attributable to <i>H pylori</i> eradication	30	0-100	[6]
Cost variables (2006USD) <sup>1</sup>			
<i>H pylori</i> serology screening	26	10-50	
<i>H pylori</i> <sup>13</sup> C-urea breath test	83	60-100	
<i>H pylori</i> eradication (triple therapy)	30	20-50	
Gastric cancer treatment per annum	4358	328-59 000	
Eradication-related adverse effects	50	5-100	
Other variables			
Annual discount rate for costs and effectiveness (%)	3	0-7	[19,29]
Life expectancy, years	77	76-80	[14]
Utility			
<i>H pylori</i> non-infected	1.00	0.95-1.00	[26]
<i>H pylori</i> infected	0.90	0.80-1.00	[26]
Gastric cancer	0.38	0.13-0.65	[26]

Triple therapy: Rabeprazole 20 mg, amoxicillin 1000 mg, and clarithromycin 500 mg, twice a day for 4 d. <sup>1</sup>All costs were estimated from the records of local public hospitals.

have 30% of excess risk reduction compared to those *H pylori* infected persons in the base-case analysis, while a wide range of excess risk reduction from 10% to 100% was tested in one-way sensitivity analysis.

## Costs

The present study was done from the public healthcare provider's perspective. Thus, the model included direct medical costs of serology screening, the UBT, and triple therapy. Adverse effects associated with the triple therapy that necessitated medical intervention were also included (Table 1). Annual direct medical costs associated with treatment of gastric cancer were estimated at the average level across different stages of the cancer<sup>[28]</sup>. Nonmedical direct costs and indirect costs were not included. The costs were accrued from the time of screening until death. All costs were reported in 2006 US dollars and annually discounted at 3% in all analyses<sup>[29]</sup>.

## Effectiveness

Three health outcomes evaluated in this model included number of gastric cancer cases prevented, life years saved,

and QALYs gained. All outcomes were also annually discounted at 3% in the base-case analysis<sup>[29]</sup>.

## Incremental cost-effectiveness ratio (ICER)

ICER was expressed as US dollars per life year saved and US dollars per QALY gained, which were calculated for the two screening strategies compared to no screening strategy, as well as the UBT compared to the serology screening. The cost-effectiveness threshold was estimated at \$28 000 per QALY gained in local context, which was derived from the conventional threshold of \$50 000 per QALY gained used in the United States by comparing the gross national income per capita between the United States and Singapore<sup>[28,30]</sup>.

## RESULTS

There were a total of 237 900 Chinese males aged between 35 and 44 in Singapore<sup>[31]</sup>. In the base-case scenario, compared to no screening and no eradication therapy, strategy 2 that implemented the serology screening on all cohort members with treatment for those with positive test

**Table 2** Incremental cost-effectiveness ratios of screening strategies at age 40 yr (compared to no screening strategy unless stated)

US\$/QALY	Base-case		Best-case <sup>1</sup>		Worst-case <sup>1</sup>	
	Serology	UBT	Serology	UBT	Serology	UBT
ICER per life year saved	16166	38792 477079 <sup>2</sup>	Dominant	Dominant Dominant <sup>2</sup>	389728	640000 5645449 <sup>2</sup>
ICER per QALY gained	13571	32525 390337 <sup>2</sup>	Dominant	Dominant Dominant <sup>2</sup>	324773	560000 Dominated <sup>2</sup>

ICER: Incremental cost-effectiveness ratio; QALY: Quality-adjusted life year; UBT: <sup>13</sup>C-Urea breath test. <sup>1</sup>Variables modified in best and worst-case analyses were, gastric cancer risk reduction by eradication (100% and 10%, respectively), relative risk (12 and 2), cost of annual gastric cancer treatment (\$59000 and \$328), cost of the serology screening (\$10 and \$50), cost of the UBT (\$60 and \$100), and annual discount rate (0% and 7%); <sup>2</sup>The ICER was calculated by comparing the UBT with the serology screening.

cost \$9.8 million, which saved 523 life years or gained 623 QALYs by preventing 272 gastric cancer cases. Strategy 3 that implemented the UBT on this cohort with treatment for those with positive test cost \$23.0 million, which saved 550 life years or gained 656 QALYs by preventing 281 gastric cancer cases. A total of 875 and 847 persons were screened for each case of gastric cancer prevented in strategy 2 and 3, respectively. The serology screening avoided \$1.4 million of discounted expenditures on treatment of gastric cancer, while the UBT avoided \$1.5 million. The ICER were \$16166 per life year saved and \$13571 per QALY gained for the serology screening, and \$38792 per life year saved and \$32525 per QALY gained for the UBT (Table 2). When compared to serology screening, the ICER was \$477079 per life year saved or \$390337 per QALY gained for the UBT.

In the one-way sensitivity analyses, the level of excess gastric cancer risk reduction attributable to *H pylori* eradication varied from 10% to 100%<sup>[6,7]</sup>. Using a \$28000 per QALY gained as a threshold, the serology screening would be cost-effective if *H pylori* eradication reduced more than 15% of excess gastric cancer risk. In contrast, the UBT could be cost-effective only when the excess gastric cancer risk was reduced by 35% or more (Figure 2A).

The ICER was sensitive to age at which population-based screening was carried out as shown in Figure 3. When screening age was more than 32 years, the ICER was less than \$28000 per QALY gained for the serology screening. The UBT appeared cost-effective when the screening age was more than 45 years (Figure 2B).

Relative risk of gastric cancer for *H pylori* infected population had a significant impact on the ICER. When *H pylori* eradication was assumed to reduce 30% of excess gastric cancer risk (as in the base-case analysis), the serology screening appeared cost-effective over the full range of the relative risk (i.e. from 2 to 12). In contrast, the UBT appeared cost-effective only with the relative risk above 5 (Figure 2C).

Cost of annual gastric cancer treatment imposed a substantial impact on the cost-effectiveness of the strategies. For both strategies, the cost had an approximately linear relation with the ICER that decreased dramatically with the increase in annual cost of the cancer treatment (Figure 2D). When the annual cost was \$30075, the one-time expenditure on serology screening and treatment of those with positive test would be fully offset by the savings in preventing gastric

cancers (Figure 2D). Cost of the serology screening and the UBT also had a moderate impact on the ICER. Each \$5 increment in cost of the serology screening and the UBT augmented the ICER by \$2000 and \$1800, respectively (data not shown).

The ICER was also sensitive to the annual discount rate. With the increase in the annual discount rate, the ICER appeared less favorable for both strategies.

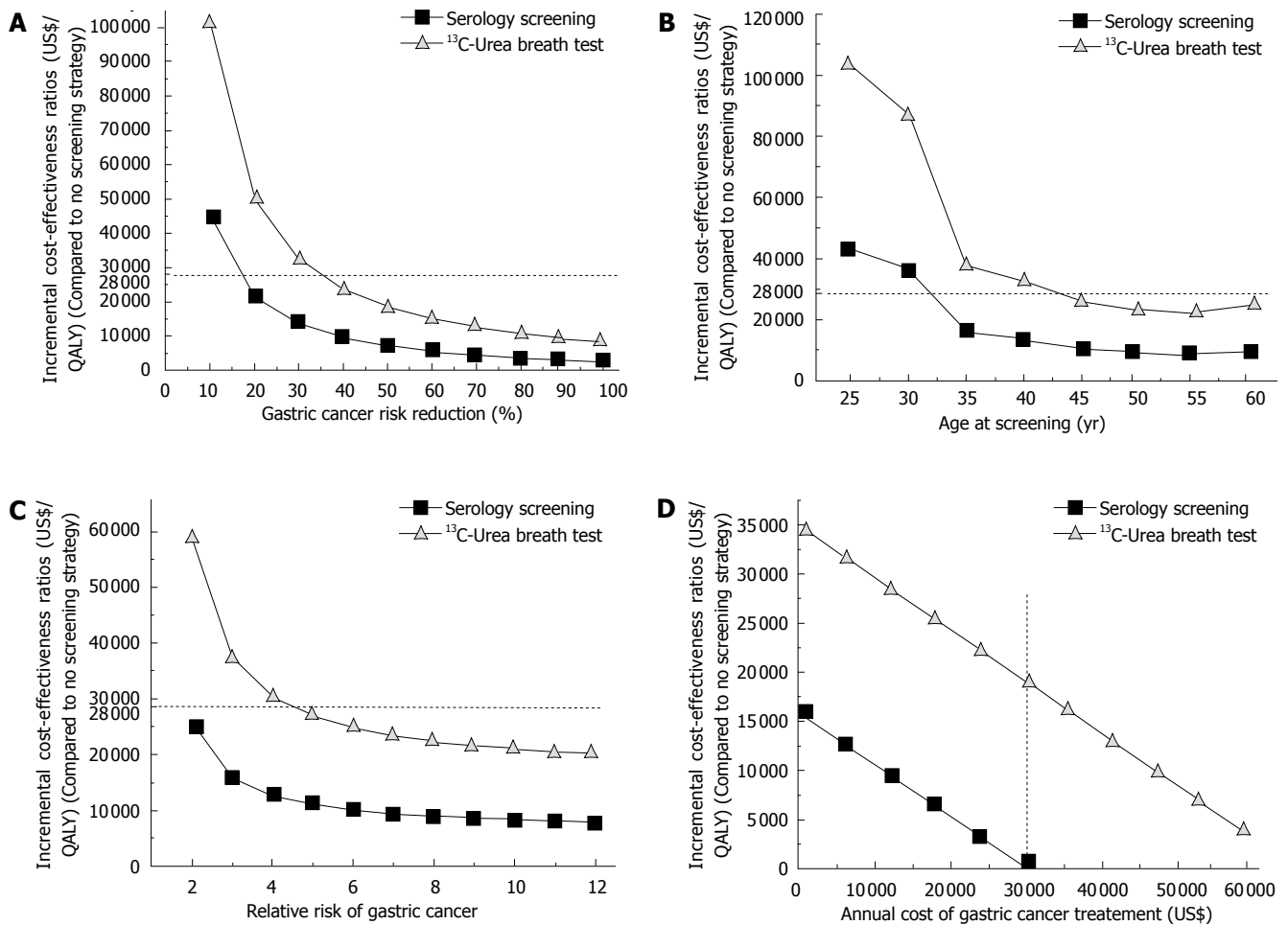
Other variables had little impact on the cost-effectiveness within the ranges listed in Table 1, which included sensitivity and specificity of the serology screening and the UBT, effectiveness of *H pylori* eradication, probability and costs of adverse effects related to eradication therapy necessitating medical intervention, and utilities of each health state.

In all these sensitivity analyses, the ICER was extremely less favorable for the UBT compared to the serology screening.

In the best-case and worst-case analyses, the most critical variables, including level of excess gastric cancer risk reduction, relative risk of gastric cancer in *H pylori* infected population, annual cost of gastric cancer treatment, cost of the serology screening and the UBT, and annual discount rate, were simultaneously varied. Both strategies achieved more health benefits (i.e. life years gained or QALYs) at a lower cost compared to no screening, and the UBT also received more health benefit at a lower cost compared to the serology screening in the best-case scenario (i.e. dominant) (Table 2). In contrast, the ICER was more than \$300000 for all comparisons in the worse-case scenario. The UBT achieved the same gaining in QALYs but at an extra cost of \$11290897 compared to the serology screening in the worst case analysis (Table 2).

## DISCUSSION

The present study modeled the life-time cost and effectiveness associated with population-based *H pylori* screening and treatment for those with positive test in Chinese males. Compared to no screening and no eradication therapy strategy, the serology screening was cost-effective, while the UBT was not cost-effective based on the threshold of \$28000 per QALY gained. The UBT gained very little extra health benefits in terms of either



**Figure 2** A: Sensitivity analysis on excess gastric cancer risk reduction attributable to *H pylori* eradication; B: Sensitivity analysis on age at time of screening; C: Sensitivity analysis on relative risk of gastric cancer in *H pylori* infected people with 30% gastric cancer risk reduction attributable to eradication; D: Sensitivity analysis on annual cost of gastric cancer treatment.

life years saved or QALYs gained but at a substantially higher cost compared to the serology screening. This suggests that the population-based serology screening for *H pylori* infection be adopted in this specific population, especially under the circumstances that the cost of gastric cancer treatment keeps arising due to the advances in new technologies. Also with this model, future clinical advances on the efficacy of *H pylori* eradication in prevention of gastric cancer can be easily translated into the cost-effectiveness ratio, which is now playing an increasingly important role in informing medical decision making.

The serology screening was found to be cost-effective in the present study, which is similar to the published studies using the similar model to estimate the economic and clinical effects of *H pylori* screening<sup>[6,7]</sup>. Nevertheless, the model used in the present study had several improvements which are worth noting. First, we have a health state to identify the persons who were *H pylori* positive and successfully eradicated by the triple therapy (i.e. '*H pylori* eradicated' in Figure 1). This is a health state in the Markov model which can allow for successful capturing of the economic and health benefits resulted from the screening strategies. Second, in line with the important assumption that the persons who survived more than 5 years after diagnosis of gastric

cancer were assumed to be cured<sup>[7,20]</sup>, we used five tunnel states, instead of a single gastric cancer health state, to represent the status for each of the first five years since diagnosed with gastric cancer. The mortalities for these tunnel states were different from each other based on the epidemiological evidence<sup>[19]</sup>. This refinement may better simulate the real progress of gastric cancer and thus obtain more accurate estimations in cost and effectiveness. Third, this model is life-time estimation and every person remained in the model until death. Thus some parameters are time-sensitive including *H pylori* incidence, gastric cancer incidence, and mortality (Table 1). Instead of fixed point estimates, age-specific estimates may be more appropriate and accurate to reflect the changes in these important parameters with the aging of the cohort in the model.

Besides, some differences between these two studies and the present study are notable. The cost and effectiveness of the screening strategies essentially stemmed from the actual number of gastric cancer cases prevented by the strategies. Therefore, given the certain level of excess gastric cancer risk reduction by the eradication, cost of gastric cancer treatment and relative risk of gastric cancer in *H pylori* infected persons are deemed to have a very important and significant impact on



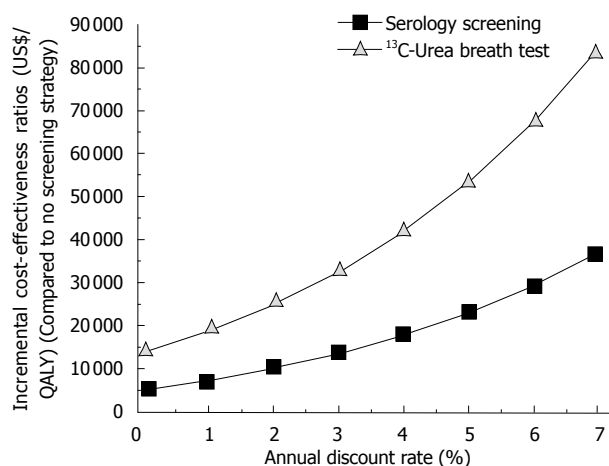


Figure 3 Sensitivity analysis on annual discount rate.

the estimated ICER. The screening strategies would save more money if the cost needed to treat a gastric cancer case increased and prevent more gastric cancer cases if relative risk of gastric cancer in *H pylori* infected persons increased. Furthermore, the economic and health benefits of prevention of gastric cancer cases may only occur in the future rather than in the present, which highlights the important role of discount rate used: the larger the discount rate used the less the benefits obtained (Figure 3). However, these parameters were not examined in some previous study<sup>[6]</sup>, or only little impact of these parameters was reported<sup>[7]</sup>.

The cost-effectiveness of the serology screening over the UBT study was robust to most of the parameters through the one-way sensitivity analyses. Nevertheless, some findings are worth attention. As shown in Figure 3, the screening strategies would be more cost-effective if the starting age increased, which might be explicitly explained by the fact that both *H pylori* infection rate and gastric cancer incidence would increase with age. However, a recent large randomized controlled trial in Chinese revealed that persons with precancerous lesions (gastric atrophy, intestinal metaplasia, and dysplasia) significantly reduced the efficacy of *H pylori* eradication in prevention of gastric cancer compared to those without the lesions<sup>[32]</sup>. As the precancerous lesions increased significantly with age in Chinese<sup>[33]</sup>, this could be important evidence to support the younger screening age. Thus, we suggested that the optimal screening age could be 35 years where there would be a substantial improvement on the ICER compared to younger age but only slight improvement compared to older age. Otherwise, if an older screening age was chosen, despite the increase in *H pylori* infection rate and gastric cancer incidence, the level of excess gastric cancer risk reduction (i.e. the efficacy of the eradication) would remain at the far lower end of the spectrum, favoring no screening against the serology screening (Figure 2).

Prevention of gastric cancer will save the medical expenditures for treatment of cancer and increase the life years and QALYs. However, this health benefit could be associated with additional medical expenditures (even the expenditures on daily living for extended life years)

incurred during the extended life years, which will not occur in case of premature death. As including this cost component remains controversial, we did not take it into consideration in the present study. We also acknowledged that some parameters used in the model (e.g. survival rate of gastric cancer) were not available for Chinese males in Singapore, which may limit the accuracy of point estimates for cost and effectiveness. Finally, the threshold for ICER used in the present study was estimated from the US threshold using the ratio of gross national income between two countries, which is relative arbitrary and warrants further empirical local studies on this important topic.

In summary, the population-based serology screening for *H pylori* infection was more cost-effective than the UBT in prevention of gastric cancer in Singapore Chinese males.

## COMMENTS

### Background

*H pylori* infection has been recognized as an important risk factor for gastric cancer. Screening for *H pylori* has been proposed as a cost-effective strategy in prevention of gastric cancer.

### Research frontiers

A number of screening strategies are currently available. However, it is unknown which screening strategy is more cost-effective in high-risk populations, especially in Asian populations.

### Innovations and breakthroughs

A separate health state was used to identify the persons who were *H pylori* positive and successfully eradicated by the triple therapy. This state can allow for successful capturing of the economic and health benefits resulted from the screening strategies. Five tunnel states, instead of a single gastric cancer health state, were used in line with the important assumption that the persons who survived more than 5 years after diagnosis of gastric cancer were assumed to be cured.

### Applications

The findings in this study will be useful and important for decision makers to efficiently allocate scarce health resources for population-based *H pylori* screening.

### Peer review

The authors studied a clinically relevant issue. The manuscript is well written and is worth of publication in the Journal as is. This study has a substantial element of novelty. There is no data in literature concerning cost-effectiveness of serology-based screening strategy, particularly in countries with high prevalence of the infection, where the gastric cancer is a problem of special importance.

## REFERENCES

- 1 Kelley JR, Duggan JM. Gastric cancer epidemiology and risk factors. *J Clin Epidemiol* 2003; **56**: 1-9
- 2 Crew KD, Neugut AI. Epidemiology of gastric cancer. *World J Gastroenterol* 2006; **12**: 354-362
- 3 Forman D, Newell DG, Fullerton F, Yarnell JW, Stacey AR, Wald N, Sitas F. Association between infection with *Helicobacter pylori* and risk of gastric cancer: evidence from a prospective investigation. *BMJ* 1991; **302**: 1302-1305
- 4 Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelstein JH, Orentreich N, Sibley RK. *Helicobacter pylori* infection and the risk of gastric carcinoma. *N Engl J Med* 1991; **325**: 1127-1131
- 5 An international association between *Helicobacter pylori* infection and gastric cancer. The EUROGAST Study Group. *Lancet* 1993; **341**: 1359-1362

- 6 **Fendrick AM**, Chernew ME, Hirth RA, Bloom BS, Bandekar RR, Scheiman JM. Clinical and economic effects of population-based *Helicobacter pylori* screening to prevent gastric cancer. *Arch Intern Med* 1999; **159**: 142-148
- 7 **Parsonnet J**, Harris RA, Hack HM, Owens DK. Modelling cost-effectiveness of *Helicobacter pylori* screening to prevent gastric cancer: a mandate for clinical trials. *Lancet* 1996; **348**: 150-154
- 8 **Roderick P**, Davies R, Raftery J, Crabbe D, Pearce R, Patel P, Bhandari P. Cost-effectiveness of population screening for *Helicobacter pylori* in preventing gastric cancer and peptic ulcer disease, using simulation. *J Med Screen* 2003; **10**: 148-156
- 9 **Miwa H**, Go MF, Sato N. *H. pylori* and gastric cancer: the Asian enigma. *Am J Gastroenterol* 2002; **97**: 1106-1112
- 10 **Kang JY**, Yeoh KG, Ho KY, Guan R, Lim TP, Quak SH, Wee A, Teo D, Ong YW. Racial differences in *Helicobacter pylori* seroprevalence in Singapore: correlation with differences in peptic ulcer frequency. *J Gastroenterol Hepatol* 1997; **12**: 655-659
- 11 **Lee HS**, Gwee KA, Teng LY, Kang JY, Yeoh KG, Wee A, Chua BC. Validation of [<sup>13</sup>C]urea breath test for *Helicobacter pylori* using a simple gas chromatograph-mass selective detector. *Eur J Gastroenterol Hepatol* 1998; **10**: 569-572
- 12 **Briggs A**, Sculpher M. An introduction to Markov modelling for economic evaluation. *Pharmacoeconomics* 1998; **13**: 397-409
- 13 **Sonnenberg FA**, Beck JR. Markov models in medical decision making: a practical guide. *Med Decis Making* 1993; **13**: 322-338
- 14 **World Health Organization**. Mortality Country Fact Sheet 2006 Singapore. Geneva: World Health Organization, 2006
- 15 **The Committee on Epidemic Diseases**. Seroprevalence of *Helicobacter pylori* infection in Singapore. *Epidemiological News Bulletin* 1996; **22**: 31-32
- 16 **Singapore Department of Statistics**. Yearbook of Statistics 2006 Singapore. Singapore: Department of Statistics 2006
- 17 **Seow A**, Koh WP, Chia KS, Shi LM, Lee HP, Shanmugaratnam K. Trends in Cancer Incidence in Singapore 1968-2002. Singapore: Singapore Cancer Registry Report No. 6, 2004
- 18 **Forman D**, Webb P, Parsonnet J. *H pylori* and gastric cancer. *Lancet* 1994; **343**: 243-244
- 19 **Tian J**, Wang XD, Chen ZC. Survival of patients with stomach cancer in Changle city of China. *World J Gastroenterol* 2004; **10**: 1543-1546
- 20 **Koga S**, Kaibara N, Kishimoto H, Nishidoi H, Kimura O, Okamoto T, Tamura H. Comparison of 5- and 10-year survival rates in operated gastric cancer patients. Assessment of the 5-year survival rate as a valid indicator of postoperative curability. *Langenbecks Arch Chir* 1982; **356**: 37-42
- 21 **Yang KC**, Wang GM, Chen JH, Chen TJ, Lee SC. Comparison of rabeprazole-based four- and seven-day triple therapy and omeprazole-based seven-day triple therapy for *Helicobacter pylori* infection in patients with peptic ulcer. *J Formos Med Assoc* 2003; **102**: 857-862
- 22 **Gambara C**, Bilardi C, Dulbecco P, Iiritano E, Zentilin P, Mansia C, Usai P, Vigneri S, Savarino V. Comparable *Helicobacter pylori* eradication rates obtained with 4- and 7-day rabeprazole-based triple therapy: a preliminary study. *Dig Liver Dis* 2003; **35**: 763-767
- 23 **Danese S**, Armuzzi A, Romano A, Cremonini F, Candelli M, Franceschi F, Ojetti V, Venuti A, Pola P, Gasbarrini G, Gasbarrini A. Efficacy and tolerability of antibiotics in patients undergoing *H pylori* eradication. *Hepatogastroenterology* 2001; **48**: 465-467
- 24 **Lam SK**, Talley NJ. Report of the 1997 Asia Pacific Consensus Conference on the management of *Helicobacter pylori* infection. *J Gastroenterol Hepatol* 1998; **13**: 1-12
- 25 **Stack WA**, Knifton A, Thirlwell D, Cockayne A, Jenkins D, Hawkey CJ, Atherton JC. Safety and efficacy of rabeprazole in combination with four antibiotic regimens for the eradication of *Helicobacter pylori* in patients with chronic gastritis with or without peptic ulceration. *Am J Gastroenterol* 1998; **93**: 1909-1913
- 26 **Wang Q**, Jin PH, Lin GW, Xu SR, Chen J. Cost-effectiveness of *Helicobacter pylori* screening to prevent gastric cancer: Markov decision analysis. *Zhonghua Liuxingbingxue Zazhi* 2003; **24**: 135-139
- 27 **Eslick GD**, Lim LL, Byles JE, Xia HH, Talley NJ. Association of *Helicobacter pylori* infection with gastric carcinoma: a meta-analysis. *Am J Gastroenterol* 1999; **94**: 2373-2379
- 28 **Dan YY**, So JB, Yeoh KG. Endoscopic screening for gastric cancer. *Clin Gastroenterol Hepatol* 2006; **4**: 709-716
- 29 **Lipscomb J**, Weinstein MC, Torrance GW. Time preference. In: Gold MR, Siegel JE, Russell LB, Weinstein MC. Cost-Effectiveness in Health and Medicine. New York: Oxford University Press 1996: 214-246
- 30 **World Bank**. World Development Indicators Database.: World Bank, 2006
- 31 **Department of Statistics**. Census of Population 2000 Statistical Release 1: Demographic Characteristics. Singapore: Department of Statistics, 2001
- 32 **Wong BC**, Lam SK, Wong WM, Chen JS, Zheng TT, Feng RE, Lai KC, Hu WH, Yuen ST, Leung SY, Fong DY, Ho J, Ching CK, Chen JS. *Helicobacter pylori* eradication to prevent gastric cancer in a high-risk region of China: a randomized controlled trial. *JAMA* 2004; **291**: 187-194
- 33 **You WC**, Blot WJ, Li JY, Chang YS, Jin ML, Kneller R, Zhang L, Han ZX, Zeng XR, Liu WD. Precancerous gastric lesions in a population at high risk of stomach cancer. *Cancer Res* 1993; **53**: 1317-1321

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CLINICAL RESEARCH

## Endoscopic ultrasound: It's accuracy in evaluating mediastinal lymphadenopathy? A meta-analysis and systematic review

Srinivas R Puli, Jyotsna Batapati Krishna Reddy, Matthew L Bechtold, Jamal A Ibdah, Daphne Antillon, Shailender Singh, Mojtaba Olyaei, Mainor R Antillon

Srinivas R Puli, Jyotsna Batapati Krishna Reddy, Matthew L Bechtold, Jamal A Ibdah, Daphne Antillon, Mainor R Antillon, Division of Gastroenterology and Hepatology, University of Missouri- Columbia, Columbia, Missouri 65212, United states  
Shailender Singh, Mojtaba Olyaei, Division of Gastroenterology and Hepatology, University of Kansas School of Medicine, Kansas City, Kansas 66160, United states

**Author contributions:** Puli SR designed the research; Puli SR and Batapati Krishna Reddy J collected the data; Puli SR contributed analytic tools; Puli SR analyzed data; Puli SR wrote the paper; and Puli SR, Batapati Krishna Reddy J, Bechtold ML, Ibdah JA, Antillon D, Singh S, Olyaei M, and Antillon MR helped edit the paper.

**Correspondence to:** Mainor R Antillon, MD, Division of Gastroenterology, One Hospital Drive, M580a, Columbia, MO 65212, United states. [antillonmr@missouri.edu](mailto:antillonmr@missouri.edu)

Telephone: +1-573-8821013 Fax: +1-573-8844595

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### Abstract

**AIM:** To evaluate the accuracy of endoscopic ultrasound (EUS), EUS-fine needle aspiration (FNA) in evaluating mediastinal lymphadenopathy.

**METHODS:** Only EUS and EUS-FNA studies confirmed by surgery or with appropriate follow-up were selected. Articles were searched in Medline, Pubmed, and Cochrane control trial registry. Only studies from which a 2 × 2 table could be constructed for true positive, false negative, false positive and true negative values were included. Two reviewers independently searched and extracted data. The differences were resolved by mutual agreement. Meta-analysis for the accuracy of EUS was analyzed by calculating pooled estimates of sensitivity, specificity, likelihood ratios, and diagnostic odds ratios. Pooling was conducted by both Mantel-Haenszel method (fixed effects model) and DerSimonian Laird method (random effects model). The heterogeneity of studies was tested using Cochran's Q test based upon inverse variance weights.

**RESULTS:** Data was extracted from 76 studies ( $n = 9310$ ) which met the inclusion criteria. Of these, 44 studies used EUS alone and 32 studies used EUS-FNA. FNA improved the sensitivity of EUS from 84.7% (95% CI: 82.9-86.4) to 88.0% (95% CI: 85.8-90.0). With FNA, the specificity of EUS improved from 84.6% (95% CI: 83.2-85.9) to 96.4% (95% CI: 95.3-97.4). The  $P$  for

chi-squared heterogeneity for all the pooled accuracy estimates was  $> 0.10$ .

**CONCLUSION:** EUS is highly sensitive and specific for the evaluation of mediastinal lymphadenopathy and FNA substantially improves this. EUS with FNA should be the diagnostic test of choice for evaluating mediastinal lymphadenopathy.

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**Key words:** Endoscopic ultrasound; EUS-fine needle aspiration; Mediastinal lymphadenopathy

**Peer reviewer:** Damian Casadesus Rodriguez, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

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### INTRODUCTION

Management of patients with mediastinal lymphadenopathy depends on the etiology of lymphadenopathy. Differentiating inflammatory from neoplastic processes in the mediastinal lymph nodes is not only important from the treatment standpoint, but also vital in predicting survival. Multiple diagnostic modalities are available to evaluate mediastinal lymphadenopathy. Computer tomography (CT) of the chest does not clearly image the aortopulmonary, subcarinal, and paraesophageal areas due to the lowering of image resolution because of the movement and partial volume effect of pulmonary vessels, aortic arch, and left atrium<sup>[1]</sup>. Also, for lesions smaller than 1 cm, the sensitivity of CT is low<sup>[2-5]</sup>, and the size-based criteria to diagnose metastatic involvement of the lymph nodes have lower accuracy<sup>[6]</sup>. Therefore, other methods were introduced, including transbronchial biopsy, CT-guided transthoracic fine-needle aspiration (FNA), mediastinoscopy, or thoracoscopic biopsy.

In the transbronchial technique, the FNA needle

is advanced blindly, reducing the yield of diagnosing subcarinal and paraesophageal nodes to approximately 50%<sup>[7,8]</sup>. Due to the potential danger of inadvertent vascular puncture, transthoracic biopsy is avoided when the mass is close to major vessels. This procedure is also associated with significant complications, including bleeding and pneumothorax in up to 25%-35% of cases<sup>[9,10]</sup>. Extended cervical mediastinoscopy or anterior mediastinoscopy can be used to access level 5 (aortopulmonary window) mediastinal nodes, which is not inspected by the standard methods<sup>[11-13]</sup>. Extended cervical mediastinoscopy has a sensitivity of 83% in examining the paraaortic and subaortic lymph node chains, but the subcarinal group is inaccessible<sup>[11]</sup>. Thoracoscopy can visualize the inferior mediastinum effectively, but it is limited only to accessing the level of major bronchi, leaving the superior mediastinum non-visualized<sup>[14]</sup>. Both procedures are invasive, require hospitalization and general anesthesia, and both have limitations.

With the introduction of endoscopic ultrasonography (EUS), it is now possible to visualize not only the gastrointestinal tract but also surrounding structures. However, EUS is limited in its ability to distinguish an inflammatory/reactive process from a malignancy, particularly within lymph nodes<sup>[15,16]</sup>. The accuracy of EUS in diagnosing mediastinal lymphadenopathy has been varied<sup>[17-21]</sup>. FNA during EUS may be performed safely in a short outpatient procedure setting without general anesthesia. It is not clear to what extent, if any, FNA adds in improving the accuracy of EUS to diagnose mediastinal lymphadenopathy<sup>[22-25]</sup>.

The goal of this meta-analysis was to evaluate the accuracy of EUS alone and EUS with FNA in correctly diagnosing mediastinal lymphadenopathy. Due to multiple studies scattered in the literature and no published meta-analysis in this area, this meta-analysis was performed in an attempt to answer this essential clinical question. This meta-analysis and systematic review was written in accordance with the proposal for reporting by the QUOROM (Quality of Reporting of Meta-analyses) statement<sup>[26]</sup>. Since this manuscript looks at diagnostic accuracy of a test, the study design for this meta-analysis and systematic review conformed to the guidelines of Standards for Reporting of Diagnostic Accuracy (STARD) initiative<sup>[27]</sup>.

## MATERIALS AND METHODS

### Study selection criteria

Only EUS-FNA studies confirmed by surgery or appropriate follow-up were selected. From this pool, only studies from which a 2 × 2 table could be constructed for true positive, false negative, false positive and true negative values were included.

### Data collection and extraction

Articles were searched in Medline, Pubmed, Ovid journals, Cumulative Index for Nursing & Allied Health Literature, ACP journal club, DARE, International Pharmaceutical Abstracts, old Medline, Medline non-indexed citations, OVID Healthstar, and Cochrane Control Trial Registry.

The search terms used were endoscopic ultrasound, EUS, ultrasound, mediastinal lymphadenopathy, nodal invasion, fine needle aspiration, FNA, staging, surgery, sensitivity, specificity, positive predictive value, and negative predictive value. 2 × 2 tables were constructed with the data extracted from each study. To give validity to the data, two authors (SP and JR) independently searched and extracted the data into an abstraction form. Any differences were resolved by mutual agreement.

### Quality of studies

Clinical trial with a control arm can be assessed for the quality of the study. A number of criteria have been used to assess this quality of a study (e.g. randomization, selection bias of the arms in the study, concealment of allocation, and blinding of outcome)<sup>[28,29]</sup>. There is no consensus on how to assess studies without a control arm. Hence, these criteria do not apply to studies without a control arm<sup>[29]</sup>. Therefore, for this meta-analysis and systematic review, studies were selected based on completeness of data and inclusion criteria.

### Statistical analysis

Meta-analysis for the accuracy of EUS in diagnosing the etiology of mediastinal lymphadenopathy was performed by calculating pooled estimates of sensitivity, specificity, likelihood ratios, and diagnostic odds ratios. EUS studies were grouped into time periods to standardize the change in EUS technology and EUS criteria for lymph node involvement<sup>[30]</sup>. These time periods were 1988 to 1994, 1995 to 1999, and 2000 to 2006. Pooling was conducted using both Mantel-Haenszel method (fixed effects model) and DerSimonian Laird method (random effects model). The confidence intervals were calculated using the F distribution method<sup>[31]</sup>. The width of the point estimates in the Forrest plots indicates the assigned weight to that study. For 0 value cells, a 0.5 was added as described by Cox<sup>[32]</sup>. The heterogeneity of the sensitivities and specificities was tested by applying the likelihood ratio test<sup>[33]</sup>. The heterogeneity of likelihood ratios and diagnostic odds ratios were tested using Cochran's *Q* test based upon inverse variance weights<sup>[34]</sup>. Heterogeneity among studies was also tested by using summary receiver operating characteristic (SROC) curves. SROC curves were used to calculate the area under the curve (AUC). The effect of publication and selection bias on the summary estimates was tested by Harbord-Egger bias indicator<sup>[35]</sup> and Begg-Mazumdar indicator<sup>[36]</sup>. Also, funnel plots were constructed to evaluate potential publication bias using the standard error and diagnostic odds ratio<sup>[37,38]</sup>.

## RESULTS

The initial search using the search terms identified 4310 reference articles. Among these, 460 relevant articles were selected and reviewed by two authors independently. Data was extracted from 76 studies (*n* = 9310) which met the inclusion criteria. Of these, 44 studies used EUS alone<sup>[17,18,39-80]</sup> and 32 studies used EUS-FNA<sup>[19-25,81-107]</sup>. Figure 1 shows the search results. Table 1 shows the characteristics for EUS studies without FNA and Table 2



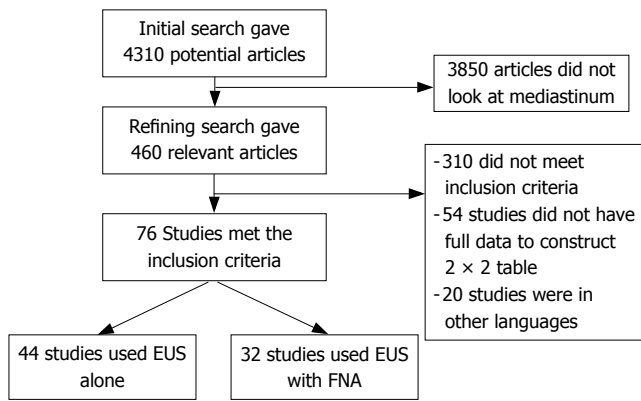


Figure 1 The search results.

Table 1 Characteristics of studies included in this meta-analysis for EUS without FNA

Author	Year of publication	No. of patients	Type of recruitment	Confirmatory procedure
Tio <i>et al</i> <sup>[71]</sup>	1986	26	Prospective	Surgery
Murata <i>et al</i> <sup>[57]</sup>	1988	173	Consecutive	Surgery
Tio <i>et al</i> <sup>[69]</sup>	1989	75	Prospective	Surgery
Vilgrain <i>et al</i> <sup>[75]</sup>	1990	51	Consecutive	Surgery
Tio <i>et al</i> <sup>[68]</sup>	1990	102	Consecutive	Surgery
Rice <i>et al</i> <sup>[63]</sup>	1991	22	Consecutive	Surgery
Heintz <i>et al</i> <sup>[52]</sup>	1991	40	Consecutive	Surgery
Botet <i>et al</i> <sup>[40]</sup>	1991	50	Consecutive	Surgery
Tio <i>et al</i> <sup>[70]</sup>	1989	74	Prospective	Surgery
Ziegler <i>et al</i> <sup>[80]</sup>	1991	52	Consecutive	Surgery
Rosch <i>et al</i> <sup>[64]</sup>	1992	44	Consecutive	Surgery
Fok <i>et al</i> <sup>[46]</sup>	1992	54	Consecutive	Surgery
Yoshikane <i>et al</i> <sup>[79]</sup>	1993	28	Consecutive	Surgery
Grimm <i>et al</i> <sup>[49]</sup>	1993	63	Prospective	Surgery
Dittler <i>et al</i> <sup>[45]</sup>	1993	167	Consecutive	Surgery
Peters <i>et al</i> <sup>[61]</sup>	1994	42	Consecutive	Surgery
Catalano <i>et al</i> <sup>[43]</sup>	1994	100	Consecutive	Surgery
McLoughlin <i>et al</i> <sup>[18]</sup>	1995	15	Consecutive	Surgery
Binmoeller <i>et al</i> <sup>[39]</sup>	1995	87	Prospective	Surgery
HunerBein <i>et al</i> <sup>[53]</sup>	1996	19	Consecutive	Surgery
Hasegawa <i>et al</i> <sup>[50]</sup>	1996	22	Consecutive	Surgery
Francois <i>et al</i> <sup>[47]</sup>	1996	29	Consecutive	Surgery
Natsugoe <i>et al</i> <sup>[58]</sup>	1996	37	Consecutive	Surgery
Milena <i>et al</i> <sup>[54]</sup>	1997	40	Prospective	Surgery
Vikers <i>et al</i> <sup>[73]</sup>	1997	50	Consecutive	Surgery
Shimizu <i>et al</i> <sup>[67]</sup>	1997	431	Consecutive	Surgery
Pham <i>et al</i> <sup>[62]</sup>	1998	28	Consecutive	Surgery
Vikers <i>et al</i> <sup>[74]</sup>	1998	50	Prospective	Surgery
Salminen <i>et al</i> <sup>[65]</sup>	1999	32	Consecutive	Surgery
Krasna <i>et al</i> <sup>[56]</sup>	1999	88	Consecutive	Surgery
Browrey <i>et al</i> <sup>[41]</sup>	1999	98	Prospective	Surgery
Catalano <i>et al</i> <sup>[42]</sup>	1999	149	Prospective	Surgery
Giovannini <i>et al</i> <sup>[48]</sup>	1999	198	Prospective	Surgery
Nishimaki <i>et al</i> <sup>[60]</sup>	1999	224	Consecutive	Surgery
Heidemann <i>et al</i> <sup>[51]</sup>	2000	68	Consecutive	Surgery
Nesje <i>et al</i> <sup>[59]</sup>	2000	68	Prospective	Surgery
Vazquez-Sequeiros <i>et al</i> <sup>[105]</sup>	2001	37	Consecutive	Surgery
Wiersema <i>et al</i> <sup>[77]</sup>	2001	82	Prospective	Surgery
Wakelin <i>et al</i> <sup>[76]</sup>	2002	36	Consecutive	Surgery
Kienle <i>et al</i> <sup>[55]</sup>	2002	117	Prospective	Surgery
Schwartz <i>et al</i> <sup>[66]</sup>	2002	188	Consecutive	Surgery
Wu <i>et al</i> <sup>[78]</sup>	2003	31	Prospective	Surgery
Arima <i>et al</i> <sup>[17]</sup>	2003	58	Consecutive	Surgery
DeWitt <i>et al</i> <sup>[44]</sup>	2005	102	Prospective	Surgery

Table 2 Characteristics of studies included in this meta-analysis for EUS with FNA

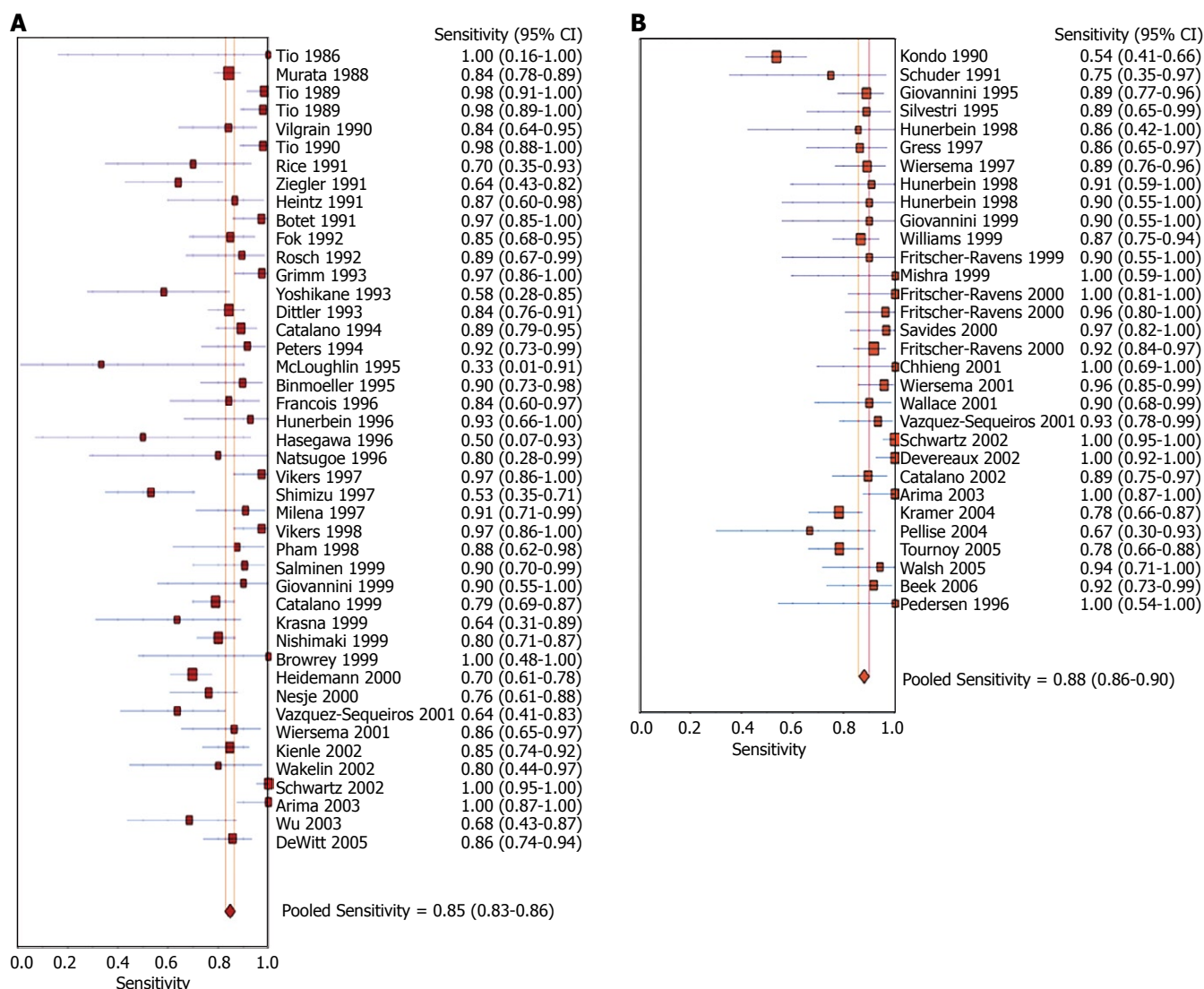
Author	Year of publication	No. of patients	Type of recruitment	Confirmatory procedure
Kondo <i>et al</i> <sup>[6]</sup>	1990	503	Consecutive	Surgery
Schuder <i>et al</i> <sup>[25]</sup>	1991	32	Consecutive	Surgery
Silvestri <i>et al</i> <sup>[83]</sup>	1995	27	Prospective	Surgery
Giovannini <i>et al</i> <sup>[82]</sup>	1995	141	Prospective	Surgery or appropriate follow-up
Pedersen <i>et al</i> <sup>[121]</sup>	1996	9	Consecutive	FNA and appropriate follow-up
HunerBein <i>et al</i> <sup>[90]</sup>	1996	19	Consecutive	Surgery
Gress <i>et al</i> <sup>[19]</sup>	1997	52	Prospective	Surgery
Wiersema <i>et al</i> <sup>[104]</sup>	1997	60	Consecutive	FNA and appropriate follow-up
HunerBein <i>et al</i> <sup>[91]</sup>	1998	15	Consecutive	Surgery
HunerBein <i>et al</i> <sup>[98]</sup>	1998	16	Consecutive	Surgery
Fritscher-Ravens <i>et al</i> <sup>[101]</sup>	1999	16	Consecutive	FNA and appropriate follow-up
Mishra <i>et al</i> <sup>[102]</sup>	1999	111	Consecutive	FNA and appropriate follow-up
Giovannini <i>et al</i> <sup>[81]</sup>	1999	198	Prospective	Surgery or appropriate follow-up
Williams <i>et al</i> <sup>[89]</sup>	1999	333	Prospective	Surgery or appropriate follow-up
Fritscher-Ravens <i>et al</i> <sup>[84]</sup>	2000	35	Prospective	Surgery
Fritscher-Ravens <i>et al</i> <sup>[98]</sup>	2000	35	Consecutive	FNA and appropriate follow-up
Savides <i>et al</i> <sup>[100]</sup>	2000	54	Consecutive	FNA and appropriate follow-up
Fritscher-Ravens <i>et al</i> <sup>[103]</sup>	2000	153	Consecutive	FNA and appropriate follow-up
Vazquez-Sequeiros <i>et al</i> <sup>[105]</sup>	2001	37	Consecutive	Surgery
Wallace <i>et al</i> <sup>[91]</sup>	2001	43	Consecutive	FNA and appropriate follow-up
Wiersema <i>et al</i> <sup>[85]</sup>	2001	82	Prospective	Surgery
Chhieng <i>et al</i> <sup>[96]</sup>	2001	103	Consecutive	Surgery
Devereaux <i>et al</i> <sup>[22]</sup>	2002	49	Consecutive	Surgery
Catalano <i>et al</i> <sup>[92]</sup>	2002	62	Consecutive	Surgery
Schwartz <i>et al</i> <sup>[66]</sup>	2002	188	Consecutive	Surgery
Arima <i>et al</i> <sup>[93]</sup>	2003	58	Consecutive	Surgery
Pellise <i>et al</i> <sup>[23]</sup>	2004	11	Consecutive	Surgery
Kramer <i>et al</i> <sup>[86]</sup>	2004	81	Prospective	Surgery
Walsh <i>et al</i> <sup>[97]</sup>	2005	27	Consecutive	Surgery or appropriate follow-up
Tournoy <i>et al</i> <sup>[88]</sup>	2005	67	Prospective	Surgery
Khoo <i>et al</i> <sup>[93]</sup>	2006	20	Prospective	Surgery
Beek <i>et al</i> <sup>[87]</sup>	2006	43	Prospective	Surgery

76 selected studies were published as full-text articles in peer review journals. The pooled estimates given are estimates calculated by the fixed effect model.

### Accuracy of EUS with and without FNA

Pooled sensitivity to diagnose the cause for mediastinal lymphadenopathy was 84.7% (95% CI: 82.9-86.4) for

depicts characteristics of EUS studies with FNA. All the



**Figure 2** Forrest plots. **A:** Sensitivity of EUS alone in diagnosing mediastinal lymphadenopathy; **B:** Sensitivity of EUS-FNA in diagnosing mediastinal lymphadenopathy.

EUS alone *versus* 88.0% (95% CI: 85.8-90.0) for EUS with FNA. The Forrest plot showing the sensitivity of EUS with and without FNA in various studies is shown in Figure 2A and B, respectively. EUS without FNA had a pooled specificity of 84.6% (95% CI: 83.2-85.9) and with FNA was 96.4% (95% CI: 95.3-97.4). Forrest plots showing specificity from various studies with and without FNA is depicted in Figure 3A and B, respectively.

The pooled positive likelihood ratio of EUS without FNA was 3.3 (95% CI: 2.6-4.3) and with FNA was 11.2 (95% CI: 5.9-21.2). The pooled negative likelihood ratio was 0.24 (95% CI: 0.1-0.3) for EUS without FNA and 0.13 (95% CI: 0.1-0.2) for EUS with FNA. The diagnostic odds ratio, the odds of having nodal metastasis in positive as compared to negative EUS studies, was 19.1 (95% CI: 12.7-28.5) for EUS without FNA and 106.9 (95% CI: 54.4-210.3) for EUS with FNA. Figure 4 shows a Forrest plot of various studies with FNA and their DOR. All the pooled estimates calculated by random effect models were similar to the estimates of fixed effect model.

SROC curves for EUS without FNA showed an area under the curve (AUC) of 0.91. EUS with FNA showed

an AUC of 0.97. Figure 5 shows the SROC curve. The *P* for Chi-squared heterogeneity for all the pooled accuracy estimates was  $> 0.10$ . Table 3 shows the accuracy estimates of EUS alone and EUS-FNA.

### Effect of technology over time

To standardize the criteria for lymph node involvement and change in technology, the studies were grouped into three time periods<sup>[30]</sup>. These time periods were 1988 to 1994, 1995 to 1999, and 2000 to 2006. During these time periods, the number of studies that met the inclusion criteria for EUS alone were 17, 17, and 10, respectively. Studies that met inclusion criteria for EUS-FNA were 4, 10, and 18, respectively. For the most recent time period, EUS alone had a sensitivity of 81.6% (95% CI: 77.8-85.1) and specificity of 82.4% (95% CI: 78.2-86.1). During the same time period, EUS-FNA had a sensitivity of 91.7% (95% CI: 89.3-93.7) and specificity of 96.8% (95% CI: 94.9-98.2). All pooled estimates during the three time periods are given in Table 4. The *P* for chi-squared heterogeneity for all the pooled accuracy estimates was  $> 0.1$ .

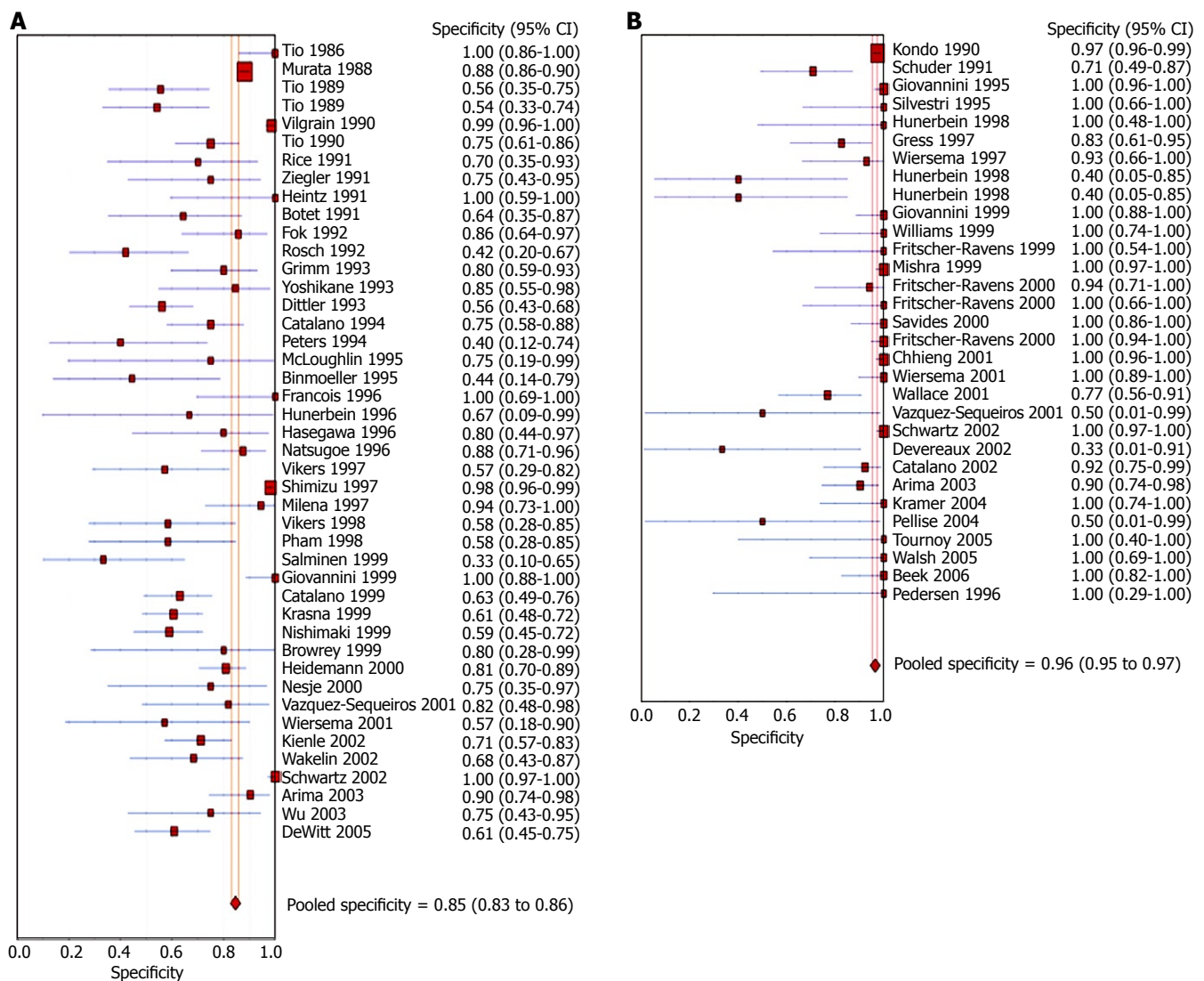


Figure 3 Forrest plots. A: Specificity of EUS alone in diagnosing mediastinal lymphadenopathy. B: Specificity of EUS-FNA alone in diagnosing mediastinal lymphadenopathy.

# Bias estimates

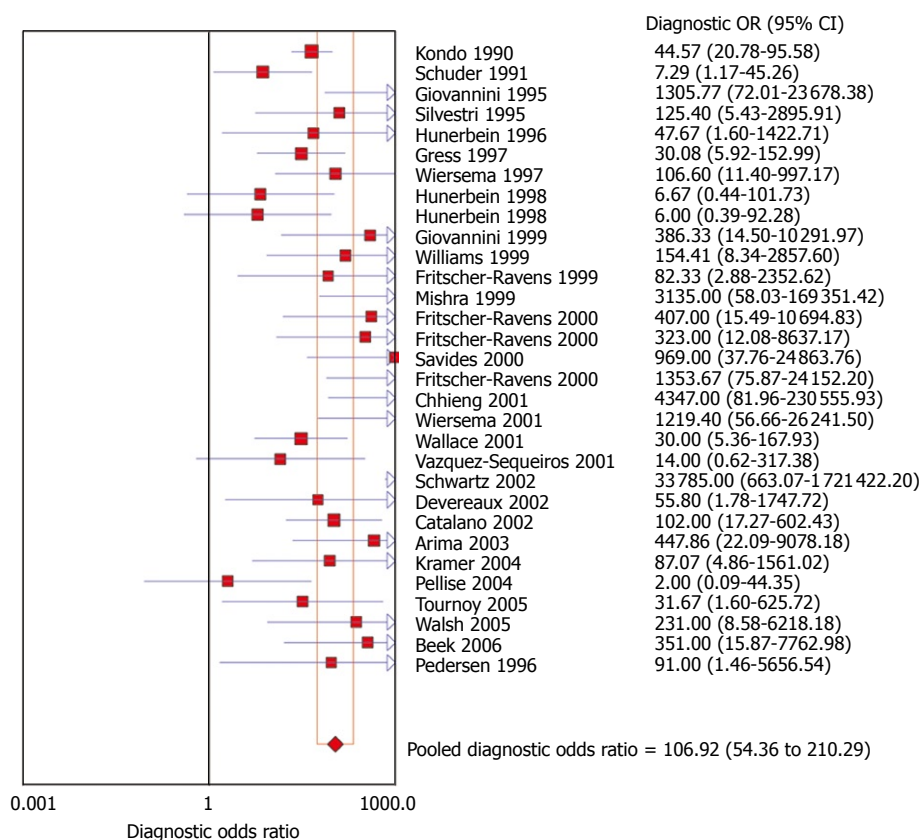
The bias calculations using Harbord-Egger bias indicator gave a value of 1.08 (95% CI: -0.79-2.95,  $P = 0.29$ ) for EUS studies without FNA and 2.02 (95% CI: 0.29-3.74,  $P = 0.04$ ) for studies with FNA. The Begg-Mazumdar indicator for bias gave a Kendall's tau  $b$  value of 0.13 ( $P = 0.36$ ) for studies without FNA and -0.19 ( $P = 0.07$ ) for studies with FNA. The funnel plots for the studies without and with FNA are shown in Figure 6A and B.

# DISCUSSION

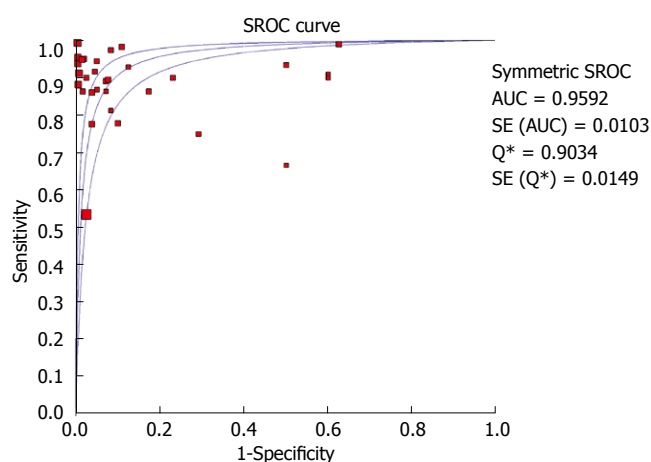
Diagnosing the correct etiology for mediastinal lymphadenopathy helps direct precise therapy and prognosis. Thoracoscopic procedures for tissue biopsy carry a risk of complications in 25%-35% of cases<sup>[9,10]</sup>. The advantage of EUS is the ability to perform FNA during the procedure for tissue diagnosis. The procedure is, in comparison with other alternative options, safe, less invasive, and does not require general anesthesia or hospitalization<sup>[107]</sup>. The complication rate is extremely low (0.5%-2.3%) with several studies reporting no complications<sup>[48,77,83,107]</sup>. Modalities using FNA, such as transbronchial, computed

tomography, or thoracoscopic procedure, cannot be used for the entire mediastinum<sup>[2-13]</sup>. EUS has the ability to image the aortopulmonary window, the subcarinal nodes, inferior mediastinum, and entire posterior part of the mediastinum.

This meta-analysis and systematic review was written in accordance with the proposal for reporting by the QUOROM (Quality of Reporting of Meta-analyses) statement<sup>[7]</sup>. This meta-analysis and systematic review shows that the pooled sensitivity of EUS for mediastinal lymphadenopathy is high and use of FNA during the procedure, further increases such sensitivity. The pooled specificity for diagnosing mediastinal lymphadenopathy is also high with substantial improvement if FNA is performed during the procedure (from 84.6% to 96.4%). Diagnostic odds ratio is defined as the odds of having a positive test in patients with true anatomic disease when compared to patients who do not have the disease. EUS has a very high diagnostic odds ratio for mediastinal lymphadenopathy. For example, if EUS indicates mediastinal lymphadenopathy and if FNA is performed on the enlarged nodes, the patient has odds of 106 times to have the correct etiology for lymph node enlargement. If EUS shows mediastinal lymphadenopathy, then the nodes



**Figure 4** Forrest plot showing diagnostic odds ratio of EUS-FNA in identifying mediastinal lymphadenopathy.



**Figure 5** SROC for EUS to diagnose mediastinal lymphadenopathy.

should be biopsied by FNA to improve the diagnostic accuracy.

The positive likelihood ratio measures how well a test identifies a disease state. The higher the positive likelihood ratio, the better the test performs in identifying the correct disease state. The negative likelihood ratio of the same test measures how well the test performs in excluding a disease state. The lower the negative likelihood ratio, the better the test performs in excluding a disease. For mediastinal lymphadenopathy, EUS has a high positive likelihood ratio and low negative likelihood ratio. This indicates that EUS performs better in diagnosing and excluding mediastinal lymphadenopathy. For mediastinal lymphadenopathy, all the pooled accuracy estimates of EUS are higher if FNA

**Table 3** Pooled diagnostic accuracy estimates of EUS alone and EUS-FNA

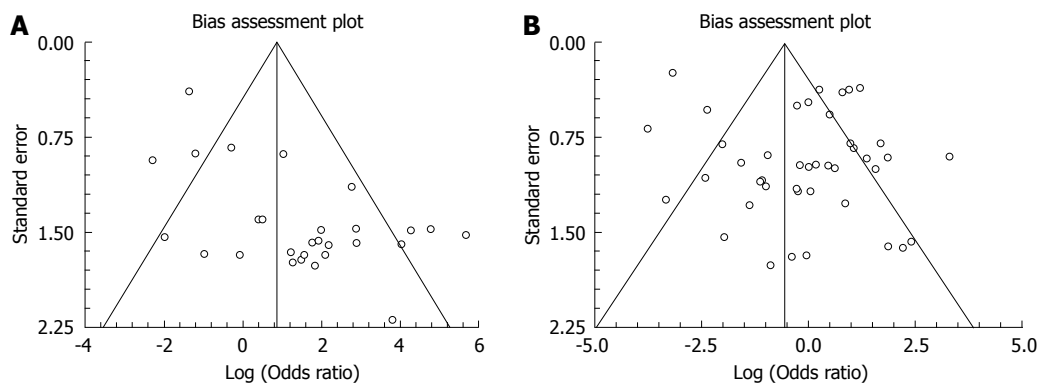
	EUS	EUS-FNA
Studies	44	32
Pooled sensitivity	84.7% (82.9-86.4)	88.0% (85.8-90.0)
Pooled specificity	84.6% (83.2-85.9)	96.4% (95.3-97.4)
Positive likelihood ratio	3.3 (2.6-4.3)	11.2 (5.9-21.2)
Negative likelihood ratio	0.24 (0.1-0.3)	0.13 (0.1-0.2)
Diagnostic odds ratio	19.1 (12.7-28.5)	106.9 (54.4-210.3)
Area under the curve	0.91	0.97

is performed during the procedure. Also, these pooled estimates give a baseline for future study comparisons.

The EUS studies with FNA were grouped into time periods and analyzed to standardize the criteria and the technology of EUS over the past two decades. Over the last two decades, the sensitivity and specificity of EUS with FNA has substantially improved.

Due to the possibility of different studies using slightly different criteria for diagnosis, heterogeneity among the studies was tested by drawing SROC curves and finding the AUC. An AUC of 1 for any test indicates that the test is excellent. SROC curves for EUS showed that the value for AUC was very close to 1, indicating that EUS is an excellent test to diagnose mediastinal lymphadenopathy. Publication bias and selection bias may affect the summary estimates. Studies with statistically significant results tend to be published and cited. Smaller studies may show larger treatment effects due to fewer case-mix differences (e.g. patients with only early or late disease) than larger trials. This bias can be estimated by bias indicators and construction of





**Figure 6** Funnel plots. **A:** Bias assessment for EUS studies without FNA in examining mediastinal lymphadenopathy; **B:** Bias assessment for EUS-FNA studies in examining mediastinal lymphadenopathy.

**Table 4** Pooled diagnostic accuracy estimates of EUS alone and EUS-FNA for different time periods with 95% CI

Time period	No. of studies	Pooled sensitivity	Pooled specificity	Pooled LR+ 1	Pooled LR-2	Pooled DOR3
EUS without FNA						
1988 to 1994	17	88.0% (85.4-90.2)	85.2% (83.4-86.9)	3.6 (2.4-5.4)	0.2 (0.1-0.3)	27.5 (14.5-52.4)
1995 to 1999	17	82.6% (78.8-85.9)	84.4% (81.6-86.9)	3.0 (2.0-4.5)	0.3 (0.2-0.4)	14.8 (7.5-29.3)
2000 to 2005	10	81.6% (77.8-85.1)	82.4% (78.2-86.1)	3.4 (2.2-5.3)	0.3 (0.2-0.4)	14.9 (6.7-33.1)
EUS-FNA						
1988 to 1994	4	71.8% (63.9-78.9)	96.8% (94.9-98.1)	15.5 (2.4-101.2)	0.3 (0.1-0.6)	61.8 (10.5-63.8)
1995 to 1999	10	88.9% (83.5-93.0)	94.7% (90.7-97.3)	8.1 (2.8-23.3)	0.1 (0.1-0.2)	57.0 (20.7-57.1)
2000 to 2005	18	91.7% (89.3-93.7)	96.8% (94.9-98.2)	12.5 (5.2-29.8)	0.1 (0.1-0.2)	17.7 (5.0-62.8)

<sup>1</sup>LR+: Positive likelihood ratio; <sup>2</sup>LR-: Negative likelihood ratio; <sup>3</sup>DOR: Diagnostic odds ratio.

funnel plots. Bias among studies can affect the shape of the funnel plot. In this meta-analysis and systematic review, bias calculations using Harbord-Egger indicator<sup>[36]</sup> and Begg-Mazumdar indicator<sup>[37]</sup> showed no statistically significant bias for EUS studies without FNA. Furthermore, funnel plot analyses showed no significant bias for EUS without FNA and EUS-FNA studies (Figure 6B).

In conclusion, EUS has high sensitivity and specificity to evaluate mediastinal lymphadenopathy. This meta-analysis demonstrates that FNA substantially improves the specificity of EUS in evaluating mediastinal lymphadenopathy. EUS with FNA should be the diagnostic test of choice for evaluating mediastinal lymphadenopathy.

## REFERENCES

- Genereux GP, Howie JL. Normal mediastinal lymph node size and number: CT and anatomic study. *AJR Am J Roentgenol* 1984; **142**: 1095-1100
- Arita T, Kuramitsu T, Kawamura M, Matsumoto T, Matsunaga N, Sugi K, Esato K. Bronchogenic carcinoma: incidence of metastases to normal sized lymph nodes. *Thorax* 1995; **50**: 1267-1269
- Izbicki JR, Thetter O, Karg O, Kreusser T, Passlick B, Trupka A, Haussinger K, Woeckel W, Kenn RW, Wilker DK. Accuracy of computed tomographic scan and surgical assessment for staging of bronchial carcinoma. A prospective study. *J Thorac Cardiovasc Surg* 1992; **104**: 413-420
- McLoud TC, Bourgouin PM, Greenberg RW, Kosiuk JP, Templeton PA, Shepard JA, Moore EH, Wain JC, Mathisen DJ, Grillo HC. Bronchogenic carcinoma: analysis of staging in the mediastinum with CT by correlative lymph node mapping and sampling. *Radiology* 1992; **182**: 319-323
- McKenna RJ Jr, Libshitz HI, Mountain CE, McMurtrey MJ. Roentgenographic evaluation of mediastinal nodes for preoperative assessment in lung cancer. *Chest* 1985; **88**: 206-210
- Kondo D, Imaizumi M, Abe T, Naruke T, Suemasu K. Endoscopic ultrasound examination for mediastinal lymph node metastases of lung cancer. *Chest* 1990; **98**: 586-593
- Harrow EM, Oldenburg FA Jr, Lingenfelter MS, Smith AM Jr. Transbronchial needle aspiration in clinical practice. A five-year experience. *Chest* 1989; **96**: 1268-1272
- Harrow EM, Wang KP. The staging of lung cancer by bronchoscopic transbronchial needle aspiration. *Chest Surg Clin N Am* 1996; **6**: 223-235
- Salazar AM, Westcott JL. The role of transthoracic needle biopsy for the diagnosis and staging of lung cancer. *Clin Chest Med* 1993; **14**: 99-110
- Gardner D, vanSonnenberg E, D'Agostino HB, Casola G, Taggart S, May S. CT-guided transthoracic needle biopsy. *Cardiovasc Intervent Radiol* 1991; **14**: 17-23
- Lopez L, Varela A, Freixinet J, Quevedo S, Lopez Pujol J, Rodriguez de Castro F, Salvatierra A. Extended cervical mediastinoscopy: prospective study of fifty cases. *Ann Thorac Surg* 1994; **57**: 555-557; discussion 557-558
- Barendregt WB, Deleu HW, Joosten HJ, Berg W, Janssen JP. The value of parasternal mediastinoscopy in staging bronchial carcinoma. *Eur J Cardiothorac Surg* 1995; **9**: 655-658
- Merav AD. The role of mediastinoscopy and anterior mediastinotomy in determining operability of lung cancer: a review of published questions and answers. *Cancer Invest* 1991; **9**: 439-442
- Landreneau RJ, Hazelrigg SR, Mack MJ, Fitzgibbon LD, Dowling RD, Acuff TE, Keenan RJ, Ferson PF. Thoracoscopic mediastinal lymph node sampling: useful for mediastinal lymph node stations inaccessible by cervical mediastinoscopy. *J Thorac Cardiovasc Surg* 1993; **106**: 554-558
- Heintz A, Mildnerberger P, Georg M, Braunstein S, Junginger T. Endoscopic ultrasonography in the diagnosis of regional lymph nodes in esophageal and gastric cancer--results of studies in vitro. *Endoscopy* 1993; **25**: 231-235
- Kaufman AR, Sivak MV Jr. Endoscopic ultrasonography in the differential diagnosis of pancreatic disease. *Gastrointest*

- Endosc* 1989; **35**: 214-219
- 17 **Arima M**, Tada M. Endoscopic ultrasound-guided fine needle aspiration biopsy in esophageal and mediastinal diseases: Clinical indications and results. *Dig Endosc* 2003; **15**: 93-99
  - 18 **McLoughlin RF**, Cooperberg PL, Mathieson JR, Stordy SN, Halparin LS. High resolution endoluminal ultrasonography in the staging of esophageal carcinoma. *J Ultrasound Med* 1995; **14**: 725-730
  - 19 **Gress FG**, Savides TJ, Sandler A, Kesler K, Conces D, Cummings O, Mathur P, Ikenberry S, Bilderback S, Hawes R. Endoscopic ultrasonography, fine-needle aspiration biopsy guided by endoscopic ultrasonography, and computed tomography in the preoperative staging of non-small-cell lung cancer: a comparison study. *Ann Intern Med* 1997; **127**: 604-612
  - 20 **Schwartz DA**, Unni KK, Levy MJ, Clain JE, Wiersema MJ. The rate of false-positive results with EUS-guided fine-needle aspiration. *Gastrointest Endosc* 2002; **56**: 868-872
  - 21 **Pedersen BH**, Vilman P, Folke K, Jacobsen GK, Krasnik M, Milman N, Hancke S. Endoscopic ultrasonography and real-time guided fine-needle aspiration biopsy of solid lesions of the mediastinum suspected of malignancy. *Chest* 1996; **110**: 539-544
  - 22 **Devereaux BM**, Leblanc JK, Yousif E, Kesler K, Brooks J, Mathur P, Sandler A, Chappo J, Lehman GA, Sherman S, Gress F, Ciaccia D. Clinical utility of EUS-guided fine-needle aspiration of mediastinal masses in the absence of known pulmonary malignancy. *Gastrointest Endosc* 2002; **56**: 397-401
  - 23 **Pellise M**, Castells A, Gines A, Agrelo R, Sole M, Castellvi-Bel S, Fernandez-Esparrach G, Llach J, Esteller M, Bordas JM, Pique JM. Detection of lymph node micrometastases by gene promoter hypermethylation in samples obtained by endosonography-guided fine-needle aspiration biopsy. *Clin Cancer Res* 2004; **10**: 4444-4449
  - 24 **Kondo D**, Imaizumi M, Abe T, Naruke T, Suemasu K. Endoscopic ultrasound examination for mediastinal lymph node metastases of lung cancer. *Chest* 1990; **98**: 586-593
  - 25 **Schuder G**, Isringhaus H, Kubale B, Seitz G, Sybrecht GW. Endoscopic ultrasonography of the mediastinum in the diagnosis of bronchial carcinoma. *Thorac Cardiovasc Surg* 1991; **39**: 299-303
  - 26 **Moher D**, Cook DJ, Eastwood S, Olkin I, Rennie D, Stroup DF. Improving the quality of reports of meta-analyses of randomised controlled trials: the QUOROM statement. Quality of Reporting of Meta-analyses. *Lancet* 1999; **354**: 1896-1900
  - 27 **Bossuyt PM**, Reitsma JB, Bruns DE, Gatsonis CA, Glasziou PP, Irwig LM, Lijmer JG, Moher D, Rennie D, de Vet HC. Towards complete and accurate reporting of studies of diagnostic accuracy: the STARD initiative. The Standards for Reporting of Diagnostic Accuracy Group. *Croat Med J* 2003; **44**: 635-638
  - 28 **Jadad AR**, Moore RA, Carroll D, Jenkinson C, Reynolds DJ, Gavaghan DJ, McQuay HJ. Assessing the quality of reports of randomized clinical trials: is blinding necessary? *Control Clin Trials* 1996; **17**: 1-12
  - 29 **Stroup DF**, Berlin JA, Morton SC, Olkin I, Williamson GD, Rennie D, Moher D, Becker BJ, Sipe TA, Thacker SB. Meta-analysis of observational studies in epidemiology: a proposal for reporting. Meta-analysis Of Observational Studies in Epidemiology (MOOSE) group. *JAMA* 2000; **283**: 2008-2012
  - 30 **Puli SR**, Singh S, Hagedorn CH, Reddy J, Olyae M. Diagnostic accuracy of EUS for vascular invasion in pancreatic and periampullary cancers: a meta-analysis and systematic review. *Gastrointest Endosc* 2007; **65**: 788-797
  - 31 **Leemis LM**, Trivedi KS. A Comparison of Approximate Interval Estimators for the Bernoulli Parameter. *Am Stat* 1996; **50**: 63-68
  - 32 **Cox DR**. The analysis of binary data. London: Methuen, 1970
  - 33 **Agresti A**. Analysis of ordinal categorical data. New York: John Wileys & Sons, 1984
  - 34 **Deeks JJ**. Systematic reviews of evaluations of diagnostic and screening tests. In: Egger M, Smith GD, Altman DG, editors. Systematic Reviews in Health Care: Meta-analysis in context. London: BMJ Books, 2001
  - 35 **Harbord RM**, Egger M, Sterne JA. A modified test for small-study effects in meta-analyses of controlled trials with binary endpoints. *Stat Med* 2006; **25**: 3443-3457
  - 36 **Begg CB**, Mazumdar M. Operating characteristics of a rank correlation test for publication bias. *Biometrics* 1994; **50**: 1088-1101
  - 37 **Sterne JA**, Egger M, Smith GD. Systematic reviews in health care: Investigating and dealing with publication and other biases in meta-analysis. *BMJ* 2001; **323**: 101-105
  - 38 **Sterne JA**, Egger M. Funnel plots for detecting bias in meta-analysis: guidelines on choice of axis. *J Clin Epidemiol* 2001; **54**: 1046-1055
  - 39 **Binmoeller KF**, Seifert H, Seitz U, Izbicki JR, Kida M, Soehendra N. Ultrasonic esophagoprobe for TNM staging of highly stenosing esophageal carcinoma. *Gastrointest Endosc* 1995; **41**: 547-552
  - 40 **Botet JF**, Lightdale CJ, Zauber AG, Gerdes H, Urmacher C, Brennan MF. Preoperative staging of esophageal cancer: comparison of endoscopic US and dynamic CT. *Radiology* 1991; **181**: 419-425
  - 41 **Bowrey DJ**, Clark GW, Roberts SA, Maughan TS, Hawthorne AB, Williams GT, Carey PD. Endosonographic staging of 100 consecutive patients with esophageal carcinoma: introduction of the 8-mm esophagoprobe. *Dis Esophagus* 1999; **12**: 258-263
  - 42 **Catalano MF**, Alcocer E, Chak A, Nguyen CC, Rajman I, Geenen JE, Lahoti S, Sivak MV Jr. Evaluation of metastatic celiac axis lymph nodes in patients with esophageal carcinoma: accuracy of EUS. *Gastrointest Endosc* 1999; **50**: 352-356
  - 43 **Catalano MF**, Sivak MV Jr, Rice T, Gragg LA, Van Dam J. Endosonographic features predictive of lymph node metastasis. *Gastrointest Endosc* 1994; **40**: 442-446
  - 44 **DeWitt J**, Kesler K, Brooks JA, LeBlanc J, McHenry L, McGreevy K, Sherman S. Endoscopic ultrasound for esophageal and gastroesophageal junction cancer: Impact of increased use of primary neoadjuvant therapy on preoperative locoregional staging accuracy. *Dis Esophagus* 2005; **18**: 21-27
  - 45 **Dittler HJ**, Siewert JR. Role of endoscopic ultrasonography in esophageal carcinoma. *Endoscopy* 1993; **25**: 156-161
  - 46 **Fok M**, Cheng SW, Wong J. Endosonography in patient selection for surgical treatment of esophageal carcinoma. *World J Surg* 1992; **16**: 1098-1103; discussion 1103
  - 47 **Francois E**, Peroux J, Mouroux J, Chazalle M, Hastier P, Ferrero J, Simon J, Bourry J. Preoperative endosonographic staging of cancer of the cardia. *Abdom Imaging* 1996; **21**: 483-487
  - 48 **Giovannini M**, Monges G, Seitz JF, Moutardier V, Bernardini D, Thomas P, Houvenaeghel G, Delperro JR, Giudicelli R, Fuentes P. Distant lymph node metastases in esophageal cancer: impact of endoscopic ultrasound-guided biopsy. *Endoscopy* 1999; **31**: 536-540
  - 49 **Grimm H**, Binmoeller KF, Hamper K, Koch J, Henne-Bruns D, Soehendra N. Endosonography for preoperative locoregional staging of esophageal and gastric cancer. *Endoscopy* 1993; **25**: 224-230
  - 50 **Hasegawa N**, Niwa Y, Arisawa T, Hase S, Goto H, Hayakawa T. Preoperative staging of superficial esophageal carcinoma: comparison of an ultrasound probe and standard endoscopic ultrasonography. *Gastrointest Endosc* 1996; **44**: 388-393
  - 51 **Heidemann J**, Schilling MK, Schmassmann A, Maurer CA, Buchler MW. Accuracy of endoscopic ultrasonography in preoperative staging of esophageal carcinoma. *Dig Surg* 2000; **17**: 219-224
  - 52 **Heintz A**, Hohn U, Schweden F, Junginger T. Preoperative detection of intrathoracic tumor spread of esophageal cancer: endosonography versus computed tomography. *Surg Endosc* 1991; **5**: 75-78
  - 53 **Hunerbein M**, Dohmoto M, Rau B, Schlag PM. Endosonography and endosonography-guided biopsy of upper-GI-tract tumors using a curved-array echoendoscope. *Surg Endosc* 1996; **10**: 1205-1209
  - 54 **Kallimanis GE**, Gupta PK, al-Kawas FH, Tio LT, Benjamin SB, Bertagnolli ME, Nguyen CC, Gomes MN, Fleischer DE. Endoscopic ultrasound for staging esophageal cancer, with or without dilation, is clinically important and safe. *Gastrointest Endosc* 1995; **41**: 540-546

- 55 **Kienle P**, Buhl K, Kuntz C, Dux M, Hartmann C, Axel B, Herfarth C, Lehnert T. Prospective comparison of endoscopy, endosonography and computed tomography for staging of tumours of the oesophagus and gastric cardia. *Digestion* 2002; **66**: 230-236
- 56 **Krasna MJ**, Mao YS, Sonett J, Gamliel Z. The role of thoracoscopic staging of esophageal cancer patients. *Eur J Cardiothorac Surg* 1999; **16** Suppl 1: S31-S33
- 57 **Murata Y**, Suzuki S, Hashimoto H. Endoscopic ultrasonography of the upper gastrointestinal tract. *Surg Endosc* 1988; **2**: 180-183
- 58 **Natsugoe S**, Yoshinaka H, Morinaga T, Shimada M, Baba M, Fukumoto T, Stein HJ, Aikou T. Ultrasonographic detection of lymph-node metastases in superficial carcinoma of the esophagus. *Endoscopy* 1996; **28**: 674-679
- 59 **Nesje LB**, Svanes K, Viste A, Laerum OD, Odegaard S. Comparison of a linear miniature ultrasound probe and a radial-scanning echoendoscope in TN staging of esophageal cancer. *Scand J Gastroenterol* 2000; **35**: 997-1002
- 60 **Nishimaki T**, Tanaka O, Ando N, Ide H, Watanabe H, Shinoda M, Takiyama W, Yamana H, Ishida K, Isono K, Endo M, Ikeuchi T, Mitomi T, Koizumi H, Imamura M, Iizuka T. Evaluation of the accuracy of preoperative staging in thoracic esophageal cancer. *Ann Thorac Surg* 1999; **68**: 2059-2064
- 61 **Peters JH**, Hoeft SF, Heimbucher J, Bremner RM, DeMeester TR, Bremner CG, Clark GW, Kiyabu M, Parisky Y. Selection of patients for curative or palliative resection of esophageal cancer based on preoperative endoscopic ultrasonography. *Arch Surg* 1994; **129**: 534-539
- 62 **Pham T**, Roach E, Falk GL, Chu J, Ngu MC, Jones DB. Staging of oesophageal carcinoma by endoscopic ultrasound: preliminary experience. *Aust N Z J Surg* 1998; **68**: 209-212
- 63 **Rice TW**, Boyce GA, Sivak MV. Esophageal ultrasound and the preoperative staging of carcinoma of the esophagus. *J Thorac Cardiovasc Surg* 1991; **101**: 536-543; discussion 543-544
- 64 **Rosch T**, Lorenz R, Zenker K, von Wichert A, Dancygier H, Hofler H, Siewert JR, Classen M. Local staging and assessment of resectability in carcinoma of the esophagus, stomach, and duodenum by endoscopic ultrasonography. *Gastrointest Endosc* 1992; **38**: 460-467
- 65 **Salminen JT**, Farkkila MA, Ramo OJ, Toikkanen V, Simpanen J, Nuutinen H, Salo JA. Endoscopic ultrasonography in the preoperative staging of adenocarcinoma of the distal oesophagus and oesophagogastric junction. *Scand J Gastroenterol* 1999; **34**: 1178-1182
- 66 **Schwartz DA**, Unni KK, Levy MJ, Clain JE, Wiersema MJ. The rate of false-positive results with EUS-guided fine-needle aspiration. *Gastrointest Endosc* 2002; **56**: 868-872
- 67 **Shimizu Y**, Mera K, Tsukagoshi H, Takamasa M, Kawarazaki M, Watanabe Y, Nakasato T, Oohara M, Hosokawa M, Fujita M, Asaka M. Endoscopic Ultrasonography for the Detection of Lymph Node Metastasis in Superficial Esophageal Carcinoma. *Dig Endosc* 1997; **9**: 178-182
- 68 **Tio TL**, Coene PP, den Hartog Jager FC, Tytgat GN. Pre-operative TNM classification of esophageal carcinoma by endosonography. *Hepatogastroenterology* 1990; **37**: 376-381
- 69 **Tio TL**, Coene PP, Schouwink MH, Tytgat GN. Esophagogastric carcinoma: preoperative TNM classification with endosonography. *Radiology* 1989; **173**: 411-417
- 70 **Tio TL**, Cohen P, Coene PP, Udding J, den Hartog Jager FC, Tytgat GN. Endosonography and computed tomography of esophageal carcinoma. Preoperative classification compared to the new (1987) TNM system. *Gastroenterology* 1989; **96**: 1478-1486
- 71 **Tio TL**, den Hartog Jager FC, Tytgat GN. The role of endoscopic ultrasonography in assessing local resectability of oesophagogastric malignancies. Accuracy, pitfalls, and predictability. *Scand J Gastroenterol Suppl* 1986; **123**: 78-86
- 72 **Vazquez-Sequeiros E**, Norton ID, Clain JE, Wang KK, Affi A, Allen M, Deschamps C, Miller D, Salomao D, Wiersema MJ. Impact of EUS-guided fine-needle aspiration on lymph node staging in patients with esophageal carcinoma. *Gastrointest Endosc* 2001; **53**: 751-757
- 73 **Vickers J**, Alderson D. Influence of luminal obstruction on oesophageal cancer staging using endoscopic ultrasonography. *Br J Surg* 1998; **85**: 999-1001
- 74 **Vickers J**. Role of endoscopic ultrasound in the preoperative assessment of patients with oesophageal cancer. *Ann R Coll Surg Engl* 1998; **80**: 233-239
- 75 **Vilgrain V**, Mompont D, Palazzo L, Menu Y, Gayet B, Ollier P, Nahum H, Fekete F. Staging of esophageal carcinoma: comparison of results with endoscopic sonography and CT. *AJR Am J Roentgenol* 1990; **155**: 277-281
- 76 **Wakelin SJ**, Deans C, Crofts TJ, Allan PL, Plevris JN, Paterson-Brown S. A comparison of computerised tomography, laparoscopic ultrasound and endoscopic ultrasound in the preoperative staging of oesophago-gastric carcinoma. *Eur J Radiol* 2002; **41**: 161-167
- 77 **Wiersema MJ**, Vazquez-Sequeiros E, Wiersema LM. Evaluation of mediastinal lymphadenopathy with endoscopic US-guided fine-needle aspiration biopsy. *Radiology* 2001; **219**: 252-257
- 78 **Wu LF**, Wang BZ, Feng JL, Cheng WR, Liu GR, Xu XH, Zheng ZC. Preoperative TN staging of esophageal cancer: comparison of miniprobe ultrasonography, spiral CT and MRI. *World J Gastroenterol* 2003; **9**: 219-224
- 79 **Yoshikane H**, Tsukamoto Y, Niwa Y, Goto H, Hase S, Shimodaira M, Maruta S, Miyata A, Yoshida M. Superficial esophageal carcinoma: evaluation by endoscopic ultrasonography. *Am J Gastroenterol* 1994; **89**: 702-707
- 80 **Ziegler K**, Sanft C, Zeitz M, Friedrich M, Stein H, Haring R, Riecken EO. Evaluation of endosonography in TN staging of oesophageal cancer. *Gut* 1991; **32**: 16-20
- 81 **Giovannini M**, Monges G, Seitz JF, Moutardier V, Bernardini D, Thomas P, Houvenaeghel G, Delperio JR, Giudicelli R, Fuentes P. Distant lymph node metastases in esophageal cancer: impact of endoscopic ultrasound-guided biopsy. *Endoscopy* 1999; **31**: 536-540
- 82 **Giovannini M**, Seitz JF, Monges G, Perrier H, Rabbia I. Fine-needle aspiration cytology guided by endoscopic ultrasonography: results in 141 patients. *Endoscopy* 1995; **27**: 171-177
- 83 **Silvestri GA**, Hoffman BJ, Bhutani MS, Hawes RH, Coppage L, Sanders-Clitte A, Reed CE. Endoscopic ultrasound with fine-needle aspiration in the diagnosis and staging of lung cancer. *Ann Thorac Surg* 1996; **61**: 1441-1445; discussion 1445-1446
- 84 **Fritscher-Ravens A**, Soehendra N, Schirrow L, Sriram PV, Meyer A, Hauber HP, Pforte A. Role of transesophageal endosonography-guided fine-needle aspiration in the diagnosis of lung cancer. *Chest* 2000; **117**: 339-345
- 85 **Wiersema MJ**, Vazquez-Sequeiros E, Wiersema LM. Evaluation of mediastinal lymphadenopathy with endoscopic US-guided fine-needle aspiration biopsy. *Radiology* 2001; **219**: 252-257
- 86 **Kramer H**, van Putten JW, Post WJ, van Dullemen HM, Bongaerts AH, Pruim J, Suurmeijer AJ, Klinkenberg TJ, Groen H, Groen HJ. Oesophageal endoscopic ultrasound with fine needle aspiration improves and simplifies the staging of lung cancer. *Thorax* 2004; **59**: 596-601
- 87 **van Beek FT**, Maas KW, Timmer R, Seldenrijk CA, de Bruin PC, Schramel FM. Oesophageal endoscopic ultrasound with fine-needle aspiration biopsy in the staging of non-small-cell lung carcinoma; results from 43 patients. *Ned Tijdschr Geneesk* 2006; **150**: 144-150
- 88 **Tournoy KG**, Praet MM, Van Maele G, Van Meerbeeck JP. Esophageal endoscopic ultrasound with fine-needle aspiration with an on-site cytopathologist: high accuracy for the diagnosis of mediastinal lymphadenopathy. *Chest* 2005; **128**: 3004-3009
- 89 **Williams DB**, Sahai AV, Aabakken L, Penman ID, van Velse A, Webb J, Wilson M, Hoffman BJ, Hawes RH. Endoscopic ultrasound guided fine needle aspiration biopsy: a large single centre experience. *Gut* 1999; **44**: 720-726
- 90 **Hunerbein M**, Dohmoto M, Rau B, Schlag PM. Endosonography and endosonography-guided biopsy of upper-GI-tract tumors using a curved-array echoendoscope. *Surg Endosc* 1996; **10**: 1205-1209
- 91 **Hunerbein M**, Ghadimi BM, Haensch W, Schlag PM. Transesophageal biopsy of mediastinal and pulmonary

- tumors by means of endoscopic ultrasound guidance. *J Thorac Cardiovasc Surg* 1998; **116**: 554-559
- 92 **Catalano MF**, Nayar R, Gress F, Scheiman J, Wassef W, Rosenblatt ML, Kochman M. EUS-guided fine needle aspiration in mediastinal lymphadenopathy of unknown etiology. *Gastrointest Endosc* 2002; **55**: 863-869
  - 93 **Khoo KL**, Ho KY, Nilsson B, Lim TK. EUS-guided FNA immediately after unrevealing transbronchial needle aspiration in the evaluation of mediastinal lymphadenopathy: a prospective study. *Gastrointest Endosc* 2006; **63**: 215-220
  - 94 **Hunerbein M**, Dohmoto M, Haensch W, Schlag PM. Endosonography-guided biopsy of mediastinal and pancreatic tumors. *Endoscopy* 1998; **30**: 32-36
  - 95 **Arima M**, Tada M. Endoscopic ultrasound-guided fine needle aspiration biopsy in esophageal and mediastinal diseases: Clinical indications and results. *Dig Endosc* 2003; **15**: 93-99
  - 96 **Chhieng DC**, Jhala D, Jhala N, Eltoum I, Chen VK, Vickers S, Heslin MJ, Wilcox CM, Eloubeidi MA. Endoscopic ultrasound-guided fine-needle aspiration biopsy: a study of 103 cases. *Cancer* 2002; **96**: 232-239
  - 97 **Walsh PR**, Williams DB. Mediastinal adenopathy: finding the answer with endoscopic ultrasound-guided fine-needle aspiration biopsy. *Intern Med J* 2005; **35**: 392-398
  - 98 **Fritscher-Ravens A**, Sriram PV, Topalidis T, Hauber HP, Meyer A, Soehendra N, Pforte A. Diagnosing sarcoidosis using endosonography-guided fine-needle aspiration. *Chest* 2000; **118**: 928-935
  - 99 **Wallace MB**, Kennedy T, Durkalski V, Eloubeidi MA, Etamad R, Matsuda K, Lewin D, Van Velse A, Hennesey W, Hawes RH, Hoffman BJ. Randomized controlled trial of EUS-guided fine needle aspiration techniques for the detection of malignant lymphadenopathy. *Gastrointest Endosc* 2001; **54**: 441-447
  - 100 **Savides TJ**, Binmoeller K and Sarlin R. Effectiveness of EUS/ FNA for diagnosing lung cancer in a managed care setting. *Gastrointest Endosc* 2005; **51**: AB143
  - 101 **Fritscher-Ravens A**, Petrasch S, Reinacher-Schick A, Graeven U, Konig M, Schmiegel W. Diagnostic value of endoscopic ultrasonography-guided fine-needle aspiration cytology of mediastinal masses in patients with intrapulmonary lesions and nondiagnostic bronchoscopy. *Respiration* 1999; **66**: 150-155
  - 102 **Mishra G**, Sahai AV, Penman ID, Williams DB, Judson MA, Lewin DN, Hawes RH, Hoffman BJ. Endoscopic ultrasonography with fine-needle aspiration: an accurate and simple diagnostic modality for sarcoidosis. *Endoscopy* 1999; **31**: 377-382
  - 103 **Fritscher-Ravens A**, Sriram PV, Bobrowski C, Pforte A, Topalidis T, Krause C, Jaeckle S, Thonke F, Soehendra N. Mediastinal lymphadenopathy in patients with or without previous malignancy: EUS-FNA-based differential cytodiagnosis in 153 patients. *Am J Gastroenterol* 2000; **95**: 2278-2284
  - 104 **Wiersema MJ**, Vilmann P, Giovannini M, Chang KJ, Wiersema LM. Endosonography-guided fine-needle aspiration biopsy: diagnostic accuracy and complication assessment. *Gastroenterology* 1997; **112**: 1087-1095
  - 105 **Vazquez-Sequeiros E**, Norton ID, Clain JE, Wang KK, Affi A, Allen M, Deschamps C, Miller D, Salomao D, Wiersema MJ. Impact of EUS-guided fine-needle aspiration on lymph node staging in patients with esophageal carcinoma. *Gastrointest Endosc* 2001; **53**: 751-757
  - 106 **Shimizu Y**, Mera K, Tsukagoshi H, Takamasa M, Kawarazaki M, Watanabe Y, Tomohiko Nakasato, Oohara M, Hosokawa M, Fujita M, Asaka M. Endoscopic ultrasonography for the detection of lymph node metastasis in superficial esophageal carcinoma. *Dig Endosc* 1997; **9**: 178-182
  - 107 **Vilmann P**. Endoscopic ultrasonography-guided fine-needle aspiration biopsy of lymph nodes. *Gastrointest Endosc* 1996; **43**: S24-S29

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RAPID COMMUNICATION

## Short-term intravenous interferon therapy for chronic hepatitis B

Hiroaki Okushin, Toru Ohnishi, Kazuhiko Morii, Koichi Uesaka, Shiro Yuasa

Hiroaki Okushin, Toru Ohnishi, Kazuhiko Morii, Koichi Uesaka, Shiro Yuasa, Department of Internal Medicine, Himeji Red Cross Hospital, Hyogo, Japan

Correspondence to: Hiroaki Okushin, MD, Department of Internal Medicine, Himeji Red Cross Hospital, 1-12-1 Shimoteno, Himeji-shi, Hyogo 670-8540,

Japan. [hiroaki\\_okushin@hotmail.co.jp](mailto:hiroaki_okushin@hotmail.co.jp)

Telephone: +81-79-2942251 Fax: +81-79-2964050

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### Abstract

**AIM:** To investigate the therapeutic efficacy of short-term, multiple daily dosing of intravenous interferon (IFN) in patients with hepatitis B e antigen (HBeAg)-positive chronic hepatitis B.

**METHODS:** IFN- $\beta$  was intravenously administered at a total dose of 102 million international units (MIU) over a period of 28 d in 26 patients positive for HBeAg and HBV-DNA. IFN-beta was administered at doses of 2 MIU and 1 MIU on d 1, 3 MIU twice daily from d 2 to d 7, and 1 MIU thrice daily from d 8 to d 28. Patients were followed up for 24 wk after the end of treatment.

**RESULTS:** Six months after the end of the treatment, loss of HBV-DNA occurred in 13 (50.0%) of the 26 patients, loss of HBeAg in 9 (34.6%), development of anti-HBe in 10 (38.5%), HBeAg seroconversion in 8 (30.8%), and normalization of alanine aminotransferase (ALT) levels in 11 (42.0%).

**CONCLUSION:** This 4-wk long IFN- $\beta$  therapy, which was much shorter than conventional therapy lasting 12 wk or even more than 1 year, produced therapeutic effects similar to those achieved by IFN- $\alpha$  or pegylated-IFN- $\alpha$  (peg-IFN). Fewer adverse effects, greater efficacy, and a shorter treatment period led to an improvement in patients' quality of life. IFN- $\beta$  is administered intravenously, whereas IFN- $\alpha$  is administered intramuscularly or subcutaneously. Because both interferons are known to bind to an identical receptor and exert antiviral effects through intracellular signal transduction, the excellent results of IFN- $\beta$  found in this study may be attributed to the multiple doses allowed by the intravenous route.

**Hepatitis B virus; Interferon beta; Multiple daily dosing; Short-term treatment; Intravenous injection**

**Peer reviewers:** Philip Abraham, Dr, Professor, Consultant Gastroenterologist & Hepatologist, P. D. Hinduja National Hospital & Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai 400 016, India; Richard A Rippe, Dr, Department of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7038, United States

Okushin H, Ohnishi T, Morii K, Uesaka K, Yuasa S. Short-term intravenous interferon therapy for chronic hepatitis B. *World J Gastroenterol* 2008; 14(19): 3038-3043 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3038.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3038>

### INTRODUCTION

The increasing prevalence of chronic hepatitis caused by hepatitis B or C virus infection represents a concern in many regions worldwide. Interferons (IFN) are widely used in the treatment of the disease. With the recent launch of lamivudine, adefovir, and entecavir, the number of treatment options for chronic hepatitis B has increased. Treatment with these oral nucleoside analogues has serious drawbacks, such as the development of resistant HBV strains<sup>[1,2]</sup> and the need for years of treatment<sup>[3,4]</sup> or even a lifetime therapy. Thus, a large number of patients still require IFN therapy, which is effective in a relatively short period of time. Recently, however, in some patients, the treatment with IFN is often prolonged up to 24-48 wk to improve efficacy<sup>[5-7]</sup>. IFN- $\alpha$  is administered intramuscularly or subcutaneously and may be associated with such adverse effects as fatigue, insomnia, anorexia, and alopecia<sup>[7,8]</sup>. These effects presumably result from prolonged elevation of blood IFN levels. Prolonged exposure to higher levels of the circulating drug may produce a greater therapeutic effect while inducing greater adverse effects<sup>[9,10]</sup>. Treatment for a higher therapeutic effect without consideration of the burden on patients is not a good therapeutic strategy.

In Japan, IFN preparations for the treatment of hepatitis B include IFN- $\alpha$  for intramuscular or subcutaneous administration and IFN- $\beta$  for intravenous administration<sup>[11]</sup>. Both IFN- $\alpha$  and IFN- $\beta$  bind to the an identical IFN receptor and induce PKR and other antiviral proteins via intracellular signal transduction systems represented by JAK/STAT<sup>[12]</sup>. Because of the intravenous route, the blood concentration of IFN- $\beta$  reaches its peak immediately after infusion and then decreases

rapidly<sup>[13]</sup>. Decrease or loss of efficacy by receptor down-regulation<sup>[14-16]</sup> and adverse effects with IFN therapy are less likely to occur because blood level of IFN- $\beta$  does not maintain after signal transduction *via* the IFN receptor. The receptor function is maintained, and thus frequent dosing of IFN- $\beta$  is likely to produce greater efficacy. Indeed, in patients with hepatitis C, we found that IFN- $\beta$  in divided doses administered in the morning and evening was more effective than that administered once daily at the same total dose<sup>[17]</sup>.

For the development of short-term IFN therapy for hepatitis B, in the present study we investigated a 4-wk, multiple daily dosing of intravenous IFN- $\beta$ , a new regimen that produced therapeutic effects similar to those achieved by 24-wk or 1-year treatment with IFN- $\alpha$ .

## MATERIALS AND METHODS

### Patients

Among Japanese adult patients with chronic hepatitis B who were positive for HBeAg and HBV-DNA and presented at our hospital from 1996 to 2002, 26 patients were enrolled in this open-label study. The study was conducted in accordance with the Declaration of Helsinki, and the patients consented to the experimental treatment of hepatitis B. Inclusion criteria were: age of 20 years or older, blood HBeAg positivity, blood HBV-DNA positivity, and persistent abnormal elevation of ALT levels. Exclusion criteria included: coinfection with hepatitis C virus or HIV, presence of hepatocellular carcinoma, symptoms caused by decompensated cirrhosis, alcoholic, autoimmune, drug-induced, or other non-viral liver disorders, and hypersensitivity to IFN- $\beta$ . Any herbal medicines were discontinued during the treatment with IFN- $\beta$ .

### Treatment methods

Human fibroblast-derived natural IFN- $\beta$  (FERON<sup>®</sup>, Toray Industries Inc., Japan) was used; 1 to 3 MIU was dissolved in 100 mL of 5% glucose or isotonic saline solution for injection and infused intravenously for about 10 minutes. The dosing schedule comprised 2 MIU in the morning and 1 MIU in the evening (twice daily) at d 1 of treatment, 3 MIU in the morning and evening (twice daily) from d 2 through d 7, and 1 MIU each in the morning, in the afternoon, and at bedtime (thrice daily) from d 8 to d 28, with a total dose of 102 MIU administered over a treatment period of 28 d. Patients were followed up for 24 wk after the end of the IFN- $\beta$  therapy.

### Laboratory methods

Blood samples were collected immediately before the start of treatment, weekly during the treatment, and monthly during the follow-up period. Biochemical and hematological tests were performed each time. HBsAg was measured by reversed passive hemagglutination (R-PHA), anti-HBs was measured by passive hemagglutination (PHA), HBeAg and anti-HBe were measured by radioimmunoassay (RIA). HBV-DNA polymerase activity was measured by radioassay. Serum HBV-DNA was

measured by branched DNA probe assay (Chiron Corp, USA) with a detection sensitivity of 0.70 megaequivalents (Meq) per milliliter. Anti-hepatitis C virus antibodies were measured by enzyme immunoassay (EIA). In addition, 2',5'-oligoadenylate synthetase (2-5AS), an indicator of IFN activity, was quantitatively measured by RIA.

### Statistical analysis

Values are given as either mean  $\pm$  SD or median and range. For comparison, Student's *t*-test or the Chi-square test were used. Statistical tests were two-sided, and a *P* value of less than 0.05 was considered as statistically significant.

## RESULTS

### Patient population

Clinical characteristics of the 26 patients with HBeAg positive chronic hepatitis B at beginning of the treatment are shown in Table 1. All patients received a total dose of 102 MIU of IFN- $\beta$  over a period of 28 d, and none of them dropped out because of adverse effects or other reasons.

### Clinical outcomes

Six months after the end of IFN administration, loss of HBV-DNA occurred in 13 (50.0%) patients, loss of HBeAg in 9 (34.6%), loss of HBV-DNA and HBeAg in 9 (34.6%), development of anti-HBe in 10 (38.5%), and HBe seroconversion in 8 (30.8%). The last parameter is a measure of the therapeutic effect, defined by the loss of HBeAg and the subsequent development of anti-HBe. ALT levels normalized in 11 (42.0%) of the 26 patients. The percentage of patients, which became negative for HBV-DNA, HBeAg, and the change in ALT levels during/after the treatment are shown in Table 2 and Figure 1, respectively.

### Baseline HBV DNA polymerase activity and virological response

Patients were stratified according to baseline DNA polymerase activity (less than 1000 cpm *vs* 1000 cpm or more), and virological responses were recorded. Among 15 patients with an activity lower than 1000 cpm, 11 (73.3%) had a complete virological response, and 4 (26.7%) had no response. Among the 11 patients with an activity of 1000 cpm or more, 2 (18.2%) had a complete virological response, and 9 (81.8%) had no response.

### 2-5AS

Figure 2 shows the change in 2-5AS levels. The level of 2-5AS at baseline was  $114.8 \pm 102.1$  (mean  $\pm$  SD). The levels at wk 1, 2, and at the end of treatment were  $389.9 \pm 205.3$ ,  $333.3 \pm 133.4$ , and  $344.3 \pm 181.2$ , respectively.

### Adverse effects

No patients discontinued treatment because of adverse effects, with a treatment completion rate of 100%. Fever was mild because antipyretic loxoprofen sodium was administered before intravenous infusion to suppress IFN-

Table 1 Clinical characteristics at the beginning of the treatment

Characteristics	Baseline
Age (yr)	31.8 ± 7.0 <sup>1</sup>
Sex (male/female)	19/7
ALT (U/L)	246.9 ± 154.2 <sup>1</sup>
HBV DNA (≥ 10/ <sup>&lt;</sup> 10 Meq/mL)	16/10
HBV DNA polymerase (cpm)	750.5 (10-10710) <sup>2</sup>
PLT (× 10 <sup>4</sup> /mm <sup>3</sup> )	19.3 ± 10.7 <sup>1</sup>

<sup>1</sup>mean ± SD; <sup>2</sup>Median (range).

Table 2 Response rate in patients with HBeAg positive chronic hepatitis B by interferon-β treatment (%)

	wk 1	wk 2	End of the treatment	6 mo after treatment
HBV-DNA negative	5/26 (19.2)	5/26 (19.2)	10/26 (38.5)	13/26 (50.0)
HBeAg and HBV-DNA negative			4/26 (15.4)	9/26 (34.6)

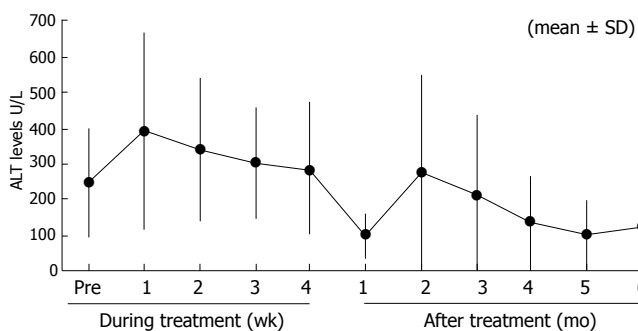


Figure 1 Change in ALT levels during treatment with interferon-β and during the follow-up.

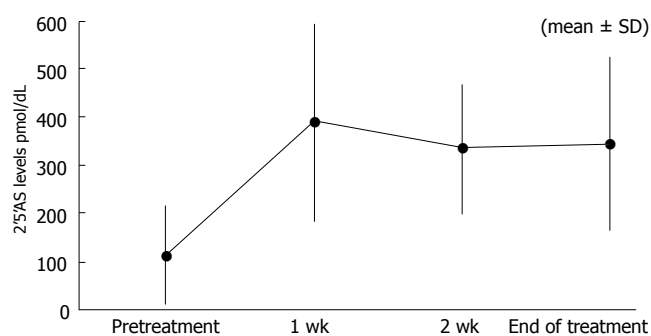


Figure 2 Change in 2'5'AS levels during treatment.

induced fever. During treatment with IFN, no patients experienced depression. There was no proteinuria, severe thrombocytopenia or leukopenia (as shown in Figure 3).

## DISCUSSION

Approximately 10 years ago, the IFN therapy for chronic hepatitis B was administered for up to 4 wk in Japan. However, a 24-wk regimen has been recently used because

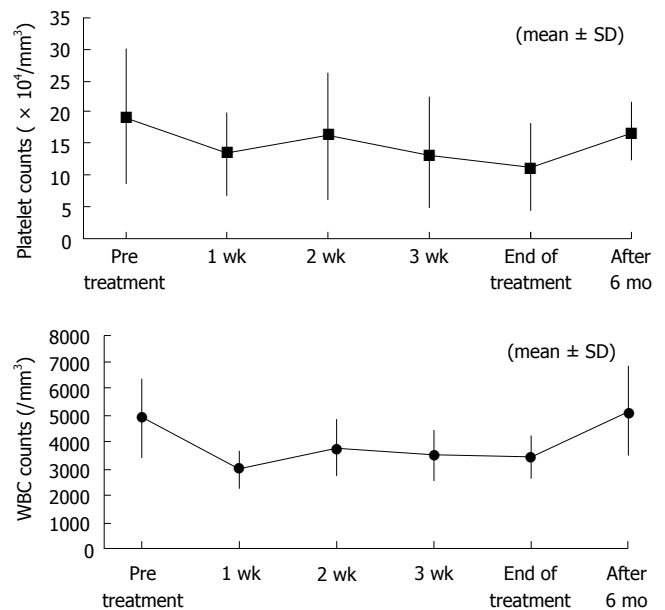
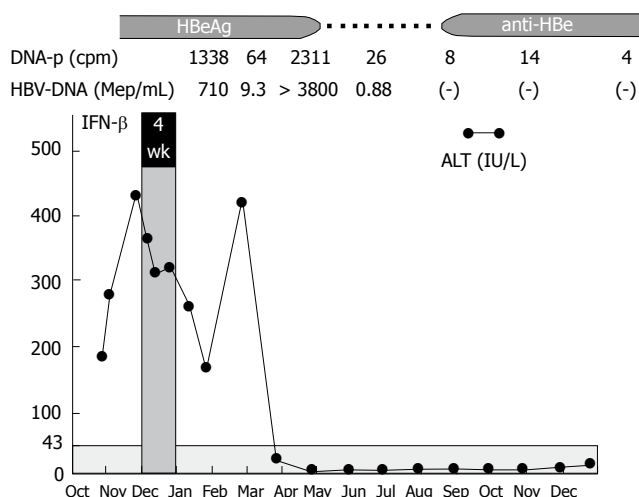


Figure 3 Changes in platelet and WBC counts.

Table 3 Comparison of response rates in patients with HBeAg positive chronic hepatitis B at 6 mo after the treatment (%)

	INF-β (iv) 4 wk	INF-α (sc or im) 12-24 wk	Lamivudine 1 yr	Adefovir dipivoxil 48 wk	Pegylated interferon-α 48 wk
Loss of serum HBV DNA	50	37	44	21	32
Loss of HBeAg	35	33	17-32	24	34
HBeAg seroconversion	31	Difference of 18	16-18	12	32
Loss of HBsAg		8	< 1	0	3
Normalization of ALT	42	Difference of 23	41-72	48	41
Histological improvement			49-56	53	38
Durability of the response		80-90	50-80		82

a longer treatment seems to improve the efficacy. In the present study, we used a short-term, intravenous therapy of 4 wk, which seems to be against the recent recommendations for long-term regimens. However, 4-wk multiple daily dosing of intravenous IFN-β used in our study produced therapeutic effects similar to those achieved by 12-wk or 24-wk IFN-α or 48-wk peg-IFN-α, which are indicated by the American Association for the Study of Liver Diseases<sup>[18,19]</sup>. The HBe seroconversion rate with IFN-β in this study was 31%, which was higher than the reported 12-18% with IFN-α<sup>[18,20]</sup>, lamivudine<sup>[18,21-23]</sup>, or adefovir<sup>[19,24]</sup>, and which was almost equal to that achieved by a 48-wk therapy with peg-IFN-α<sup>[25]</sup> (Table 3). In the United States, the distribution of HBV genotypes was reported as genotypes A (33%), B (21%), C (34%), D (9%), E (1%), F (1%), and G (1%)<sup>[26]</sup>. Given that the majority (about 80%) of Japanese patients infected with HBV has IFN-resistant genotype C<sup>[27]</sup>, the multiple daily dosing of intravenous IFN-β used in this study appears to be a beneficial treatment.



**Figure 4** Typical pattern of clinical course with transient increase in ALT level after treatment with interferon- $\beta$ .

Our results suggest that HBV DNA polymerase activity at baseline before the treatment may be used to predict the therapeutic effect of IFN to some degree. Multiple daily dosing of IFN- $\beta$  may be the regimen of first-line choice in patients with baseline HBV DNA polymerase activity less than 1000 cpm because 73.3% of those patients had a complete virological response. We believe that the direct antiviral effect of IFN on HBV is enough to achieve a complete response in those patients, whereas an appropriate host immune response are also needed in patients with a polymerase activity of 1000 cpm or more indicating rapid proliferation of HBV. A typical example is shown in Figure 4. The patient had an HBV DNA polymerase activity of 1338 cpm and an HBV-DNA level of 710 Meq/mL before the IFN therapy. After the end of IFN- $\beta$  administration, an increase in HBV-DNA and subsequent rapid increase in ALT levels (so-called Schub) occurred, followed by the loss of HBeAg, HBV-DNA, and DNA polymerase, normalization of ALT levels, and development of anti-HB. The rapid increase in ALT levels probably resulted from the host's immune response to the rapid increase in the HBV proliferation following the regimen and the subsequent rapid elimination of infected hepatocytes in an appropriate manner.

Our dosing regimen had a good safety profile with a low incidence of mild adverse effects and no serious adverse effects. This may be attributed to lower daily doses of 3 MIU from d 8 onward and a short treatment period of 1 mo. Although platelet and leukocyte counts decreased at wk 1 compared with baseline levels, the counts remained unchanged thereafter until the end of treatment and almost returned to baseline levels after completion of therapy. Our previous experience suggested that thrombocytopenia and proteinuria should be closely monitored during treatment with IFN- $\beta$  at doses of 3 MIU twice daily. However, cytopenia did not worsen because of switching to 1 MIU thrice daily from d 8. The levels of 2-5AS in blood (mean  $\pm$  SD) at baseline and wk 1, 2, and 4 of treatment were  $133.9 \pm 122.2$ ,  $445.0 \pm 209.7$ ,  $335.0 \pm 139.9$ , and  $387.8 \pm 200.7$ , respectively, and

remained elevated during treatment, suggesting that the dose regimen produced a potent and durable antiviral effect despite a modest cytopenia.

In general, the pharmacokinetics of an intravenously administered drug are characterized by a higher blood elimination rate, higher peak blood concentration, and greater tissue distribution than an intramuscularly administered drug, and these are also true of IFN. Different types of IFN formulations are available for therapy, and human fibroblast IFN- $\beta$  is applicable to intravenous administration for the treatment of hepatitis in Japan.

We chose intravenous administration and multiple daily dosing because of the following three reasons. First, intravenously administered IFN- $\beta$  is rapidly eliminated from the blood and below the detection limit shortly after administration<sup>[13]</sup>. Compared with intramuscularly or subcutaneously administered IFN- $\alpha$ , IFN- $\beta$  accumulates to a lesser degree and is likely to have less adverse effects<sup>[28]</sup>. Second, blood concentrations of IFN administered intravenously in multiple daily doses fluctuate with high blood levels and rapid elimination rates. Accordingly, this regimen is likely to avoid persistently elevated blood IFN levels and resultant downregulation of the IFN receptor<sup>[14-16]</sup>, which is likely to occur after intramuscular or subcutaneous administration. The avoidance of the receptor downregulation allows effective binding of IFN and its receptor, and triggers the host defense mechanisms a few times a day to eliminate the virus. Third, the drug administered intravenously is more extensively distributed into organs than that administered intramuscularly. For elimination of HBV present in hepatocytes, intravenous dosing is considered as an effective route of administration, which allows extensive delivery of IFN to the liver. When IFN- $\alpha$ , which was induced by treating human leukocytes with the Sendai virus, was administered intravenously or intramuscularly to rats, IFN- $\alpha$  was detectable in the liver at 10 and 30 min but not at 1 h after intravenous administration whereas IFN levels remained below the detection limit for 4 h in rats receiving an intramuscular administration<sup>[29]</sup>. In patients with hepatitis, a transient increase in ALT levels is often observed after intravenous administration of IFN<sup>[30]</sup>. Because IFN distributes in the liver at high concentrations after intravenous administration, extensive loss of infected hepatocytes may occur, resulting in an increase in ALT levels.

When IFN or any other cytokine that exerts a pharmacological effect *via* receptor binding is administered, it is important to choose an appropriate route of administration that ensures effective delivery of the drug to the target-cells. An ideal pharmacokinetic profile should include a rapid increase to effective blood concentrations and a rapid elimination after receptor binding to avoid downregulation of the receptor. We believe that intravenous IFN therapy can also be used effectively for the treatment of other diseases including cancer, infection with HIV, and SARS. However, intravenous IFN is now available only in Japan. For further promotion of research on the establishment of intravenous IFN therapy as a convenient, general way of treating these diseases,



intravenous IFN should preferably be available in other countries.

Oral nucleoside analogues, such as lamivudine, adefovir, and entecavir, have a potent effect in suppressing hepatitis B virus; however, most patients relapse and become positive for the virus after discontinuation of treatment. Thus, these drugs should be taken for a few years or the rest of patients' lives. These agents also cause problems including development of resistant strains and fetotoxicity, which discourages physicians from administering these agents in pregnant, parturient, and nursing women. Meanwhile, IFN therapy tends to continue for more than 6 months, and increased adverse effects associated with prolonged therapy have become a significant problem. In Japan, both physicians and patients have great difficulty coping with these problems and they are waiting for new effective treatments that ensure improvement in the quality of life for patients.

Short-term treatment with multiple daily dosing of IFN- $\beta$  used in the present pilot study has fewer adverse effects, good therapeutic effects, and reproducibility to some degree. Further studies and randomized clinical trials are required to confirm our promising results.

## COMMENTS

### Background

Hepatitis B virus (HBV) is a major cause of liver disease worldwide, ranging from acute and chronic hepatitis to cirrhosis and hepatocellular carcinoma. Therefore, in order to improve the hepatitis and cirrhosis, and decrease the risk of hepatocellular carcinoma on the patients of chronic hepatitis B, it is extremely important to achieve sustained suppression of HBV replication, normalize serum alanine aminotransferase (ALT) level, and induce seroconversion by therapies. Recently, interferon (IFN)- $\alpha$  (conventional and pegylated) with or without nucleoside analogues or nucleoside analogues only are used for therapy. However, available therapies are suboptimal.

### Research frontiers

Therapies using IFN- $\alpha$  and/or nucleotide analogues are needed a long period to treat. Furthermore, those therapies are associated with some side effects. So, the authors tried to establish the new therapeutic protocol using IFN- $\beta$ , because IFN- $\beta$  belongs to type I IFN family like IFN- $\alpha$  and there were some reports that indicated the treatment of IFN- $\beta$  twice a day was more effective than that of IFN- $\alpha$  or IFN- $\beta$  once a day in chronic hepatitis C patients.

### Innovations and breakthroughs

In this study, the author's have evaluated the efficacy of a short term (4 wk), multiple daily dosing therapeutic protocol using IFN- $\beta$  for chronic hepatitis B patients. As a result, the therapeutic efficacy of that regimen is similar to that of PEG-IFN- $\alpha$  treatment for 24 wk or 1 year. Furthermore, the side effects of IFN- $\beta$  treatment in this study were less than those of PEG-IFN- $\alpha$  or IFN- $\alpha$  treatment for 24 wk or 1 year. Therefore, this treatment method of IFN- $\beta$  few times a day is more effective than standard therapeutic protocols on chronic hepatitis B patients for the first time.

### Applications

In the present pilot study, the authors indicated that the treatment protocol of IFN- $\beta$  in this study could improve a rate of side effects compare with the standard IFN- $\alpha$  or PEG-IFN- $\alpha$  treatment protocol without loss of therapeutic effects. Further studies and randomized clinical trials are required to confirm the indication of short term therapy for chronic hepatitis B.

### Terminology

It has reported that the treatment of IFN- $\beta$  twice a day is more effective than that of IFN- $\alpha$  or IFN- $\beta$  once a day in chronic hepatitis C patients. However, there is

no investigation that described the efficacy of treatment of IFN- $\beta$  twice a day on chronic hepatitis B patients.

### Peer review

The authors may want to provide end-of-treatment data as well, in addition to the SVR data that they have provided. Overall, I feel this is a novel approach that needs wider consideration.

## REFERENCES

- 1 **Honkoop P**, Niesters HG, de Man RA, Osterhaus AD, Schalm SW. Lamivudine resistance in immunocompetent chronic hepatitis B. Incidence and patterns. *J Hepatol* 1997; **26**: 1393-1395
- 2 **Hoofnagle JH**. Therapy of viral hepatitis. *Digestion* 1998; **59**: 563-578
- 3 **Liaw YF**, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Chien RN, Dent J, Roman L, Edmundson S, Lai CL. Effects of extended lamivudine therapy in Asian patients with chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *Gastroenterology* 2000; **119**: 172-180
- 4 **Leung NW**, Lai CL, Chang TT, Guan R, Lee CM, Ng KY, Lim SG, Wu PC, Dent JC, Edmundson S, Condreay LD, Chien RN. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. *Hepatology* 2001; **33**: 1527-1532
- 5 **Janssen HL**, Gerken G, Carreno V, Marcellin P, Naoumov NV, Craxi A, Ring-Larsen H, Kitis G, van Hattum J, de Vries RA, Michielsen PP, ten Kate FJ, Hop WC, Heijtkink RA, Honkoop P, Schalm SW. Interferon alfa for chronic hepatitis B infection: increased efficacy of prolonged treatment. The European Concerted Action on Viral Hepatitis (EUROHEP). *Hepatology* 1999; **30**: 238-243
- 6 **Sakai T**, Shiraki K, Inoue H, Okano H, Deguchi M, Sugimoto K, Ohmori S, Murata K, Nakano T. Efficacy of long-term interferon therapy in chronic hepatitis B patients with HBV genotype C. *Int J Mol Med* 2002; **10**: 201-204
- 7 **Cooksley WG**, Piratvisuth T, Lee SD, Mahachai V, Chao YC, Tanwandee T, Chutaputti A, Chang WY, Zahm FE, Pluck N. Peginterferon alpha-2a (40 kDa): an advance in the treatment of hepatitis B e antigen-positive chronic hepatitis B. *J Viral Hepat* 2003; **10**: 298-305
- 8 **Wong JB**, Koff RS, Tine F, Pauker SG. Cost-effectiveness of interferon-alpha 2b treatment for hepatitis B e antigen-positive chronic hepatitis B. *Ann Intern Med* 1995; **122**: 664-675
- 9 **Sagir A**, Wettstein M, Heintges T, Haussinger D. Autoimmune thrombocytopenia induced by PEG-IFN-alpha2b plus ribavirin in hepatitis C. *Dig Dis Sci* 2002; **47**: 562-563
- 10 **Lambotte O**, Gelu-Simeon M, Maigne G, Kotb R, Buffet C, Delfraissy JF, Goujard C. Pegylated interferon alpha-2a-associated life-threatening Evans' syndrome in a patient with chronic hepatitis C. *J Infect* 2005; **51**: e113-e115
- 11 **Suzuki F**, Arase Y, Akuta N, Tsubota A, Suzuki Y, Sezaki H, Hosaka T, Someya T, Kobayashi M, Saitoh S, Ikeda K, Kobayashi M, Matsuda M, Satoh J, Kumada H. Efficacy of 6-month interferon therapy in chronic hepatitis B virus infection in Japan. *J Gastroenterol* 2004; **39**: 969-974
- 12 **Stark GR**, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. *Annu Rev Biochem* 1998; **67**: 227-264
- 13 **Hino K**, Kondo T, Yasuda K, Fukuhara A, Fujioka S, Shimoda K, Niwa H, Iino S, Suzuki H. Pharmacokinetics and biological effects of beta interferon by intravenous (iv) bolus administration in healthy volunteers as compared with iv infusion. *Jpn J Clin Pharmacol Ther* 1998; **19**: 625-635
- 14 **Lau AS**, Hannigan GE, Freedman MH, Williams BR. Regulation of interferon receptor expression in human blood lymphocytes in vitro and during interferon therapy. *J Clin Invest* 1986; **77**: 1632-1638
- 15 **Nakajima S**, Kuroki T, Kurai O, Kobayashi K, Yamamoto S. Interferon receptors during treatment of chronic hepatitis B

- with interferon. *J Gastroenterol Hepatol* 1989; **4**: 419-427
- 16 **Nakajima S**, Kuroki T, Shintani M, Kurai O, Takeda T, Nishiguchi S, Shiomi S, Seki S, Kobayashi K. Changes in interferon receptors on peripheral blood mononuclear cells from patients with chronic hepatitis B being treated with interferon. *Hepatology* 1990; **12**: 1261-1265
  - 17 **Okushin H**, Morii K, Kishi F, Yuasa S. Efficacy of the combination therapy using twice-a-day IFN-beta followed by IFN-alpha-2b in treatment for chronic hepatitis C. *Kanzo* 1997; **38**: 11-18
  - 18 **Lok AS**, McMahon BJ. Chronic hepatitis B. *Hepatology* 2001; **34**: 1225-1241
  - 19 **Lok AS**, McMahon BJ. Chronic hepatitis B: update of recommendations. *Hepatology* 2004; **39**: 857-861
  - 20 **Wong DK**, Cheung AM, O'Rourke K, Naylor CD, Detsky AS, Heathcote J. Effect of alpha-interferon treatment in patients with hepatitis B e antigen-positive chronic hepatitis B. A meta-analysis. *Ann Intern Med* 1993; **119**: 312-323
  - 21 **Lai CL**, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; **339**: 61-68
  - 22 **Dienstag JL**, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, Crowther L, Condreay LD, Woessner M, Rubin M, Brown NA. Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999; **341**: 1256-1263
  - 23 **Schalm SW**, Heathcote J, Cianciara J, Farrell G, Sherman M, Willems B, Dhillon A, Moorat A, Barber J, Gray DF. Lamivudine and alpha interferon combination treatment of patients with chronic hepatitis B infection: a randomised trial. *Gut* 2000; **46**: 562-568
  - 24 **Marcellin P**, Chang TT, Lim SG, Tong MJ, Sievert W, Shiffman ML, Jeffers L, Goodman Z, Wulfsohn MS, Xiong S, Fry J, Brosgart CL. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med* 2003; **348**: 808-816
  - 25 **Lau GK**, Piratvisuth T, Luo KX, Marcellin P, Thongsawat S, Cooksley G, Gane E, Fried MW, Chow WC, Paik SW, Chang WY, Berg T, Flisiak R, McCloud P, Pluck N. Peginterferon Alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2005; **352**: 2682-2695
  - 26 **Chu CJ**, Lok AS. Clinical significance of hepatitis B virus genotypes. *Hepatology* 2002; **35**: 1274-1276
  - 27 **Orito E**, Ichida T, Sakugawa H, Sata M, Horiike N, Hino K, Okita K, Okanoue T, Iino S, Tanaka E, Suzuki K, Watanabe H, Hige S, Mizokami M. Geographic distribution of hepatitis B virus (HBV) genotype in patients with chronic HBV infection in Japan. *Hepatology* 2001; **34**: 590-594
  - 28 **Festi D**, Sandri L, Mazzella G, Roda E, Sacco T, Staniscia T, Capodicasa S, Vestito A, Colecchia A. Safety of interferon beta treatment for chronic HCV hepatitis. *World J Gastroenterol* 2004; **10**: 12-16
  - 29 **Mura N**, Matsuzawa H, Ueda H, Sakashita K, Nakamura K, Uemura H, Arai S, Hamanaka N, Chisaka T, Yagi N, Araki H, Koga J, Matsuo A. Pharmacokinetics of FPI-31. *Jpn Pharmacol Ther* 1993; **21**: 2211-2226
  - 30 **Fujimori K**, Mochida S, Matsui A, Ohno A, Fujiwara K. Possible mechanisms of elevation of serum transaminase levels during interferon-beta therapy in chronic hepatitis C patients. *J Gastroenterol* 2002; **37**: 40-46

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RAPID COMMUNICATION

## Discrepancies between the responses to skin prick test to food and respiratory antigens in two subtypes of patients with irritable bowel syndrome

Rosa LS Soares, Hamilton N Figueiredo, Jose M Santos, Rita F Oliveira, Raquel L Godoy, Felipe AP Mendonça

Rosa LS Soares, Internal Medicine Department, Faculty of Medicine-Federal Fluminense University, Rio de Janeiro 24030-210, Brazil

Hamilton N Figueiredo, Internal Medicine Department, Faculty of Medicine, Federal Fluminense University, Rio de Janeiro 24030-210, Brazil

Jose M Santos, Medical school of University Hospital Antonio Pedro, Federal Fluminense University, Rio de Janeiro 24030-210, Brazil

Rita F Oliveira, Raquel L Godoy, Felipe AP Mendonça, Medical school of Faculty of Medicine, Federal Fluminense University, Rio de Janeiro 24030-210, Brazil

**Author contributions:** Soares RLS and Figueiredo HN designed the research and analyzed the data; Santos JM, Oliveira RF, Godoy RL and Mendonça FAP performed the research and Soares RLS wrote the paper.

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**Correspondence to:** Rosa LS Soares, Internal Medicine Department, Faculty of Medicine-Federal Fluminense University, Rua Marques do Paraná 189 /1002, Rio de Janeiro 24030-210, Brazil. [rsalerno@openlink.com.br](mailto:rsalerno@openlink.com.br)

Telephone: +55-21-27179192 Fax: +55-21-27179926  
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FA responses differed significantly from those for the other two groups ( $P < 0.01$ ).

**CONCLUSION:** Despite the small number of cases studied, the higher reactivity to FAs in Group I compared to Groups II and III adds new information, and suggests the presence of a possible alteration in intestinal epithelial function.

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**Key words:** Atopy; Constipation; Diarrhea; Food intolerance; Irritable bowel syndrome; Skin prick test

**Peer reviewers:** Javier S Martin, Chief, Gastroenterology and Endoscopy, Sanatorio Cantegril, Av. Roosevelt y P 13, Punta del Este 20100, Uruguay; Heitor Rosa, Professor, Department of Gastroenterology and Hepatology, Federal University School of Medicine, Rua 126 n.21, Goiania-GO 74093-080, Brazil

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### Abstract

**AIM:** To compare the response to skin prick tests (SPTs) to food antigens (FAs) and inhalant allergens (IAs) in patients with two subtypes of irritable bowel syndrome (IBS) and healthy controls.

**METHODS:** We compared the results of SPTs for IAs and FAs in 87 volunteers divided into three groups: diarrhea predominant IBS (D-IBS) Group I ( $n = 19$ ), constipation predominant IBS (C-IBS) Group II ( $n = 17$ ), and normal controls Group III ( $n = 51$ ).

**RESULTS:** Of the 285 tests (171 for FAs and 114 for IAs) performed in Group I we obtained 45 (26.3%) positive responses for FA and 23 (20.1%) for IA. Of the 153 tests for FA in Group II, we obtained 66 (20.1%) positive responses, and of the 102 tests for IA, we obtained 20 (19.6%) positive responses. Of the 459 tests for FA performed in Group III, we obtained 39 (8.4%) positive responses, and of the 306 for IA, we obtained 52 (16.9%) positive responses. The numbers of positive responses were not significantly different between the three groups, but in the D-IBS group, the number of SPT

### INTRODUCTION

Irritable bowel syndrome (IBS) is an extremely common disorder that affects about one in every 5-10 persons. Estimates of prevalence range from 9% to 22% depending upon population group studied<sup>[1-7]</sup>.

The exact pathophysiology of IBS remains unknown, although various mechanisms including gastrointestinal dysmotility and visceral hypersensitivity have been well studied in IBS<sup>[8-10]</sup>. Recent interest has also been directed to the possible participation of the mucosa in the pathophysiology of IBS<sup>[11-13]</sup>. Inflammatory mediators cause intestinal dysfunction and a consequent increase in permeability<sup>[14-17]</sup>. However the role and interaction of inflammatory mediators with IBS remains to be determined<sup>[17]</sup>.

IBS is defined by symptomatic criteria rather than biological markers. No diagnostic tests are available, clinical subtypes of IBS are based on the predominant symptom:

IBS diarrhea predominant (D-IBS), IBS constipation predominant (C-IBS), and mixed (m-IBS), and treatment is selected based on the predominant symptom<sup>[3,18,19]</sup>.

Clinically, the frequency of IBS is associated with psychological stress, food intolerance (adverse reaction to a specific food or ingredient that is not immune mediated or associated with psychological phenomena), intestinal infections, and even previous abdominal surgery<sup>[3,7,12,20-25]</sup>.

Dunlop *et al.*<sup>[16]</sup> have reported that patients with sub-type D-IBS (pos-infectious and non-infectious origin) have a more pronounced permeability increase in the proximal intestine compared with controls and those with C-IBS. Another aspect of that study was the detection of a significant correlation between atopy and increased intestinal permeability, which suggests that at least a subset of IBS patients may have a systemic immunological disorder. Other studies have reported a correlation between asthma, food allergy and IBS<sup>[26-29]</sup>, but the role of allergic reactions in the pathophysiology of IBS remains controversial<sup>[7,13,27,29-31]</sup>.

We have previously observed that volunteers with a diagnosis of IBS have reported higher cutaneous reactivity to food antigens than to inhalant allergens, when compared to patients with functional dyspepsia and normal controls<sup>[30]</sup>. The association between food hypersensitivity and IBS symptoms is still open to question<sup>[7,17,20,22-25]</sup>. New information is useful for a better understanding of the relationship between increased intestinal permeability, mucosal barrier defects, and intestinal inflammation in IBS patients.

The aim of this study was to compare the response to skin prick tests (SPTs) with food and inhalant antigens in two subtypes of IBS and healthy controls.

## MATERIALS AND METHODS

We studied the response to SPTs with inhalant and food extracts<sup>[32]</sup> in 87 volunteers, 36 patients with IBS (evaluated by a pre-designed questionnaire based on the Rome III criteria<sup>[18]</sup> for functional gastrointestinal diseases), and 51 normal volunteers (school employees and medical students at Antonio Pedro University Hospital). They were evaluated between September 2006 and January 2007. The volunteers were first evaluated in outpatient clinics for functional gastrointestinal diseases at Antonio Pedro University Hospital. Subjects completed a questionnaire which included Rome III criteria for IBS, and were submitted to a clinical evaluation that included a careful history (age, duration of symptoms, psychosocial factors, alarm symptoms, personal history of atopy, family history of gastrointestinal disease), examination, and stool examination for ova and parasites (Brazil is an endemic area for parasitic infections). The inclusion criteria were age > 18 years old and a diagnosis of IBS, or volunteers from the general population. The exclusion criteria were clinical suspicion or diagnosis of organic disease of the gastrointestinal tract (including positives stool examination for ova and parasites) at least 12 mo prior to the study after clinical evaluation.

The subjects were divided into three groups. D-IBS,

Group I ( $n = 19$ ; 14 female, five male; mean age 32.6 years), with IBS ROMA III Criteria for recurrent abdominal discomfort or pain at least 3 d per month in the last 3 mo, associated with two or more of the following: (1) improvement with defecation; (2) onset associated with a change in stool frequency; or (3) onset associated with a change in stool form (appearance). C-IBS, Group II ( $n = 17$  subjects; 12 female, five male; mean age 31.8 years. Controls, Group III ( $n = 51$  subjects; 31 female, 20 male; mean age 26.3 years) without previous or current significant gastrointestinal symptoms. The three groups, after informed consent (approved by the local Ethical Committee: number CAAE 009025800007) were submitted to SPTs with nine food extracts (ovalbumin, egg yolk, nuts, peanuts, wheat flour, cow's milk, soya, crustaceans and chocolate), and six inhalant extracts (*Dermatophagoides* spp., *Blomia tropicalis*, air dust, *Dermatophagoides pteronissimus*, house dust and *Dermatophagoides farinaceus*)<sup>[14]</sup>.

The contents of glycerinated food extracts (1:20), the positive control substance, histamine, and the negative control substance, saline, were commercially available (M Queiroz Laboratory, Rio de Janeiro, Brazil). They were applied by the prick technique (percutaneous) puncture through the standardized punter (discarded after use to avoid cross reaction), which allowed allergen absorption at multiple points in the skin. The test reading, done at the 20 min after the beginning were made by the measures of the wheel diameter eliciting by the test, obtained in millimeters. A wheal that was 3 mm greater than that of the negative control was considered positive. Anything less was considered negative<sup>[14]</sup>.

## Statistical analysis

Data were analyzed using Pearson's  $\chi^2$  test.  $P < 0.05$  was considered significant.

## RESULTS

A total of 1305 SPTs (783 for the FAs and 522 for the IAs) (nine FAs and six IAs for each volunteer) were accomplished in the three groups. In the D-IBS Group I, we obtained 45 positive responses for food extracts, which corresponded to 26.3% of the 171 tests performed and 23 positive responses for IAs, which corresponded to 20.1% of the 114 tests performed. In the C-IBS Group II, we obtained 21 positives responses (13.7% of the 153 tests) for FAs and 20 positive responses (19.6%) for IAs. In the control Group III, 39 (8.4%) positive responses were obtained in 459 tests performed for FAs, and 52 (16.9%) for IAs.

The positive responses were not concentrated in one or two subjects, but dispersed throughout the populations examined. The numbers of positive responses to SPT for each antigen did not differ significantly between the three groups ( $P > 0.05$ ).

Nine (52.9%) C-IBS and 11 (57.8%) D-IBS patients reported intolerance to several foods, but we did not find any correlation between positive SPTs and specific food intolerance in these subjects. Ten (58.8%) C-IBS and 12 (63.1%) D-IBS patients reported a personal history of



**Table 1** Personal history of allergies and food intolerance, and SPT response to FAs and IAs in IBS patients and normal controls (%)

	D-IBS ( <i>n</i> = 19)	C-IBS ( <i>n</i> = 17)	Without gastrointestinal symptoms ( <i>n</i> = 51)
Reported food intolerance	11 (57.8)	9 (52.9)	3 (5.8)
Personal history of allergies	12 (63.1)	10 (58.8)	26 (50.9)
No. of tests for FAs	171	153	459
Positive SPT for FAs	45 (26.3) <sup>b</sup>	21 (13.7)	39 (8.4)
No. of tests for IAs	114	102	306
Positive SPT for IAs	23 (20.1)	20 (19.6)	52 (16.9)

Papule 3 mm larger than the negative control was considered to be a positive response. <sup>b</sup>*P* < 0.01 compared to the other groups ( $\chi^2$ ).

allergies. Three (5.8%) volunteers without gastrointestinal symptoms reported food intolerance and 26 (50.9%) had a personal history of allergies. A personal history of allergies and the number of positive SPTs to IAs did not differ significantly between the three groups (*P* > 0.05). However, the number of positive SPTs to FAs in the D-IBS group differed significantly from that in the other two groups (*P* < 0.01) (Table 1).

## DISCUSSION

In the present study, we observed that patients with a diagnosis of D-IBS had higher cutaneous reactivity to FAs than to IAs, when compared with those with C-IBS and healthy controls. An association between IBS and sensitivity to several foods was identified in two groups, but the SPT response was not specific for any type of food. None of the volunteers with IBS reported intolerance to an isolated food, and positive SPT responses also were not correlated significantly with a history of intolerance to a specific food. A positive SPT response for a specific food was not associated with the crises of IBS in any of the patients in Groups I and II.

IBS is a common disorder worldwide, but its exact pathophysiology remains unknown<sup>[3]</sup>. Various mechanisms, including gastrointestinal dysmotility and visceral hypersensitivity, have been extensively studied in IBS<sup>[6-10]</sup>, but recent interest has also been associated with, or directed to the possible participation of the intestinal mucosa in the pathophysiology of IBS<sup>[11-13,17]</sup>. Several lines of evidence suggest that IBS may be associated with inflammation in the ileal or colonic mucosa, and at least in a subset of patients with IBS, the mucosal immune system seems to be activated<sup>[11,15-17]</sup>. Mucosal inflammation and immune system activation in IBS can be caused by many factors, including gastrointestinal infections, changes in the resident microflora, bile salts and FAs<sup>[15-17]</sup>.

FAs can activate the mucosal system when there is disruption of the gut barrier<sup>[31,33-36]</sup>. It is hypothesized that mucosal immune activation by FAs may contribute to the development of food allergy and IBS<sup>[9,11,13,15-17,21,25]</sup>. Clinically, the role of food-intolerance-induced symptoms in IBS frequently contrasts with that in food allergy<sup>[20,22-25,29,37-42]</sup>, and dietary elimination may be associated with symptom improvement<sup>[31]</sup>. However, the interaction of food with

the gastrointestinal system is not completely understood<sup>[13,17,20,22,23,25,29,33-37,39]</sup>.

Our results demonstrate that patients with IBS symptoms have non-specific intolerance to foods, probably associated with generalized hypersensitivity. The lack of specificity suggests that people with IBS symptoms, associated or not with food intolerance, have difficulties with food in general and specific foods may not be involved in the pathogenesis of this condition. In agreement with other studies, we suspect that IBS causes food sensitivity rather than vice-versa<sup>[3,22,37,38,41]</sup>.

The mechanisms underlying these inflammatory responses are unclear, but recent studies have suggested that an alteration in the mucosal barrier function and a consequent increase in intestinal permeability are the basis for the increased inflammation in IBS<sup>[14-17]</sup>. Dunlop *et al*<sup>[16]</sup> have reported that patients with D-IBS have a pronounced permeability increase in the proximal intestine compared with controls, and those without a history of infection onset have a more severe defect. The increase in intestinal permeability could conceivably activate the release of neurotransmitters that stimulate afferent neurons<sup>[13]</sup>.

Scientific evidence of the functional interface between the immune and sensory motor systems of the gut and respiratory systems has been reported<sup>[34,35,39]</sup>. Recent studies have reported that the prevalence of asthma and respiratory bronchial hyper-responsiveness are more common in IBS patients than in controls, and have suggested that at least a subset of IBS patients may have a systemic immunological disorder<sup>[27,28]</sup>. We have previously noticed that IBS patients have greater cutaneous reactivity to FAs than to IAs, when compared to patients with functional dyspepsia or normal controls. An association between IBS and sensitivity to multiple foods and non-specific response to SPTs has also been identified<sup>[30,42]</sup>.

In the current study, the presence of diarrhea in IBS was a significant contributor to the greater cutaneous reactivity response to FAs. No patients with IBS, with or without diarrhea, presented with gastrointestinal infections over the 12 mo that preceded the study, including positive stool examination for ova and parasites. We did not find a significant association between personal history of allergies, IBS sub-type, food intolerance and SPT response. The discrepancies between the response to SPTs to FAs and IAs in the two subtypes of patients with IBS suggest disruption of the gut barrier in patients with D-IBS. Our findings are in agreement with other studies<sup>[13,16,17,37,38,41]</sup>.

We conclude that the lack of specificity to food SPT response and the greater cutaneous reactivity to FAs than to IAs may be associated with altered epithelial function and increase in intestinal permeability in D-IBS. Further studies are needed to clarify the potential pathogenic mechanisms underlying the association between IBS and allergy, and to determine if IBS is one or several disorders.

## COMMENTS

### Background

Irritable bowel syndrome (IBS) symptoms are frequently associated with the reporting of many food sensitivities. The role of food-intolerance-induced symptoms in IBS frequently contrasts with that in food allergy, but the pathogenesis

of this association is not completely understood. Food antigens (FAs) can activate the mucosal system when there is disruption of the gut barrier. The report of a significant correlation among atopy and increased intestinal permeability suggests that at least a subset of IBS patients may have a systemic immunological disorder.

### Research frontiers

New information is useful for a better understanding of the relationship between increased intestinal permeability, mucosal barrier defects, and intestinal inflammation in IBS patients. Studies are needed to clarify the potential pathogenic mechanisms underlying the association between IBS and allergy, and to determine if IBS is one or several disorders.

### Innovations and breakthroughs

The results of skin prick tests (SPTs) for IAs and FAs in two sub-types of IBS patients were compared. They confirmed a functional interface between the immune and sensory motor systems in the gut and suggest that in D-IBS, the epithelial function (intestinal permeability) in particular can be altered. Few studies regarding the subject are available in literature. This study provides valuable information about clinical and epidemiological aspects of IBS in Brazil.

### Applications

The underlying cause of the pathophysiological changes encountered in IBS remains unclear. In clinical practice, the type and severity of symptoms determines the treatment of IBS. Our results add new information in answer to the question. Is IBS one or several disorders? Future clinical investigations will be useful for a better understanding of the results obtained here.

### Peer review

The article gives a clear delineation of the research background and provides important data about pathophysiological changes in IBS. The references are appropriate and updated.

## REFERENCES

- 1 Jones R, Lydeard S. Irritable bowel syndrome in the general population. *BMJ* 1992; **304**: 87-90
- 2 Locke GR 3rd. The epidemiology of functional gastrointestinal disorders in North America. *Gastroenterol Clin North Am* 1996; **25**: 1-19
- 3 Saito YA, Talley NJ. Irritable Bowel Syndrome. In: Talley NJ, Locke RG III, Saito YA, editors. *GI Epidemiology*, 1st ed. USA: Blackwell Publishing Press, 2007: 176-183
- 4 Soares RL, dos Santos JM, Rocha VR. Prevalence of irritable bowel syndrome in a Brazilian Amazon community. *Neurogastroenterol Motil* 2005; **17**: 883
- 5 Talley NJ, Zinsmeister AR, Melton LJ 3rd. Irritable bowel syndrome in a community: symptom subgroups, risk factors, and health care utilization. *Am J Epidemiol* 1995; **142**: 76-83
- 6 Toner BB, Akman D. Gender role and irritable bowel syndrome: literature review and hypothesis. *Am J Gastroenterol* 2000; **95**: 11-16
- 7 Uz E, Turkay C, Aytac S, Bavbek N. Risk factors for irritable bowel syndrome in Turkish population: role of food allergy. *J Clin Gastroenterol* 2007; **41**: 380-383
- 8 Cooke HJ. Neurotransmitters in neuronal reflexes regulating intestinal secretion. *Ann N Y Acad Sci* 2000; **915**: 77-80
- 9 Downing JE, Miyan JA. Neural immunoregulation: emerging roles for nerves in immune homeostasis and disease. *Immunol Today* 2000; **21**: 281-289
- 10 Mayer EA, Collins SM. Evolving pathophysiologic models of functional gastrointestinal disorders. *Gastroenterology* 2002; **122**: 2032-2048
- 11 Barbara G, De Giorgio R, Stanghellini V, Cremon C, Corinaldesi R. A role for inflammation in irritable bowel syndrome? *Gut* 2002; **51** Suppl 1: i41-i44
- 12 McKeown ES, Parry SD, Stansfield R, Barton JR, Welfare MR. Postinfectious irritable bowel syndrome may occur after non-gastrointestinal and intestinal infection. *Neurogastroenterol Motil* 2006; **18**: 839-843
- 13 Unno N, Fink MP. Intestinal epithelial hyperpermeability. Mechanisms and relevance to disease. *Gastroenterol Clin North Am* 1998; **27**: 289-307
- 14 Park MI, Camilleri M. Is there a role of food allergy in irritable bowel syndrome and functional dyspepsia? A systematic review. *Neurogastroenterol Motil* 2006; **18**: 595-607
- 15 Camilleri M, Gorman H. Intestinal permeability and irritable bowel syndrome. *Neurogastroenterol Motil* 2007; **19**: 545-552
- 16 Dunlop SP, Hebden J, Campbell E, Naesdal J, Olbe L, Perkins AC, Spiller RC. Abnormal intestinal permeability in subgroups of diarrhea-predominant irritable bowel syndromes. *Am J Gastroenterol* 2006; **101**: 1288-1294
- 17 Barbara G. Mucosal barrier defects in irritable bowel syndrome. Who left the door open? *Am J Gastroenterol* 2006; **101**: 1295-1298
- 18 Drossman DA, Corazziari E, Talley NJ. Rome III-A multinational consensus document on functional gastrointestinal disorders. *Gastroenterology* 2006; **130**: 1480-1491
- 19 Drossman DA, Corazziari E, Talley NJ, Thompson WG, Whitehead WE. Rome. The Functional Gastrointestinal Disorders. 2nd ed. McLean, VA: Degnon Associates, 2000
- 20 Niec AM, Frankum B, Talley NJ. Are adverse food reactions linked to irritable bowel syndrome? *Am J Gastroenterol* 1998; **93**: 2184-2190
- 21 Rhodes DY, Wallace M. Post-infectious irritable bowel syndrome. *Curr Gastroenterol Rep* 2006; **8**: 327-332
- 22 Locke GR 3rd, Zinsmeister AR, Talley NJ, Fett SL, Melton LJ. Risk factors for irritable bowel syndrome: role of analgesics and food sensitivities. *Am J Gastroenterol* 2000; **95**: 157-165
- 23 Petitpierre M, Gumowski P, Girard JP. Irritable bowel syndrome and hypersensitivity to food. *Ann Allergy* 1985; **54**: 538-540
- 24 Jones VA, McLaughlan P, Shorthouse M, Workman E, Hunter JO. Food intolerance: a major factor in the pathogenesis of irritable bowel syndrome. *Lancet* 1982; **2**: 1115-1117
- 25 Zwetchkenbaum J, Burakoff R. The irritable bowel syndrome and food hypersensitivity. *Ann Allergy* 1988; **61**: 47-49
- 26 Yazar A, Atis S, Konca K, Pata C, Akbay E, Calikoglu M, Hafta A. Respiratory symptoms and pulmonary functional changes in patients with irritable bowel syndrome. *Am J Gastroenterol* 2001; **96**: 1511-1516
- 27 Jun DW, Lee OY, Yoon HJ, Lee HL, Yoon BC, Choi HS, Lee MH, Lee DH, Kee CS. Bronchial hyperresponsiveness in irritable bowel syndrome. *Dig Dis Sci* 2005; **50**: 1688-1691
- 28 Roussos A, Koursarakos P, Patsopoulos D, Gerogianni I, Philippou N. Increased prevalence of irritable bowel syndrome in patients with bronchial asthma. *Respir Med* 2003; **97**: 75-79
- 29 Ozol D, Uz E, Bozalan R, Turkay C, Yildirim Z. Relationship between asthma and irritable bowel syndrome: role of food allergy. *J Asthma* 2006; **43**: 773-775
- 30 Soares RLS, Santos JM, Figueiredo HN, Rocha VRSR, Loyola RG. Respiratory allergy and the response to the inhalant allergens skin prick test in patients with Irritable Bowel Syndrome (IBS). 2006 Joint International Society Meeting in Neurogastroenterology and GI Motility Neurogastroenterology & Motility 2006; **18**: 663-798
- 31 Atkinson W, Sheldon TA, Shaath N, Whorwell PJ. Food elimination based on IgG antibodies in irritable bowel syndrome: a randomised controlled trial. *Gut* 2004; **53**: 1459-1464
- 32 Bernstein IL, Storms WW. Practice parameters for allergy diagnostic testing. Joint Task Force on Practice Parameters for the Diagnosis and Treatment of Asthma. The American Academy of Allergy, Asthma and Immunology and the American College of Allergy, Asthma and Immunology. *Ann Allergy Asthma Immunol* 1995; **75**: 543-625
- 33 Ahmed T, Fuchs GJ. Gastrointestinal allergy to food: a review. *J Diarrhoeal Dis Res* 1997; **15**: 211-223
- 34 Brandtzaeg PE. Current understanding of gastrointestinal immunoregulation and its relation to food allergy. *Ann N Y Acad Sci* 2002; **964**: 13-45
- 35 Crowe SE, Perdue MH. Gastrointestinal food hypersensitivity: basic mechanisms of pathophysiology. *Gastroenterology* 1992; **103**: 1075-1095

- 36 **Read NW**. Food and hypersensitivity in functional dyspepsia. *Gut* 2002; **51** Suppl 1: i50-i53
- 37 **Dainese R**, Galliani EA, De Lazzari F, Di Leo V, Naccarato R. Discrepancies between reported food intolerance and sensitization test findings in irritable bowel syndrome patients. *Am J Gastroenterol* 1999; **94**: 1892-1897
- 38 **Jun DW**, Lee OY, Yoon HJ, Lee SH, Lee HL, Choi HS, Yoon BC, Lee MH, Lee DH, Cho SH. Food intolerance and skin prick test in treated and untreated irritable bowel syndrome. *World J Gastroenterol* 2006; **12**: 2382-2387
- 39 **Simonato B**, De Lazzari F, Pasini G, Polato F, Giannattasio M, Gemignani C, Peruffo AD, Santucci B, Plebani M, Curioni A. IgE binding to soluble and insoluble wheat flour proteins in atopic and non-atopic patients suffering from gastrointestinal symptoms after wheat ingestion. *Clin Exp Allergy* 2001; **31**: 1771-1778
- 40 **Zar S**, Benson MJ, Kumar D. Food-specific serum IgG4 and IgE titers to common food antigens in irritable bowel syndrome. *Am J Gastroenterol* 2005; **100**: 1550-1557
- 41 **Zuo XL**, Li YQ, Li WJ, Guo YT, Lu XF, Li JM, Desmond PV. Alterations of food antigen-specific serum immunoglobulins G and E antibodies in patients with irritable bowel syndrome and functional dyspepsia. *Clin Exp Allergy* 2007; **37**: 823-830
- 42 **Soares RL**, Figueiredo HN, Maneschy CP, Rocha VR, Santos JM. Correlation between symptoms of the irritable bowel syndrome and the response to the food extract skin prick test. *Braz J Med Biol Res* 2004; **37**: 659-662

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# Managing injuries of hepatic duct confluence variants after major hepatobiliary surgery: An algorithmic approach

Georgios Fragulidis, Athanasios Marinis, Andreas Polydorou, Christos Konstantinidis, Georgios Anastasopoulos, John Contis, Dionysios Voros, Vassilios Smyrniotis

Georgios Fragulidis, Athanasios Marinis, Andreas Polydorou, Christos Konstantinidis, Georgios Anastasopoulos, John Contis, Dionysios Voros and Vassilios Smyrniotis, Second Department of Surgery, Areteion University Hospital, Athens Medical School, University of Athens, 76 Vassilisis Sofia's Ave., Athens 11528, Greece

**Author contributions:** Fragulidis G, Marinis A and Smyrniotis V wrote the paper; Konstantinidis C and Anastasopoulos G contributed equally to this work; Polydorou A, Fragulidis G, Contis J, Voros D and Smyrniotis V performed the operations, Polydorou A and Smyrniotis V reviewed the paper.

**Correspondence to:** Athanasios Marinis, MD, Second Department of Surgery, Areteion University Hospital, Athens Medical School, University of Athens, 40 Ptolemaidos str, 13674, Acharnes, Athens 11528, Greece. [sakisdoc@yahoo.com](mailto:sakisdoc@yahoo.com)  
Telephone: +30-697-2335748 Fax: +30-210-2441689

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## Abstract

**AIM:** To investigate injuries of anatomy variants of hepatic duct confluence during hepatobiliary surgery and their impact on morbidity and mortality of these procedures. An algorithmic approach for the management of these injuries is proposed.

**METHODS:** During a 6-year period 234 patients who had undergone major hepatobiliary surgery were retrospectively reviewed in order to study postoperative bile leakage. Diagnostic workup included endoscopic and magnetic retrograde cholangiopancreatography (E/MRCP), scintigraphy and fistulography.

**RESULTS:** Thirty (12.8%) patients who developed postoperative bile leaks were identified. Endoscopic stenting and percutaneous drainage were successful in 23 patients with bile leaks from the liver cut surface. In the rest seven patients with injuries of hepatic duct confluence, biliary variations were recognized and a stepwise therapeutic approach was considered. Conservative management was successful only in 2 patients. Volume of the liver remnant and functional liver reserve as well as local sepsis were used as criteria for either resection of the corresponding liver segment or construction of a biliary-enteric anastomosis. Two deaths occurred in this group of patients with hepatic duct confluence variants (mortality rate 28.5%).

**CONCLUSION:** Management of major biliary fistulae

that are disconnected from the mainstream of the biliary tree and related to injury of variants of the hepatic duct confluence is extremely challenging. These patients have a grave prognosis and an early surgical procedure has to be considered.

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**Key words:** Biliary aberrations; Bile duct injury; Postoperative bile leakage; Hepatic duct confluence; Hepatectomy

**Peer reviewers:** Mitsuo Shimada, Professor, Department of Digestive and Pediatric Surgery, Tokushima University, Kuramoto 3-18-15, Tokushima 770-8503, Japan; Tadatoshi Takayama, Professor, Department of Digestive Surgery, Nihon University School of Medicine, 30-1 Oyaguchikami-machi, Itabashi-ku, Tokyo 173-8610, Japan; Kazuhiro Hanazaki, MD, Professor and Chairman, Department of Surgery, Kochi Medical School, Kochi University, Kohasu, Okochi, Nankoku, Kochi 783-8505, Japan

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## INTRODUCTION

Dissection of the biliary tract constitutes the most crucial step in liver resections and every effort should aim to secure integrity and normal bile flow of the liver remnant. However, up to 40% of the patients are lacking the conventional biliary branching pattern and are more often exposed to sectorial bile duct transection during liver resection<sup>[1]</sup>.

The most frequent biliary variants are found in the right liver. The right posterior sectorial duct (RPSD) and the right anterior sectorial duct (RASD) may be joining the left hepatic duct (LHD), the common hepatic duct (CHD) or even the cystic duct<sup>[2-10]</sup>. Although biliary complications in liver resections occur approximately in 10%, they are responsible for one third of the postoperative mortality<sup>[11,12]</sup>. Fortunately, the majority is amenable to non surgical treatment, but when reoperation is necessitated mortality rate may reach up to 70%<sup>[13,14]</sup>. This high



mortality rate is due to the fact that accurate diagnosis is delayed and surgical treatment is influenced by the ongoing intra-abdominal septic process. Our study aims to present our experience in managing injuries of hepatic duct confluence variants proposing an algorithmic approach.

## MATERIALS AND METHODS

During a 6-year period (Jan, 2001-Dec, 2006) 234 patients who underwent major hepatobiliary surgery were retrospectively reviewed, in order to evaluate postoperative biliary complications due to anatomic variations of hepatic duct confluence. The hepatectomy procedures performed included segmentectomy, lobectomy and extended lobectomy (trisegmentectomy). Our technique of hepatectomy has been described previously<sup>[15]</sup>, using the Pringle maneuver, vascular outflow obstruction and sharp parenchymal dissection. For major hepatectomies (lobectomy-extended lobectomy) hilar dissection was performed to divide the correspondent vessels<sup>[16]</sup>. The diagnosis of a biliary complication was based upon the presence of a persistent bile leakage *via* the drain, the surgical incision, or the development of an intra-abdominal biloma confirmed by imaging studies. In our department, we do not perform routine preoperative imaging studies of the biliary tree anatomy. Postoperative diagnostic imaging studies included endoscopic retrograde and magnetic resonance cholangiopancreatography (ERCP-MRCP) and occasionally scintigraphy (Tc99m-HIDA) or fistulography.

Thirty patients (12.8%) who developed postoperative bile leaks were identified and conservative treatment (endoscopic stenting and percutaneous drainage) resolved the problem in 23 patients. In the remaining 7 patients, a biliary variant injury of hepatic duct confluence was diagnosed. One of these seven patients underwent an initial laparoscopic cholecystectomy and was also included in this group.

Clinical characteristics, type of operation performed, type of biliary variant, treatment and outcome of these patients with variant injuries are presented in Table 1. Classification of bile duct injuries was based on that used for hepatic duct confluence by Ayuso *et al*<sup>[17]</sup>. Treatment involved a conservative (percutaneous drainage of bilomas and perihepatic abscesses, antibiotics, *etc*) and a surgical (liver resection, biliary-enteric anastomosis) approach. Timing of surgical intervention was based upon criteria of non-responsiveness to external drainage and/or persistence of intra-abdominal sepsis. The type of procedure was based upon the estimated volume of the liver remnant and the functional reserve of the liver (Child-Pugh classification) and intraoperative factors (local inflammatory process, aberrant bile duct features, *etc*).

## RESULTS

Injuries of variants of the hepatic duct confluence were retrospectively found in 7 patients (three males and four females) with an age range from 36 to 76 years old. Six patients had undergone initially major liver resections (2 for hydatid cyst and 4 for carcinoma) and in one patient a laparoscopic cholecystectomy was performed. All

patients developed a major biliary fistula postoperatively that was disconnected from the mainstream of the biliary tree. According to the classification and imaging workup previously mentioned, 1 type C, 3 type D, 2 type E and 1 type F injuries were recognized. This simply means that the most common injury involved the right posterior sectorial duct (RPSD) in four patients (cases 1, 2, 6 and 7), while injuries of the right anterior sectorial duct (RASD) were recognized in two patients (cases 3 and 4; Figure 1). In one patient (case 5), during an extended right lobectomy, the sectorial bile duct draining liver segment I, joining separately the common bile duct (type E injury), was transected with a consequent biliary fistula (Figure 2).

All patients were initially treated conservatively, but only two (cases 3 and 4) had an uneventful outcome with resolution of the bile leak 2 mo and 4 mo, respectively, after the initial operation. One patient (case 2) refused surgical therapy and died from septic shock. The remaining four patients were approached surgically; two underwent a delayed biliary-enteric (B-E) anastomosis (cases 1 and 7) while the other two had a resection of the compromised liver segments (cases 5 and 6).

In case 1, 14 mo after the initial procedure (left lateral sectionectomy) exploratory laparotomy revealed a complex situation; liver segment's IV duct was found transected and draining in the abdomen and the RPSD was ligated near its junction with the LHD. Resection of segment IV and B-E anastomosis of the dilated RPSD with a Roux-en-Y intestinal loop were carried out and the patient had an uneventful postoperative course. In contrast, the delayed B-E anastomosis in case 7, performed 10 mo after the initial procedure (laparoscopic cholecystectomy), failed and resection of liver segments VI & VII was carried out. Unfortunately, the postoperative course was complicated by overwhelming sepsis due to accidental injury of the duodenum and the patient died.

The last 2 patients (cases 5 and 6) underwent liver resections of the compromised liver segments, 6 mo and 8 mo, respectively, after initial operation with an uneventful postoperative course. An algorithmic approach for these injuries is depicted in Figure 3.

Overall, hospital stay ranged from 20-150 d (mean, 63 d) and the mortality rate in this group of patients with injuries of variants of the hepatic duct confluence was 28.5% (2/7).

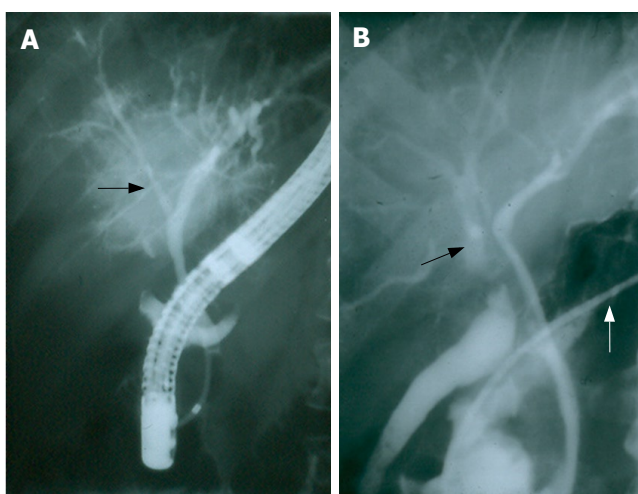
## DISCUSSION

Prevailing strategy in hepatobiliary surgery should always be the ascertainment of the integrity of normal bile flow from the liver remnant; otherwise, life-threatening complications may occur. Biliary complications in hepatobiliary surgery vary between 3%-15% and share a significant portion of the postoperative morbidity and mortality<sup>[11-14,18]</sup>. The cause of biliary leakage is usually due to unsutured collateral biliary branches of the cut surface and a non-surgical treatment settles the problem in the majority of the patients<sup>[19]</sup>. However, aberrant biliary anatomy is frequently encountered during hepatobiliary surgery and represents a totally different problem from that aforementioned.

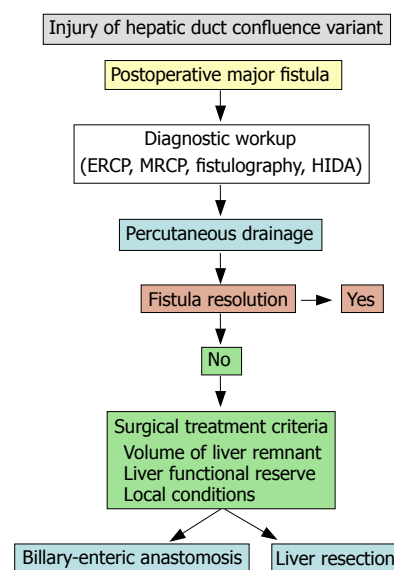
Table 1 Characteristics of patients with injury of aberrant bile ducts

No	Sex	Age	Initial operation	Biliary variant injury <sup>1</sup>	Treatment	Outcome
1	F	51	Left lateral sectionectomy (Hydatid cyst)	RPSD (type D)	Resection of segment IV and biliary-enteric (B-E) anastomosis	Uneventful
2	F	76	Left Hepatectomy (CHD carcinoma)	RPSD (type C)	Denied liver resection	Died
3	M	65	Left hepatectomy (Liver carcinoma)	RASD (type D)	Conservative (external drainage)	Resolved after 2 mo
4	M	71	Left hepatectomy (Liver carcinoma)	RASD (type D)	Conservative (external drainage)	Resolved after 4 mo
5	M	51	Right extended lobectomy (cholangiocarcinoma)	Segment's I duct (type E)	Resection of segment I	Uneventful
6	F	49	Resection of segment V (Hydatid cyst)	RPSD (type E)	Liver resection (VI, VII)	Uneventful
7	F	36	Laparoscopic cholecystectomy	RPSD (type F)	1. B-E anastomosis failed 2. Liver resection (VI, VII)	Died

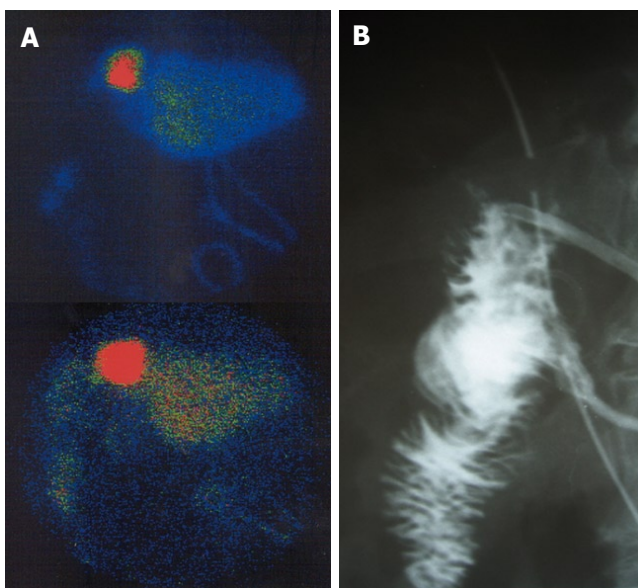
<sup>1</sup>Types of biliary variants<sup>[17]</sup>: Type A: Right hepatic duct (RHD) joins the left hepatic duct (LHD); Type B: Triple confluence of right posterior sectorial duct (RPSD) and right anterior sectorial duct (RASD) and LHD; Type C: RASD or the RPSD joins the common bile duct (CBD); Type D: RASD or RPSD joins separately the LHD; Type E: Absence of confluence; sectorial ducts join separately at the common hepatic duct (CHD); Type F: RPSD joins the cystic duct.



**Figure 1** A: Retrograde cholangiogram demonstrating the left hepatic duct and its confluence with the right anterior sectorial duct (arrow, case 7); B: Fistulogram via the drain tube resulted in a retrograde cholangiogram through the transected right posterior sectorial duct (black arrow). Presence of a nasobiliary tube (white arrow) draining the left hepatic duct (case 7).



**Figure 3** An algorithmic approach for the management of patients with injuries of hepatic duct confluence variants.



**Figure 2** A: HIDA scan demonstrating bile leakage after right extended hepatectomy (case 5); B: Stentogram with intact hepatico-jejunal anastomosis in the same patient (case 5).

Preoperative assessment of biliary anatomy and possible variations in order to prevent intraoperative injury is currently performed by means of three dimensional helical computed tomography cholangiography<sup>[20-23]</sup> and various magnetic resonance imaging techniques<sup>[24-27]</sup>. If a biliary variant is assumed to be injured intraoperatively, the surgeon should perform an intraoperative cholangiography through the injured bile duct in order to estimate the type and extent of injury. In case of a disconnected from the biliary tree sectorial bile duct the decision of the surgical approach should be based on criteria of the volume of the liver remnant and liver functional reserve in case of additional hepatectomy; otherwise a Roux-en-Y biliary-enteric anastomosis must be carried out in order to drain bile to the gut.

Several efforts have been made in order to reduce or manage postoperative bile leaks. A randomized trial using an intraoperative leakage test, injecting isotonic sodium chloride solution *via* the cystic duct had no advantage on reducing postoperative bile leak<sup>[28]</sup>, while the application of a fibrin glue sealant on the cut surface of the liver seems not to be justified<sup>[29]</sup>. A case report describing infusion

of pure ethanol in an injured sectorial duct resulting in atrophy of the corresponding liver segment and cessation of postoperative bile leak seems to be a minimally invasive approach to this devastating complication needing, however, further evaluation<sup>[30]</sup>. Postoperative persistent major biliary fistula that has been attributed from diagnostic workup to an injury of a variant of the hepatic duct confluence is initially treated conservatively by means of percutaneous drainage and management of ensuing sepsis usually in a critical care environment. This approach was effective in two of our seven patients and the bile leak resolved 2 mo and 4 mo after initial operation.

Unfortunately, conservative treatment may not settle the problem and ongoing intra-abdominal sepsis fuelled by the major bile leak is associated with high morbidity and mortality. Despite adequate biliary drainage and critical care support, surgical treatment should be instituted in order to manage this problem. A planned approach based upon patient's general status, volume of future liver remnant and liver functional reserve, type and extent of injury and the volume of the corresponding liver segment draining through the injured sectorial bile duct are crucial for decision making. Surgical treatment includes either a resection of the corresponding liver segment or a biliary-enteric anastomosis with a Roux-en-Y limb. In our series, two patients underwent a biliary-enteric anastomosis, which was not successful in one of them and resection of the corresponding liver segment was additionally carried out. Resection of the liver segment, drained by the injured sectorial bile duct, was carried out successfully in two more patients. Therefore, in the case of injury of a variant of the hepatic duct confluence an algorithmic approach is proposed and depicted schematically in Figure 3.

In conclusion, variants of hepatic duct confluence are frequently involved and injured during major hepatic surgery and seriously complicate postoperatively all patients due to delay of diagnosis and ongoing intra-abdominal sepsis. Preoperative imaging of the biliary branching pattern (ERCP, MRCP) remains the only way to recognize and address properly the problem posed by the variant of biliary anatomy. MRCP offers a reliable and non-invasive visualization of the biliary tree in a manner for the surgical approach to be planned and adapted to prevent an injury of a variant of the hepatic duct confluence. However, if this occurs, conservative treatment is the initial approach in managing these patients. Failure to resolve the problem conservatively leads to a planned re-operation which includes either a biliary-enteric anastomosis or a resection of the corresponding to the injury liver segment.

## COMMENTS

### Background

Hepatobiliary surgery is frequently encountered with variations in biliary anatomy. Injuries of variants of the hepatic duct confluence add significant morbidity and mortality after liver resections, due to the development of major bile leakage and ensuing septic sequelae.

### Research frontiers

Preoperative evaluation of anatomical variants seems to be critical in avoiding inadvertent injury. Conservative treatment by means of minimally invasive techniques of injuries of variants of hilar biliary anatomy requires further

evaluation. Surgical treatment is still debatable whether to resect the compromised liver segment or to restore bile drainage to the gut by performing a biliary-enteric anastomosis.

### Innovations and breakthroughs

The proposal of an algorithmic approach to manage postoperatively the injuries of the variants of hepatic duct confluence.

### Applications

The implications of this study are for further evaluation of newer conservative therapeutic techniques and/or decision-making regarding surgical management.

### Peer review

The author's proposed an algorithm to manage the injury of the biliary tract after hepatobiliary surgery. Their recommendation is of clinical value for the patients who may have biliary anomaly. However, it is most important for avoiding bile duct injuries during hepatic resection to evaluate accurate anatomy of bile duct preoperatively.

## REFERENCES

- 1 Choi JW, Kim TK, Kim KW, Kim AY, Kim PN, Ha HK, Lee MG. Anatomic variation in intrahepatic bile ducts: an analysis of intraoperative cholangiograms in 300 consecutive donors for living donor liver transplantation. *Korean J Radiol* 2003; **4**: 85-90
- 2 Couinaud C, Le foie. Etudes anatomiques et chirurgicales. Paris: Masson, 1957: 187-208
- 3 Poston GJ, Blumgart LH. Surgical anatomy of the liver and bile ducts. In: Poston GJ, Blumgart LH eds. Surgical management of hepatobiliary and pancreatic disorders. London: Martin Dunitz, 2003: 1-18
- 4 Icoz G, Kilic M, Zeytinlu M, Celebi A, Ersoz G, Killi R, Memis A, Karasu Z, Yuzer Y, Tokat Y. Biliary reconstructions and complications encountered in 50 consecutive right-lobe living donor liver transplantations. *Liver Transpl* 2003; **9**: 575-580
- 5 Cheng YF, Huang TL, Chen CL, Chen YS, Lee TY. Variations of the intrahepatic bile ducts: application in living related liver transplantation and splitting liver transplantation. *Clin Transplant* 1997; **11**: 337-340
- 6 Ohkubo M, Nagino M, Kamiya J, Yuasa N, Oda K, Arai T, Nishio H, Nimura Y. Surgical anatomy of the bile ducts at the hepatic hilum as applied to living donor liver transplantation. *Ann Surg* 2004; **239**: 82-86
- 7 Heloury Y, Leborgne J, Rogez JM, Robert R, Lehur PA, Pannier M, Barbin JY. Radiological anatomy of the bile ducts based on intraoperative investigation in 250 cases. *Anat Clin* 1985; **7**: 93-102
- 8 Yoshida J, Chijiwa K, Yamaguchi K, Yokohata K, Tanaka M. Practical classification of the branching types of the biliary tree: an analysis of 1,094 consecutive direct cholangiograms. *J Am Coll Surg* 1996; **182**: 37-40
- 9 Varotti G, Gondolessi GE, Goldman J, Wayne M, Florman SS, Schwartz ME, Miller CM, Sukru E. Anatomic variations in right liver living donors. *J Am Coll Surg* 2004; **198**: 577-582
- 10 Hribernik M, Gadzijev EM, Mlakar B, Ravnik D. Variations of intrahepatic and proximal extrahepatic bile ducts. *Hepatogastroenterology* 2003; **50**: 342-348
- 11 Shimada M, Matsumata T, Akazawa K, Kamakura T, Itasaka H, Sugimachi K, Nose Y. Estimation of risk of major complications after hepatic resection. *Am J Surg* 1994; **167**: 399-403
- 12 Bismuth H, Chiche L, Castaing D. Surgical treatment of hepatocellular carcinomas in noncirrhotic liver: experience with 68 liver resections. *World J Surg* 1995; **19**: 35-41
- 13 Pace RF, Blenkarn JL, Edwards WJ, Orloff M, Blumgart LH, Benjamin IS. Intra-abdominal sepsis after hepatic resection. *Ann Surg* 1989; **209**: 302-306
- 14 Lam CM, Lo CM, Liu CL, Fan ST. Biliary complications during liver resection. *World J Surg* 2001; **25**: 1273-1276
- 15 Smyrniotis V, Arkadopoulos N, Kostopanagiotou G, Farantos

- C, Vassiliou J, Contis J, Karvouni E. Sharp liver transection versus clamp crushing technique in liver resections: a prospective study. *Surgery* 2005; **137**: 306-311
- 16 **Smyrniotis V**, Arkadopoulos N, Theodoraki K, Voros D, Vassiliou I, Polydorou A, Dafnios N, Gamaletsos E, Daniilidou K, Kannas D. Association between biliary complications and technique of hilar division (extrahepatic vs. intrahepatic) in major liver resections. *World J Surg Oncol* 2006; **4**: 59
  - 17 **Ayuso JR**, Ayuso C, Bombuy E, De Juan C, Llovet JM, De Caralt TM, Sanchez M, Pages M, Bruix J, Garcia-Valdecasas JC. Preoperative evaluation of biliary anatomy in adult live liver donors with volumetric mangafodipir trisodium enhanced magnetic resonance cholangiography. *Liver Transpl* 2004; **10**: 1391-1397
  - 18 **Nery JR**, Fragulidis GP, Scagnelli T, Weppler D, Webb MG, Khan MF, Tzakis AG. Donor biliary variations: an overlooked problem? *Clin Transplant* 1997; **11**: 582-587
  - 19 **Reed DN Jr**, Vitale GC, Wrightson WR, Edwards M, McMasters K. Decreasing mortality of bile leaks after elective hepatic surgery. *Am J Surg* 2003; **185**: 316-318
  - 20 **Cheng YF**, Lee TY, Chen CL, Huang TL, Chen YS, Lui CC. Three-dimensional helical computed tomographic cholangiography: application to living related hepatic transplantation. *Clin Transplant* 1997; **11**: 209-213
  - 21 **Kitami M**, Takase K, Murakami G, Ko S, Tsuboi M, Saito H, Higano S, Nakajima Y, Takahashi S. Types and frequencies of biliary tract variations associated with a major portal venous anomaly: analysis with multi-detector row CT cholangiography. *Radiology* 2006; **238**: 156-166
  - 22 **Izuishi K**, Toyama Y, Nakano S, Goda F, Usuki H, Masaki T, Maeta H. Preoperative assessment of the aberrant bile duct using multislice computed tomography cholangiography. *Am J Surg* 2005; **189**: 53-55
  - 23 **Wang ZJ**, Yeh BM, Roberts JP, Breiman RS, Qayyum A, Coakley FV. Living donor candidates for right hepatic lobe transplantation: evaluation at CT cholangiography--initial experience. *Radiology* 2005; **235**: 899-904
  - 24 **Khalid TR**, Casillas VJ, Montalvo BM, Centeno R, Levi JU. Using MR cholangiopancreatography to evaluate iatrogenic bile duct injury. *AJR Am J Roentgenol* 2001; **177**: 1347-1352
  - 25 **Lee VS**, Krinsky GA, Nazzaro CA, Chang JS, Babb JS, Lin JC, Morgan GR, Teperman LW. Defining intrahepatic biliary anatomy in living liver transplant donor candidates at mangafodipir trisodium-enhanced MR cholangiography versus conventional T2-weighted MR cholangiography. *Radiology* 2004; **233**: 659-666
  - 26 **Fulcher AS**, Szucs RA, Bassignani MJ, Marcos A. Right lobe living donor liver transplantation: preoperative evaluation of the donor with MR imaging. *AJR Am J Roentgenol* 2001; **176**: 1483-1491
  - 27 **Goldman J**, Florman S, Varotti G, Gondolesi GE, Gerning A, Fishbein T, Kim L, Schwartz ME. Noninvasive preoperative evaluation of biliary anatomy in right-lobe living donors with mangafodipir trisodium-enhanced MR cholangiography. *Transplant Proc* 2003; **35**: 1421-1422
  - 28 **Ijichi M**, Takayama T, Toyoda H, Sano K, Kubota K, Makuuchi M. Randomized trial of the usefulness of a bile leakage test during hepatic resection. *Arch Surg* 2000; **135**: 1395-1400
  - 29 **Figueras J**, Llado L, Miro M, Ramos E, Torras J, Fabregat J, Serrano T. Application of fibrin glue sealant after hepatectomy does not seem justified: results of a randomized study in 300 patients. *Ann Surg* 2007; **245**: 536-542
  - 30 **Shimizu T**, Yoshida H, Mamada Y, Taniai N, Matsumoto S, Mizuguchi Y, Yokomuro S, Arima Y, Akimaru K, Tajiri T. Postoperative bile leakage managed successfully by intrahepatic biliary ablation with ethanol. *World J Gastroenterol* 2006; **12**: 3450-3452

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RAPID COMMUNICATION

# Inhibitory effect of dimeric $\beta$ peptide on the recurrence and metastasis of hepatocellular carcinoma *in vitro* and in mice

Song-Mei Wang, Jun Zhu, Luan-Feng Pan, Yin-Kun Liu

Song-Mei Wang, Luan-Feng Pan, Laboratory of Molecular Biology, Shanghai Medical College, Fudan University, Shanghai 200032, China

Jun Zhu, Department of Pharmacy, Shanghai Chest Hospital, Shanghai 200031, China

Yin-Kun Liu, Liver Cancer Institute, Fudan University, Shanghai 200032, China

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Correspondence to: Professor Jun Zhu, Department of Pharmacy, Shanghai Chest Hospital, Shanghai 200031, China. [jone\\_zhu@126.net](mailto:jone_zhu@126.net)

Telephone: +86-21-62821990-20111 Fax: +86-21-62801109

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thepectomy *in vivo*. Thus,  $\beta 2$  should be further studied as a new anti-tumor drug.

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**Key words:**  $\beta$  peptide; Hepatocellular carcinoma; Anti-adhesion; Invasion; Metastasis; Recurrence

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Wang SM, Zhu J, Pan LF, Liu YK. Inhibitory effect of dimeric  $\beta$  peptide on the recurrence and metastasis of hepatocellular carcinoma *in vitro* and in mice. *World J Gastroenterol* 2008; 14(19): 3054-3058 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3054.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3054>

## Abstract

**AIM:** To block the adhesion of tumor cells to the extracellular matrix, and prevent tumor metastasis and recurrence, the dimer of the  $\beta$  peptide (DLYYLMDSLMSMKGDLYYLMDSLMSMK,  $\beta 2$ ) was designed and synthesized and its anti-adhesion and anti-invasion effects on hepatocellular carcinoma cells were assessed. Additionally, its influence on the metastasis and recurrence of mouse hepatocellular carcinoma was measured.

**METHODS:** The anti-adhesion effect of  $\beta 2$  on the highly metastatic hepatocellular carcinoma cell line HCCLM6 cells and fibronectin (FN) was assayed by the MTT assay. The inhibition of invasion of HCCLM6 cells by  $\beta 2$  was observed using a Transwell (modified Boyden chamber) and matrigel. Using the hepatocellular carcinoma metastasis model and LCI-D20 nude mice, the influence of  $\beta 2$  on the metastasis and recurrence of hepatocellular carcinoma after early resection was investigated.

**RESULTS:** HCCLM6 cells co-incubated with 100  $\mu\text{mol/L}$ , 50  $\mu\text{mol/L}$ , 20  $\mu\text{mol/L}$  or 10  $\mu\text{mol/L}$   $\beta 2$  for 3 h showed an obvious decrease in adhesion to FN. The adhesion inhibition ratios were 11.8%, 21.7%, 29.6% and 48.7%, respectively. Additionally, HCCLM6 cells cultured with 100  $\mu\text{mol/L}$   $\beta 2$  had a dramatic decrease in cell invasion.  $\beta 2$  was also observed to inhibit the incisional edge recurrence and the distant metastasis of nude mice hepatocellular carcinoma after early resection ( $P < 0.05$ ).

**CONCLUSION:** The  $\beta 2$  peptide can specifically block the adhesion and invasion of HCCLM6 cells, and can inhibit HCC recurrence and metastasis of LCI-D20 model pos-

## INTRODUCTION

Despite significant advances in the treatment of human hepatocellular carcinoma (HCC) and the prevention of postoperative metastasis, the 5-year postoperative recurrence rate of HCC is still very high<sup>[1,2]</sup>. Many efforts have been made to develop a more efficient treatment to inhibit and prevent tumor metastasis, as the recurrence and metastasis of HCC is still a large problem in clinical practice. It is well known that the metastatic process is very complex, including tumor cells dissociating from the primary locus, invading the surrounding tissue, entering and extravasating from the circulation, and growing in distant organs<sup>[3,4]</sup>. During this process, cell adhesion is one of the most important events<sup>[5]</sup>. Many studies have been focused on the synthesized anti-adhesion peptides<sup>[6-8]</sup>. However, the application of these short peptides is limited due to their short half-life and high dosage required. To prolong the peptide's half-life, the polymer and a derivative of synthesized peptides were designed<sup>[9-11]</sup>. The anti-tumor metastasis effect of the repeat sequence of synthesized peptides was stronger than that of non-repeat peptides<sup>[12,13]</sup>.

Integrins are a family of adhesion molecules located on cells and in the extracellular matrix. The expression level of integrins is related closely to a cell's migration ability<sup>[14,15]</sup>. The anti-adhesion peptide  $\beta$  (DLYYLMDSLMSMK,  $\beta 1$ ) was designed by Liu *et al*<sup>[16]</sup>, according to the conserved sequence of the integrin  $\alpha$  and  $\beta$  unit. This peptide can block the

interaction between tumor cells and the extracellular matrix and can also inhibit intrahepatic and pulmonary metastases after carcinosectomy in a nude mouse model with human HCC of high metastatic potential (LCI-D20)<sup>[17-21]</sup>. On the basis of these studies, here we have designed and synthesized the dimeric peptide  $\beta$  ( $\beta$ 2). The effects of  $\beta$ 2 on the adhesion of human liver cancer cell line HCCLM6 cells to fibronectin (FN), the invasion of HCCLM6 cells to reconstituted basement membrane, as well as liver cancer recurrence and metastasis after hepatectomy in a nude mouse model were investigated.

## MATERIALS AND METHODS

### Cell culture

The highly metastatic hepatocellular carcinoma cell line HCCLM6, initially established and preserved by the Liver Cancer Institute, Fudan University, was cultured in Dulbecco's modified eagle's medium (DMEM, Gibco, UK), supplemented with 10% fetal bovine serum, 100 U/mL penicillin and grown at 37°C under an atmosphere of 5% CO<sub>2</sub>. The medium was replenished every three days to maintain cell growth.

### Coating the 96 well high bind microplate with FN

Ten  $\mu$ g/mL FN (Sigma, USA) solution (containing 10  $\mu$ g/mL FN, 20 mmol/L Tris-Cl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L MgCl<sub>2</sub>, 1 mmol/L CaCl<sub>2</sub>, 1 mmol/L MnCl<sub>2</sub>) was added to a 96-well high bind microplate (Corning, USA) (100  $\mu$ L per well), and allowed to incubate at 4°C overnight. The plate was then incubated with blocking buffer (10 mmol/L Hepes, pH 7.4, 140 mmol/L NaCl, 5.4 mmol/L KCl, 5.56 mmol/L glucose, 3% BSA, 1 mmol/L MgCl<sub>2</sub>, 2 mmol/L CaCl<sub>2</sub>, 1 mmol/L MnCl<sub>2</sub>) at 37°C for 2 h and air dried for further use.

### Cell adhesion assay

$\beta$ 2 peptide was designed in our laboratory using the sequence DLYYLMDSLYSMKGGDLYYLMDSLYS MK. The peptide was synthesized by Shanghai Sangon Bioengineering Company. 100  $\mu$ L of a HCCLM6 suspension ( $2 \times 10^5$ /mL) was plated in each well of an FN coated 96-well high bind microplate. 100  $\mu$ L DMEM medium containing  $\beta$ 2 at a concentration of 200  $\mu$ mol/L, 100  $\mu$ mol/L, 40  $\mu$ mol/L or 20  $\mu$ mol/L was added to the cells concomitantly. The same volume of cell culture medium in place of  $\beta$ 2 was added to the control group. 200  $\mu$ L of cell culture medium only was added in the plate for the blank group. The assay was conducted in quintuplicate for each sample. After incubation for 3 h at 37°C, under an atmosphere of 5% CO<sub>2</sub>, the unattached cells were gently washed away with HANKS buffer. The attached cell number in each well was measured by MTT. The inhibition rate of  $\beta$ 2 on cell adhesion to FN was calculated with the following equation: Cell adhesion inhibitory rate = (average OD of control well-average OD of  $\beta$ 2-treated well)/(average OD of control well-average OD of blank well)  $\times$  100%

### MTT assay

The number of attached cells in each well was examined by

the MTT assay, as previously described<sup>[22]</sup>, and quantified by a micro-titer plate reader (Amersham, USA). Briefly, after incubation for 3 h at 37°C in 5% CO<sub>2</sub>, the unattached cells were removed by gentle washing with HANKS buffer. 100  $\mu$ L DMEM and 20  $\mu$ L MTT (5 mg/mL) (Sigma, USA) were added to each well. After incubation at 37°C for 4 h, the medium was discarded. 200  $\mu$ L of 0.04 mol/L hydrochloric acid in isopropanol was added to each well. The amount of MTT formazan product, which reflects the number of cells adhering to FN, was determined by measuring absorbance with a microplate reader at a test wavelength of 570 nm and a reference wavelength of 630 nm.

### Invasion assay

Invasion assays were performed as described previously<sup>[23]</sup>. Briefly, the upper portion of Transwell chambers (Corning, USA) were coated with 75  $\mu$ L of Matrigel (BD, USA) diluted 1:10 in serum-free DMEM and incubated at 37°C for 2 h. The supernatants of HCCLM6 cells containing DMEM with 10% FCS were harvested after the cells had grown to confluence, and after adding FN at a final concentration of 5  $\mu$ g/mL, resulting in conditioned medium. The trypsinized cells were harvested and diluted to a  $2 \times 10^6$ /mL cell suspension with serum-free DMEM. 100  $\mu$ L of the cell suspension and 100  $\mu$ L of 200  $\mu$ mol/L  $\beta$ 2 peptides in serum-free DMEM or serum-free DMEM only as a control were added in the upper chambers. Concurrently, 600  $\mu$ L of conditioned medium was added to the bottom chamber of the Transwell plate. After incubation at 37°C for 48 h under a 5% CO<sub>2</sub> atmosphere, the non-invading cells and the gel were gently removed from the upper chamber with cotton-tipped swabs. Cells were rinsed with PBS, and the cells on the filters were fixed with Formaldehyde and stained in Giemsa staining solution for 30 min. The number of invaded cells on the filters was counted in 5 randomly selected high-powered ( $\times$  200) fields per filter under a microscope (Leica, Switzerland). Invasion inhibitory rate was expressed as the following equation: Invasion inhibitory rate = [1 - (invaded cell number in  $\beta$ 2 chamber/invaded cell number in control chamber)]  $\times$  100%.

### Animal model and treatment

Twelve 5-wk-old male nude mice (BALB/cA) weighing 17-20 g were obtained from the Shanghai Institute of Materia Medica, Chinese Academy of Sciences. The nude mouse model of human hepatocellular carcinoma with high metastatic potential (LCI-D20), which was established in Zhongshan Hospital Liver Cancer Institute, Fudan University, was used in this study. A tumor block of LCI-D20 nude mice human liver cancer metastasis model was implanted into the left lobe of the nude mouse liver as described previously<sup>[24]</sup>. Briefly, a left upper abdominal transverse incision was made under anesthesia; the left lobe of the liver was exposed and a part of the liver surface was mechanically injured with scissors. Next, a tumor block of 0.2 cm  $\times$  0.2 cm  $\times$  0.2 cm was fixed within the liver tissue. After the operation, mice were kept in laminar-flow cabinets under specific-pathogen-free conditions and given free access to mouse chow. Liver cancer early resection

**Table 1** The inhibitory effects of  $\beta 2$  on the invasion ability of HCCLM6 cells ( $n = 5$ )

Group	Mean of invaded cell (SD)	Invasion inhibitory rate (%)
Control group	19.30 (9.3)	-
$\beta 2$ group	12.20 (6.2)	36.80%

**Table 2** Liver cancer recurrences in incisional margins in nude mouse models after early resection

Group	Number of mice tested	Mean weight of recurrent lesion (g) (SD)	Number of mice with recurrent lesion
Control group	6	2.31 (0.64)	6
$\beta 2$ group	6	0.50 (0.41) <sup>a</sup>	4

<sup>a</sup> $P < 0.05$  vs control group.

was performed 0.2 cm from the edge of the cancer at day 10 after implantation, prior to metastasis. At day 1 after resection, the animals were subcutaneously administrated 100  $\mu$ L of 1 mg/mL of  $\beta 2$  or NS as a control every other day for 10 doses. Mice were harvested at day 55 postimplantation, and lungs were fixed in 10% formalin, embedded in paraffin, cut into 5  $\mu$ m slides and metastatic nodes were observed and counted under a microscope. If recurrence of the incisional margin of cancer was found, the lesion would be resected and weighed.

All of the animal experiments were conducted in strict accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals.

### Statistical analysis

All data were entered into Excel spreadsheets (Excel, Microsoft, Seattle, USA). We used the SAS program (SAS Institute Inc., Cary, NC, USA) for statistical analysis. Comparisons for dimensional outcomes employed the Student's *t*-test, or the Mann Whitney *U* test when the data were not normally distributed. Values of  $P < 0.05$  in a two-tailed fashion were considered to be statistically significant.

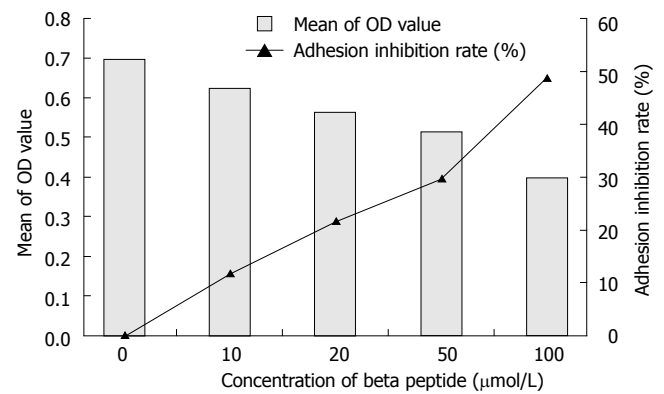
## RESULTS

### The inhibitory effect of $\beta 2$ on the adhesion of HCCLM6 cells to FN

The inhibitory effect of  $\beta 2$  on the adhesion of HCCLM6 cells to FN is shown in Figure 1. HCCLM6 cells co-incubated with 100  $\mu$ mol/L, 50  $\mu$ mol/L, 20  $\mu$ mol/L and 10  $\mu$ mol/L  $\beta 2$  for 3 h led to an obvious decrease in cellular adhesion. The adhesion inhibition ratios were 11.8%, 21.7%, 29.6% and 48.7%, respectively. This observation indicates that  $\beta 2$  is able to inhibit the adhesion of HCCLM6 cells to FN, and thus  $\beta 2$  might obstruct the invasion of HCC cells to paratumor liver parenchyma.

### The inhibitory effect of $\beta 2$ on the invasion ability of HCCLM6 cells

After incubation with 100  $\mu$ mol/L  $\beta 2$ , the number of invaded HCCLM6 cells was decreased. The inhibitory rate

**Figure 1** The inhibitory effect of  $\beta 2$  on the adhesion of HCCLM6 cells to fibronectin ( $n = 5$ ).

was 36.8% (Table 1). Thus,  $\beta 2$  might block HCC cells from invading the surrounding tissue and entering and extravasating from the circulation *in vivo*.

### The influence of $\beta 2$ on the intrahepatic recurrence of the LCI-D20 model after early resection

On the 10th d post-tumor-implantation, LCI-D20 tumors were resected, and  $\beta 2$  or the same volume of saline was subcutaneously injected. On day 55, mice were sacrificed to check for intrahepatic recurrence. The recurrent tumor was located around the incisional margins. Compared with the control group, the weight of the intrahepatic recurrent tumor of the  $\beta 2$  group was markedly decreased and statistically significant. There were 4 (4/6) mice with intrahepatic recurrent tumor in the  $\beta 2$  group, while there were 6 (6/6) mice with an intrahepatic recurrent tumor in the control group (Figure 1 and Table 2). These results indicate that  $\beta 2$  have inhibitory effects on tumor recurrence in the incisional margin.

### The inhibitory effects of $\beta 2$ on metastasis of liver cancer in nude mouse models after early resection

On the 55th day after tumor implantation, the number of metastatic nodes was calculated under a microscope. The result showed that there were fewer metastatic nodes in the  $\beta 2$  treatment group compared to the control group, and there was a statistical difference between the  $\beta 2$  group and the control group. Furthermore, all of the 6 mice in the control group (6/6) had metastatic nodes, but only 4 (4/6) mice had metastatic nodes in the  $\beta 2$  group. These results indicate that  $\beta 2$  have a significant preventive and therapeutic effect on the metastasis of liver cancer (Table 3).

## DISCUSSION

The adhesion molecules on the surface of both tumor cells and endothelial cells are associated with tumor metastasis and recurrence. Blocking the interaction between tumor cell adhesion molecules and their ligands is a major target in the prevention of cancer metastasis<sup>[25,26]</sup>. Many studies have focused on the synthesized anti-adhesion peptides<sup>[27,28]</sup>. One such peptide is RGD<sup>[29,30]</sup>, derived from the common conserved sequence of the main matrix

**Table 3** The lung metastasis in liver cancer nude mouse models after early resection

	Number ( <i>n</i> )	The total number of metastatic nodes in lung	The number of mice with lung metastatic nodes
Control group	6	30	4
β2 group	6	11 <sup>a</sup>	2

<sup>a</sup>*P* < 0.05 *vs* control group.

proteins such as fibronectin, collagen and fibrinogen. A second peptide is YIGSR<sup>[31]</sup>, which originated from the basement membrane protein laminin. The third peptide is EILDV<sup>[32]</sup>, which stemmed from the core sequence of fibronectin. The application of these short peptides was limited due to their short half-life, the ease with which they are degraded and the requirement for a high dosage. To prolong the peptides' half-life, the polymer and derivative of synthesized peptides were designed. The anti-tumor metastatic effect of repeat sequence of synthesized peptides was stronger compared to non-repeat peptides. The more times the sequence is repeated, the stronger the anti-metastasis effect is.

FN is an important cell adhesion molecule in the extracellular matrix. It mediates cell adhesion and migration, and plays a significant role in tumor invasion and metastasis. Assaying FN adhesion to tumor cells is a method commonly used for studying tumor cell metastasis. In this study, the extracellular matrix was simulated by coating cell culture plates with FN, after which the inhibitory effects of β2 peptide on FN adhesion to liver cancer cells were investigated. The results demonstrated that after co-culturing the peptides with HCCLM6 cells for 3 h, a distinct and specific inhibitory effect of β2 peptide on FN adhesion to tumor cells was observed.

Tumor cells must penetrate the basement membrane for at least three times during metastasis; i.e. dislodging from the original site, entering blood circulation, and migrating from blood flow into remote sites. Matrigel, used as a basement membrane matrix, is produced from mouse Engelbreth-Holm-Swarm sarcoma rich in extracellular matrix protein. The artificial basement membrane is plated on a Millipore filter in Transwell culture chambers, and forms a membrane structure similar to natural basement membrane. Invasive, metastatic tumor cells can penetrate the membrane under the induction of chemotactics, simulating tumor cells' invasion of the basement membrane *in vivo*. The results indicated that β2 exerted significant inhibitory effects on the invasion of HCCLM6 cells.

Metastasis and recurrence of liver cancer is a major determinant for the prognosis and long-term survival of liver cancer patients. Polypeptide therapy is a newly developed treatment for tumors<sup>[31]</sup>, but its clinical application is restricted by the degradation of these peptides. β peptides can inhibit the metastasis and recurrence of human liver cancer in nude mouse models after early excision, and can also block the recurrence of cancer at the incisional margins.

The β peptide blocked tumor cell adhesion to FN through two possible mechanisms. First, the β peptide took up the integrin binding site competently through

binding to the RGD sequence of the matrix protein. Next, the β peptide also interacted with integrin because the β peptide was designed according to the conserved sequence of the integrin α and β unit.

Taken together, these cell and animal studies demonstrated that the β2 peptide can prevent and treat liver cancer adhesion and metastasis and recurrence. Therefore, the β peptide is worthy of further investigation, as it is a potential drug for blocking tumor metastasis and recurrence.

## COMMENTS

### Background

Despite significant advances in the treatment of human hepatocellular carcinoma (HCC), metastasis and recurrence remain the main obstacles for HCC patients gaining a better outcome and long-term survival. It is well known that during the metastatic process, cell adhesion is one of the most important events. The adhesion molecules on the surface of both tumor cells and endothelial cells are associated with tumor metastasis and recurrence. So, blocking the interaction between tumor cell adhesion molecules and their ligands has become a major target in prevention cancer metastasis.

### Research frontiers

To prevent tumor metastasis and recurrence through inhibiting the adhesion of tumor cells, many studies have focused on the synthesized anti-adhesion peptides such as RGD, YIGSR and EILDV. These peptides are derived from the common conserved sequence of the main matrix proteins such as fibronectin, collagen, fibrinogen and laminin. Liu *et al* designed a new anti-adhesion peptide β (DLYYLMDSLYSYMK, β1) according to the conserved sequence of the α and β unit of integrins. These peptides can inhibit the adhesion of tumor cells and cancer metastasis and recurrence. But their application is limited due to the short half-life and high dosage required.

### Innovations and breakthroughs

On the basis of Liu's study, to prolong the peptide's half-life, the dimer of β peptide (DLYYLMDSLYSYMKGGDLYYLMDSLYSYMK, β2) was designed and synthesized and the anti-adhesion and anti-invasion effect of it on hepatocellular carcinoma cells, as well as its influence to the metastasis and recurrence of mouse hepatocellular carcinoma were measured. The result showed that β2 can inhibit the adhesion of HCCLM6 cells to FN in dose-effect manner. And the number of invaded HCCLM6 cells was decreased when incubated together with 100 μmol/L β2. Compared with the control group, the weight of the intrahepatic recurrent tumor and the number of metastatic nodes in lung of the β2 group were markedly decreased.

### Applications

β2 might obstruct the invasion of HCC cells to paratumor liver parenchyma and block HCC cells from invading the surrounding tissue and entering and extravasating from the circulation *in vivo*. In addition, β2 have inhibitory effects on tumor recurrence in the incisional margin and a significant preventive and therapeutic effect on the metastasis of liver cancer. Taken together, these cell and animal studies demonstrated that the β2 peptide can prevent and treat liver cancer adhesion, metastasis and recurrence.

### Peer review

On the basis of previous work, the β2 peptide (DLYYLMDSLYSYMKGGDLYYLMDSLYSYMK, β2) was designed and synthesized. After co-culturing with HCCLM6 cells for 3 h, a distinct and specific inhibitory effect of β2 peptide on FN adhesion to tumor cells was observed. And also β2 showed significant inhibitory effects on the invasion of HCCLM6 cells. Furthermore, β2 peptides can inhibit the metastasis and recurrence of human liver cancer in nude mouse models after early excision, and can also block the recurrence of cancer at the incisional margins. These results indicate that β2 have a significant preventive and therapeutic effect on the metastasis of liver cancer.

## REFERENCES

- 1 Fang WQ, Li SP, Zhang CQ, Xu L, Shi M, Chen MS, Li JQ.



- [Prophylaxis and clinical treatment for surgical margin recurrence of small primary hepatocellular carcinoma] *Ai Zheng* 2005; **24**: 834-836
- 2 **Lee WC**, Jeng LB, Chen MF. Estimation of prognosis after hepatectomy for hepatocellular carcinoma. *Br J Surg* 2002; **89**: 311-316
  - 3 **Wyke JA**. Overview--burgeoning promise in metastasis research. *Eur J Cancer* 2000; **36**: 1589-1594
  - 4 **Liotta LA**, Steeg PS, Stetler-Stevenson WG. Cancer metastasis and angiogenesis: an imbalance of positive and negative regulation. *Cell* 1991; **64**: 327-336
  - 5 **Chu XY**, Chen LB. Cellular adhesive molecular and the invasion and metastasis of neoplasm. *Yixue Yanjiusheng Xuebao* 2000; **13**: 42-45
  - 6 **Li FH**. The inhibitory effect of bioactive peptides on neoplasm metastasis. *Kouqiang Hemian Waike Zazhi* 1999; **9**: 231-234
  - 7 **Liu LY**, Chen ZY, Zhao TH. Investigations of a peptide with RGD and YIGSR fragments: synthesis and its anti-tumor invasion activities. *Zhongguo Xinyao Zazhi* 2005; **14**: 729-731
  - 8 **Saiki I**, Yoneda J, Kobayashi H, Igarashi Y, Komazawa H, Ishizaki Y, Kato I, Azuma I. Antimetastatic effect by anti-adhesion therapy with cell-adhesive peptide of fibronectin in combination with anticancer drugs. *Jpn J Cancer Res* 1993; **84**: 326-335
  - 9 **Zhang HQ**, Shinohara H, Gu N, Sasaki H, Sisido M. Cell Adhesion Inhibition by RGD Peptides Linked with a Photoisomerizable Nonnatural Amino Acid. *J Southeast Univ* 2001; **17**: 22-26
  - 10 **Liu LY**, Chen ZY, Zhao TH. Synthesis of RGD identical-fork-peptide derivative with inhibitive effect on adhesiveness of advanced metastatic tumor cells. *Zhongguo Xinyao Zazhi* 2006; **15**: 1661-1663
  - 11 **Zhao M**, Wang C, Jiang X, Pen S. Synthesis of RGD containing peptides and their bioactivities. *Prep Biochem Biotechnol* 2002; **32**: 363-380
  - 12 **Cao K**, Zhao TH, Chen ZY, Gao W, Yang HS, Shi B. The invasive capacity of human lung great cellular xancerous PG cells on reformed basement membrane and inhibition of synthetic peptides. *Zhongliu Fangzhi Yanjiu* 2002; **29**: 20-22
  - 13 **Okroj M**, Dobrzaska-Paprocka Z, Rolka K, Bigda J. In vitro and in vivo analyses of the biological activity of RGD peptides towards Ab Bomirski melanoma. *Cell Mol Biol Lett* 2003; **8**: 873-884
  - 14 **Heyder C**, Gloria-Maercker E, Hatzmann W, Niggemann B, Zanker KS, Dittmar T. Role of the beta1-integrin subunit in the adhesion, extravasation and migration of T24 human bladder carcinoma cells. *Clin Exp Metastasis* 2005; **22**: 99-106
  - 15 **Liu YK**, Wu WZ, Wu X, Jiang Y, Zhou XD. Liver cancer metastasis and signal transduction. In: Tang ZY. Metastasis and recurrence of hepatocellular carcinoma--basic and clinical studies. Shanghai: Shanghai scientific and technological education public house, 2003: 93-104
  - 16 **Liu YK**, Nemoto A, Feng Y, Uemura T. The binding ability to matrix proteins and the inhibitory effects on cell adhesion of synthetic peptides derived from a conserved sequence of integrins. *J Biochem* 1997; **121**: 67-74
  - 17 **Uemura T**, Nemoto A, Liu YK. Synthetic peptide derived from a conserved sequence of integrin  $\beta$  subunit. *Res. Adv in Biosci & Bioeng* 2000; **23**: 65-83
  - 18 **Sun JJ**, Zhou XD, He JY, Liu YK, Tang ZY. Inhibition of the nude mice liver cancer metastasis and recurrence by beta peptide. *Zhonghua Shiyian Waike Zazhi* 2000; **17**: 418-420
  - 19 **Sun JJ**, Zhou XD, Liu YK, Tang ZY, Shi JY, Bao WH, Xue Q. An experimental study on preventing and treating metastasis and recurrence of human liver cancer with anti-adhesive drugs in nude mice. *Zhonghua Xiaohua Zazhi* 2000; **20**: 53-54
  - 20 **Sun JJ**, Zhou XD, Liu YK, Tang ZY. An experimental study of the effect of  $\beta$  peptide on liver cancer recurrence and metastasis. *Zhonghua Putong Waike Zazhi* 2000; **15**: 27-31
  - 21 **Sun JJ**, Zhou XD, Liu YK, Tang ZY, Sun RX, Zhao Y, Uemura T. Inhibitory effects of synthetic beta peptide on invasion and metastasis of liver cancer. *J Cancer Res Clin Oncol* 2000; **126**: 595-600
  - 22 **Sun DX**, Zhang L, Chen XQ. In vitro test of cell proliferation and cytotoxic. In: Zhu LP, Chen XQ. General methods of immunologic experiment. Beijing: People's Military Medical Press, 2000: 193
  - 23 **Knutson JR**, Iida J, Fields GB, McCarthy JB. CD44/chondroitin sulfate proteoglycan and alpha 2 beta 1 integrin mediate human melanoma cell migration on type IV collagen and invasion of basement membranes. *Mol Biol Cell* 1996; **7**: 383-396
  - 24 **Sun FX**, Tang ZY, Lui KD, Ye SL, Xue Q, Gao DM, Ma ZC. Establishment of a metastatic model of human hepatocellular carcinoma in nude mice via orthotopic implantation of histologically intact tissues. *Int J Cancer* 1996; **66**: 239-243
  - 25 **Syrgios KN**, Karayiannakis AJ. Adhesion molecules as targets for the treatment of neoplastic diseases. *Curr Pharm Des* 2006; **12**: 2849-2861
  - 26 **Jiang CG**, Xu HM. Research and application of anti-adhesion therapy in cancer metastasis. *Guowai Yixue (Zhongliuxue Fence)* 2005; **32**: 31-34
  - 27 **Okroj M**, Dobrzaska-Paprocka Z, Rolka K, Bigda J. In vitro and in vivo analyses of the biological activity of RGD peptides towards Ab Bomirski melanoma. *Cell Mol Biol Lett* 2003; **8**: 873-884
  - 28 **Wang YH**, Liu YK, Li WC, Ye SL, Tang ZY. Inhibitory effect of anti-adhesion peptides on invasion/metastasis ability of hepatocellular carcinoma cells. *Zhonghua Shiyian Waike Zazhi* 2004; **21**: 1168-1169
  - 29 **Liu J**, Guo SX, Tang JG. Research progress of RGD-peptide for cancer therapy. *Guowai Yixue (Zhongliuxue Fence)* 2003; **30**: 193-197
  - 30 **Maeda M**, Izuno Y, Kawasaki K, Kaneda Y, Mu Y, Tsutsumi Y, Nakagawa S, Mayumi T. Amino acids and peptides. XXXI. Preparation of analogs of the laminin-related peptide YIGSR and their inhibitory effect on experimental metastasis. *Chem Pharm Bull (Tokyo)* 1998; **46**: 347-350
  - 31 **Kaneda Y**, Yamamoto Y, Okada N, Tsutsumi Y, Nakagawa S, Kakiuchi M, Maeda M, Kawasaki K, Mayumi T. Antimetastatic effect of synthetic Glu-Ile-Leu-Asp-Val peptide derivatives containing D-amino acids. *Anticancer Drugs* 1997; **8**: 702-707
  - 32 **Feng ZH**, Huang B, Zhang GM, Li D, Wang HT. Inducement of antitumor-immunity by DC activated by Hsp70-H22 tumor antigen peptide. *Chin J Cancer Res* 15: 79-85

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## A case-control study of the relationship between hepatitis B virus DNA level and risk of hepatocellular carcinoma in Qidong, China

Tao-Tao Liu, Ying Fang, Hui Xiong, Tao-Yang Chen, Zheng-Pin Ni, Jian-Feng Luo, Nai-Qing Zhao, Xi-Zhong Shen

Tao-Tao Liu, Xi-Zhong Shen, Department of Gastroenterology, Zhongshan Hospital, Fudan University, Shanghai 200032, China  
Ying Fang, Endoscopy Center, Huadong Hospital, Fudan University, Shanghai 200040, China

Hui Xiong, Chinese National Human Genome Center at Shanghai, Shanghai 201203, China

Tao-Yang Chen, Zheng-Pin Ni, Qidong Liver Cancer Institute, Qidong 226200, Jiangsu Province, China

Jian-Feng Luo, Nai-Qing Zhao, Department of Health Statistics and Community Medicine, Fudan University, Shanghai 200032, China

**Author contributions:** Liu TT, Xiong H and Shen XZ designed the research; Liu TT and Fang Y performed the research; Chen TY and Ni ZP contributed samples and materials; Zhao NQ and Luo JF analyzed the data; and Liu TT and Shen XZ wrote the paper.

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**Correspondents:** Professor Xi-Zhong Shen, MD, Department of Gastroenterology, Zhongshan Hospital, Fudan University, 180# Fenglin Road, Shanghai 200032, China. [shenxz99@yahoo.com](mailto:shenxz99@yahoo.com)  
Telephone: +86-21-64041990 Fax: +86-21-64038038

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**CONCLUSION:** The findings of this study provide strong longitudinal evidence of an increased risk of HCC associated with persistent elevation of serum HBV DNA level in the  $10^4$ - $10^7$  range.

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**Key words:** Hepatitis B surface antigen; Viral replication; Asymptomatic carriers; Viral load

**Peer reviewer:** Xin-Xin Zhang, Department of Infectious Disease, Ruijin Hospital, 197, Ruijin Er Road, Shanghai 200025, China

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### Abstract

**AIM:** To investigate the role of hepatitis B virus (HBV) replication in the development of hepatocellular carcinoma (HCC), a nested case-control study was performed to study the relationship between HBV DNA level and risk of HCC.

**METHODS:** One hundred and seventy cases of HCC and 276 control subjects free of HCC and cirrhosis were selected for this study. Serum HBV DNA level was measured using fluorescein quantitative polymerase chain reaction at study entry and the last visit.

**RESULTS:** In a binary unconditional logistic regression analysis adjusted for age, cigarette smoking, alcohol consumption and family history of chronic liver diseases, the adjusted odds ratios (95% confidence intervals) of HCC in patients with increasing HBV DNA level were 2.834 (1.237-6.492), 48.403 (14.392-162.789), 42.252 (14.784-120.750), and 14.819 (6.992-31.411) for HBV DNA levels  $\geq 10^4$  to  $< 10^5$ ;  $\geq 10^5$  to  $< 10^6$ ;  $\geq 10^6$  to  $< 10^7$ ;  $\geq 10^7$  copies/mL, respectively. Forty-six HCC cases were selected to compare the serums viral loads of HBV DNA at study entry with those at the last visit. The HBV DNA levels measured at the two time points did not differ significantly.

### INTRODUCTION

Chronic hepatitis B virus (HBV) infection is still a worldwide health problem<sup>[1]</sup>, with approximate 400 million patients persistently infected<sup>[2,3]</sup>. Although most of the HBV carriers are asymptomatic, about one-third (25%-40%) die from cirrhotic complications or hepatocellular carcinoma (HCC)<sup>[4]</sup>. The relative risk of HBV carriers for the development of HCC is up to 200:1, which is one of the highest relative risks known for a human malignancy<sup>[5]</sup>. Due to the high incidence of recurrence and secondary primary tumor, the survival rate of HCC after any treatment is still low<sup>[6]</sup>. Therefore, looking for the predictive factors for HCC in patients with chronic Hepatitis B will have a profound impact on the prevention and treatment of chronic HBV infection.

The precise mechanisms by which chronic Hepatitis B leads to HCC are not clearly understood. Viral, host (sex, age and genetic susceptibility) and environmental factors may play interactive roles in hepatocarcinogenesis<sup>[7-12]</sup>. Recent studies have indicated that serum level of HBV DNA may be a risk factor for HCC<sup>[13-16]</sup>. Tang *et al*<sup>[17]</sup> have previously reported that adult HBV carriers who maintain high-titer serum HBV DNA are at higher risk for development of HCC. In Taiwan, a 12-year follow-up study of 4841 men who were Hepatitis B surface antigen

(HBsAg) positive has demonstrated that the risk of HCC is 2.7-10.7-fold higher in patients with baseline HBV DNA levels of  $4.0 \log_{10}$  copies/mL to  $\geq 6.0 \log_{10}$  copies/mL<sup>[18]</sup>. However, it is important to study different endemic regions to verify the relationship between active HBV replication and development of HCC, because there is a geographic distribution of HBV genotypes. In particular, the data are largely lacking in mainland of China, where chronic HBV infection is highly endemic and accounts for half of the chronic hepatitis B in the world.

The township of Qidong, at the mouth of the Yangtze River, is one of the highest endemic regions for chronic HBV infection and HCC in China<sup>[19]</sup>. Between October 1996 and February 2006, we followed a total of 2387 HBsAg-positive adult residents in Qidong city. The aims of this study were to determine whether chronic HBV carriers who maintain high serum HBV DNA level are at higher risk for development of HCC in Chinese patients with chronic Hepatitis B.

## MATERIALS AND METHODS

### Study population

In October 1996, about 18000 male residents between the ages of 20 and 65 yr living in 17 townships in Qidong county, China were invited to participate in a prospective study. All of those invited were tested for serum HBsAg, alanine aminotransferase (ALT) and  $\alpha$ -fetoprotein (AFP). A total of 2387 participants who were seropositive for HBsAg and confirmed to be free of HCC by AFP level and abdominal ultrasonography were followed up with abdominal ultrasonography and serological tests including ALT, AFP, HBV serological markers (HBsAg) and anti-Hepatitis C virus (HCV) antibody until February 2006. Each study participant provided informed written consent and a structured questionnaire on sociodemographic characteristics, habits of alcohol and tobacco consumption and family histories. A serum specimen was collected from each participant at every interview. All of the serum samples were stored at  $-30^{\circ}\text{C}$  before analysis. This study was approved by the research ethics committee at Zhongshan Hospital, Fudan University, Shanghai, China.

### Laboratory testing

Serum HBsAg and anti-HCV antibody were tested by commercially available enzyme immunoassay kits (Shanghai Kehua Bio-engineering Co. Ltd., China). Serum ALT level was determined by ultraviolet-lactate dehydrogenase (UV-LDH) method and serum AFP level was determined by ELISA (Shanghai Kehua Bio-engineering Co. Ltd.).

### Fluorescein quantitative polymerase chain reaction (FQ-PCR)

The serum HBV DNA levels were determined using the FQ-PCR detection system (Taqmen; Roche USA), according to the manufacturer's instructions. HBV DNA was extracted using the commercial Kit (Shanghai Shenyong Biotech Company) from 50  $\mu\text{L}$  serum. The PCR reaction was carried out as follows:  $37^{\circ}\text{C}$  for 120 s,  $94^{\circ}\text{C}$  for 180 s, followed by 40 cycles of  $94^{\circ}\text{C}$  for 10 s,  $55^{\circ}\text{C}$  for 30 s and

$72^{\circ}\text{C}$  for 40 s. The lower limit of detection of this assay was 500 copies/mL with a linear range of up to  $10^8$  copies/mL.

### Statistical analysis

The  $\chi^2$  test was used to compare baseline characteristics between patients and controls subjects. Wilcoxon signed ranks test has been used to compare the constancy of the viral replication at two time points. For statistical comparisons, a value of 500 copies/mL was assigned, the detection limit of the assay, to samples that had undetectable levels of HBV DNA. Samples of the two groups were divided into six subgroups, according to the level of serum HBV DNA expressed as the logarithmic equivalent (LGE) per milliliter, subgroup 1 ( $< 500$  copies/mL, undetectable), subgroup 2 ( $2.69 \log_{10}$  to  $3.99 \log_{10}$  copies/mL), subgroup 3 ( $4.0 \log_{10}$  to  $4.99 \log_{10}$  copies/mL), subgroup 4 ( $5.0 \log_{10}$  to  $5.99 \log_{10}$  copies/mL), subgroup 5 ( $6.0 \log_{10}$  to  $6.99 \log_{10}$  copies/mL), and subgroup 6 ( $\geq 7.0 \log_{10}$  copies/mL). Binary unconditional logistic regression analysis was used to evaluate relative risks. Potential confounders including age, cigarette smoking, alcohol consumption and family history of chronic liver diseases were adjusted. SPSS 13.0 for Windows was used for all statistical analyses.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Demographic characteristic of HCC and control patients

No participants had any clinical evidence of HCC at study entry. By December 31, 2004, 243 participants died of HCC. The data were obtained from medical records and searches of computer files of death certification and cancer registry systems. To ensure complete ascertainment, we also contacted relatives by mail to identify cases. HCC was diagnosed on the basis of either surgical biopsy or an elevated serum AFP level ( $\geq 400$  ng/mL), combined with at least one positive image on sonography, computed tomography and/or magnetic resonance imaging. Seventy-three patients diagnosed with HCC within the first two years of our study probably had subclinical HCC at study entry, and were therefore, excluded from the analysis, which left 170 cases of HCC. The paired serum samples were available only in 46 cases, both at study entry and at the time of HCC, for determining the change in serum HBV DNA level over time. Two hundred and seventy-six subjects with chronic Hepatitis B infection and normal ALT level at each follow-up, and free of evidence of cirrhosis or HCC, were selected as controls.

At baseline, there were no significant differences in age, cigarette smoking and alcohol consumption between HCC and control patients, while the family histories of HBV-associated chronic liver diseases were significantly different between the two groups. 85/170 (50%) of cases had a family history, while only 92/276 (33.3%) of control subjects had (Table 1).

### Baseline serum HBV DNA level in HCC patients and controls

186/276 (67.4%) samples of control subjects had undetectable levels of serum HBV DNA. Compared with those with undetectable levels of serum HBV DNA, the adjusted odds ratios of HCC for subjects with increasing

Table 1 Demographic data in HCC and control patients *n* (%)

	HCC patients ( <i>n</i> = 170)	Control patients ( <i>n</i> = 276)	$\chi^2$	<i>P</i> value
Age at recruitment (yr)			8.347	<i>P</i> > 0.05
20-29	6 (3.5)	15 (5.4)		
30-39	52 (30.6)	88 (31.9)		
40-49	72 (42.4)	87 (31.5)		
50-59	35 (20.6)	68 (24.6)		
≥ 60	5 (2.9)	18 (6.5)		
Smoking			0.131	<i>P</i> > 0.05
Yes	88 (51.8)	138 (50.0)		
No	82 (48.2)	138 (50.0)		
Alcohol use			0.989	<i>P</i> > 0.05
Yes	103 (60.6)	154 (55.8)		
No	67 (39.4)	122 (44.2)		
Family history			12.209	<i>P</i> < 0.01
Yes	85 (50.0)	92 (33.3)		
No	85 (50.0)	184 (66.7)		

Table 2 Association between HBV DNA level at study entry and subsequent risk of HCC *n* (%)

HBV DNA level (log <sub>10</sub> copies/ mL)	HCC patients ( <i>n</i> = 170)	Control patients ( <i>n</i> = 276)	Adjusted odds ratio (95% CI)
1 undetectable	44 (25.9)	186 (67.4)	1.000 (reference)
2 (2.69-3.99)	5 (2.9)	46 (16.7)	0.465 (0.172-1.259)
3 (4.00-4.99)	12 (7.1)	19 (6.9)	2.834 (1.237-6.492)
4 (5.00-5.99)	30 (17.6)	4 (1.4)	48.403 (14.392-162.789)
5 (6.00-6.99)	38 (22.4)	5 (1.8)	42.252 (14.784-120.750)
6 (≥ 7.00)	41 (24.1)	16 (5.8)	14.819 (6.992-31.411)

Adjusted for age at enrollment (continuous variable), cigarette smoking, alcohol consumption and family history of chronic liver diseases.

HBV DNA level were 0.465 (95% CI 0.172-1.259), 2.834 (1.237-6.492), 48.403 (14.392-162.789), 42.252 (14.784-120.750), and 14.819 (6.992-31.411). The analysis has been adjusted for age, cigarette smoking, alcohol consumption and family history of chronic liver diseases. The risk of HCC was increased with increasing HBV viral load in 4.0 log<sub>10</sub> to 7.0 log<sub>10</sub> copies/mL (Table 2).

### Change of serum HBV DNA level over time

All the control subjects in our study were followed up for 10 years with persistently normal ALT level, and had no history of interferon- $\alpha$  or nucleoside analogue therapy. HBV DNA levels were compared between entry and last visit in asymptomatic HBV carriers (controls). There was a statistically significant difference in serum HBV DNA level at the two time points (Table 3). For the 46 patients for whom the serum samples were collected both at study entry and at or after the time of HCC diagnosis, the time interval between collection of the two samples ranged from 24 to 94 mo. The log HBV DNA levels measured at the two time points did not have a statistically significant difference.

## DISCUSSION

Family history of liver carcinoma is one of the main risk

Table 3 Comparison of serum levels of HBV DNA at study entry and at last visit in asymptomatic HBV carriers (controls) *n* (%)

HBV DNA level (log <sub>10</sub> copies/mL)	At study entry ( <i>n</i> = 276)	At last visit ( <i>n</i> = 276)	<i>Z</i>	<i>P</i> value
1 undetectable	186 (67.4)	221 (80.1)	-4.904	<i>P</i> < 0.01
2 (2.69-3.99)	46 (16.7)	30 (10.9)		
3 (4.00-4.99)	19 (6.9)	9 (3.3)		
4 (5.00-5.99)	4 (1.4)	6 (2.2)		
5 (6.00-6.99)	5 (1.8)	3 (1.1)		
6 (≥ 7.00)	16 (5.8)	7 (2.5)		

factors for HCC, especially in the Chinese population<sup>[20-22]</sup>. In our study, 85/170 (50%) of cases had a family history of HBV-associated chronic liver diseases. However, only 92/276 (33.3%) of control subjects did.

In China, HBV DNA levels > 5.0 log<sub>10</sub> copies/mL have been considered clinically significant, and are suggested by clinical practice guidelines for making a decision on antiviral therapy in chronic carriers of Hepatitis B. The guidelines are supported by the findings of a meta-analysis of 26 trials of statistical significance and consistent correlations between viral load and histological grading, and biochemical and serological response<sup>[23]</sup>. However, the relationship between different levels, especially lower levels, of HBV DNA and risk of HCC remains uncertain.

During the past 10 years, longitudinal studies have been used to evaluate HBV DNA level as risk factors of HCC in HBV carriers. A significant biological gradient of HCC risk by serum HBV DNA level from 4.0 log<sub>10</sub> to 7.0 log<sub>10</sub> copies/mL was observed in our cohort. Similar to previous results<sup>[24]</sup>, the HCC risk started to increase significantly at a serum HBV DNA level of 4.0 log<sub>10</sub> copies/mL, which is much lower than the level of 5.0 log<sub>10</sub> copies/mL suggested by clinical practice guidelines for making decisions on antiviral therapy in carriers of chronic Hepatitis B. Viral loads < 4.0 log<sub>10</sub> copies/mL have been thought to be characteristic of an inactive carrier state and a much lower risk of HCC. Moreover, it is important to know that compared to viral loads between 5.0 log<sub>10</sub> and 7.0 log<sub>10</sub> copies/mL, patients with HBV DNA levels > 7.0 log<sub>10</sub> copies/mL were at lower risk of developing HCC. Chronic HBV carriers with mid-high viral loads (4.0 log<sub>10</sub> to 7.0 log<sub>10</sub> copies/mL) tended to be in the phase of immune clearance, while the majority of those with viral load levels of 7.0 log<sub>10</sub> copies/mL were immunotolerant and at lower risk of HCC. Our findings are partly consistent with studies in different areas. In Japan, Ohata *et al.*<sup>[25]</sup> have investigated the risk factors for HCC in 73 patients with HBV-associated liver disease. A high viral load of HBV DNA, together with age and histological fibrosis, were found to be linked to the occurrence of HCC. Yang *et al.*<sup>[26]</sup> have reported that HCC risk increased with the increasing HBV viral load above 7.5 log<sub>10</sub> copies/mL. They have also found that HCC risk is associated with Hepatitis B e antigen (HBeAg) positivity among HBsAg-positive men in Taiwan. Based on these results, the serum level of HBV DNA may be used as a prominent risk predictor for HCC, independent of age, histological



fibrosis and HBeAg status.

To the best of our knowledge, there have been few studies on longitudinal stability of HBV DNA level in HBV carriers over time in mainland China. In the 276 control subjects in our study, all the HBV DNA levels in samples at the last visit were compared with those collected at study entry. 186/276 (67.4%) samples of control subjects had undetectable levels of serum HBV DNA at study entry, while 221/276 (80.1%) samples had undetectable levels of serum HBV DNA at the last visit. During a follow-up period of 10 years, the HBV DNA levels of those asymptomatic carriers tended to decrease. Forty-six case patients were selected whose serum samples were collected both at study entry and after the time of HCC diagnosis. Compared with serum HBV DNA levels at study entry, viral load after HCC onset remained at high levels. This implied that for chronic HBV carriers free of antiviral therapy, HCC was preceded by persistently high replication activity of HBV and viral levels did not decline with progression of HCC.

It is generally agreed that antiviral treatment is suitable in patients with active HBV replication ( $\geq 5.0 \log_{10}$  copies/mL) and elevated ALT level (at least twice the upper limit of the normal range)<sup>[27]</sup> or advanced fibrosis present upon liver biopsy. In clinical trials, among patients with chronic Hepatitis B and advanced stage fibrosis, longer term lamivudine therapy reduces the risk of HCC<sup>[28,29]</sup>. Although individuals with low viral load ( $< 4.0 \log_{10}$  copies/mL) are at decreased risk for HCC, continued monitoring is essential because of the fluctuating nature of chronic HBV infection. Treatment choices for patients with serum HBV DNA levels  $< 5.0 \log_{10}$  copies/mL are still controversial. In our study, HBV carriers with HBV DNA levels  $> 4.0 \log_{10}$  copies/mL have 2.834 times excess risk of HCC compared with HBV carriers with lower HBV DNA levels. Therefore, among patients with HBV DNA levels  $> 4.0 \log_{10}$  copies/mL, liver tests should be carefully monitored at 3-4-mo intervals, irrespective of age and ALT levels. Antiviral treatment should be advised when hepatitis flares and/or advanced fibrosis is present upon liver biopsy.

In conclusion, serum HBV DNA levels were found to be associated with increased risk of HCC. For chronic HBV carriers without antiviral therapy, HBV DNA levels changed little with the progression of HCC. Based on these findings, it is conceivable that patients with a high viral load have a high potential for hepatocarcinogenesis, and should be subjected to closer clinical monitoring<sup>[30]</sup> and even antiviral treatment.

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## COMMENTS

### Background

Chronic Hepatitis B virus (HBV) infection is still a worldwide health problem. The precise mechanisms by which chronic Hepatitis B leads to hepatocellular carcinoma (HCC) are not clearly understood. Viral, host (sex, age and genetic susceptibility) and environmental factors may play interactive roles in

hepatocarcinogenesis. Recent studies have indicated that serum level of HBV DNA may be a risk factor for HCC. However, the data are largely lacking in mainland China, where chronic HBV infection is highly endemic and accounts for half of the chronic Hepatitis B in the world. It is important to study different endemic regions to verify the relationship between active HBV replication and development of HCC, because there is geographic distribution of HBV genotypes.

### Research frontiers

Study on the prognostic factors in patients with chronic Hepatitis B, the relationship between Hepatitis B virus genotype and HBV DNA level, and HCC and treatment of chronic Hepatitis B patients who are resistant to antiviral therapy.

### Innovations and breakthroughs

The township of Qidong, at the mouth of the Yangtze River, is one of the highest endemic regions for chronic HBV infection and HCC in China. However, this is believed to be the first study of the relationship between HBV replication and development of HCC in that region.

### Applications

Base on our current findings, it is conceivable that patients with a high viral load have a high potential for hepatocarcinogenesis, and should be subjected to closer clinical monitoring and even antiviral treatment. The results provide a data-supported approach to patients with Hepatitis B.

### Peer review

This case-control study examined the relationship of HBV DNA quantitative levels and the risk of HCC in Qidong, China. They confirm other studies from Taiwan and elsewhere that demonstrate the risk of HCC occurs across a gradient of HBV DNA levels. This study is important.

## REFERENCES

- 1 **Safioleas M**, Lygidakis NJ, Manti C. Hepatitis B today. *Hepatogastroenterology* 2007; **54**: 545-548
- 2 **McMahon BJ**. Epidemiology and natural history of hepatitis B. *Semin Liver Dis* 2005; **25** Suppl 1: 3-8
- 3 **Mast EE**, Mahoney FJ, Alter MJ, Margolis HS. Progress toward elimination of hepatitis B virus transmission in the United States. *Vaccine* 1998; **16** Suppl: S48-S51
- 4 **Farrell GC**, Teoh NC. Management of chronic hepatitis B virus infection: a new era of disease control. *Intern Med J* 2006; **36**: 100-113
- 5 **Feitelson MA**, Duan LX. Hepatitis B virus X antigen in the pathogenesis of chronic infections and the development of hepatocellular carcinoma. *Am J Pathol* 1997; **150**: 1141-1157
- 6 **Kaibori M**, Matsui Y, Saito T, Kamiyama Y. Risk factors for different patterns of recurrence after resection of hepatocellular carcinoma. *Anticancer Res* 2007; **27**: 2809-2816
- 7 **Tong MJ**, Blatt LM, Kao JH, Cheng JT, Corey WG. Basal core promoter T1762/A1764 and precore A1896 gene mutations in hepatitis B surface antigen-positive hepatocellular carcinoma: a comparison with chronic carriers. *Liver Int* 2007; **27**: 1356-1363
- 8 **Kao JH**, Chen PJ, Lai MY, Chen DS. Basal core promoter mutations of hepatitis B virus increase the risk of hepatocellular carcinoma in hepatitis B carriers. *Gastroenterology* 2003; **124**: 327-334
- 9 **Jee SH**, Ohrr H, Sull JW, Samet JM. Cigarette smoking, alcohol drinking, hepatitis B, and risk for hepatocellular carcinoma in Korea. *J Natl Cancer Inst* 2004; **96**: 1851-1856
- 10 **London WT**, Evans AA, McGlynn K, Buetow K, An P, Gao L, Lustbader E, Ross E, Chen G, Shen F. Viral, host and environmental risk factors for hepatocellular carcinoma: a prospective study in Haimen City, China. *Intervirology* 1995; **38**: 155-161
- 11 **Evans AA**, Chen G, Ross EA, Shen FM, Lin WY, London WT. Eight-year follow-up of the 90000-person Haimen City cohort: I. Hepatocellular carcinoma mortality, risk factors, and gender differences. *Cancer Epidemiol Biomarkers Prev* 2002; **11**: 369-376
- 12 **Fattovich G**. Natural history and prognosis of hepatitis B. *Semin Liver Dis* 2003; **23**: 47-58
- 13 **Ikeda K**, Arase Y, Kobayashi M, Someya T, Hosaka T, Saitoh S, Sezaki H, Akuta N, Suzuki F, Suzuki Y, Kumada H.

- Hepatitis B virus-related hepatocellular carcinogenesis and its prevention. *Intervirology* 2005; **48**: 29-38
- 14 **Liaw YF**. Hepatitis B virus replication and liver disease progression: the impact of antiviral therapy. *Antivir Ther* 2006; **11**: 669-679
  - 15 **Mahmood S**, Niiyama G, Kamei A, Izumi A, Nakata K, Ikeda H, Suehiro M, Kawanaka M, Togawa K, Yamada G. Influence of viral load and genotype in the progression of Hepatitis B-associated liver cirrhosis to hepatocellular carcinoma. *Liver Int* 2005; **25**: 220-225
  - 16 **Tong MJ**, Blatt LM, Kao JH, Cheng JT, Corey WG. Precore/basal core promoter mutants and hepatitis B viral DNA levels as predictors for liver deaths and hepatocellular carcinoma. *World J Gastroenterol* 2006; **12**: 6620-6626
  - 17 **Tang B**, Kruger WD, Chen G, Shen F, Lin WY, Mboup S, London WT, Evans AA. Hepatitis B viremia is associated with increased risk of hepatocellular carcinoma in chronic carriers. *J Med Virol* 2004; **72**: 35-40
  - 18 **Yu MW**, Yeh SH, Chen PJ, Liaw YF, Lin CL, Liu CJ, Shih WL, Kao JH, Chen DS, Chen CJ. Hepatitis B virus genotype and DNA level and hepatocellular carcinoma: a prospective study in men. *J Natl Cancer Inst* 2005; **97**: 265-272
  - 19 **Ming L**, Thorgeirsson SS, Gail MH, Lu P, Harris CC, Wang N, Shao Y, Wu Z, Liu G, Wang X, Sun Z. Dominant role of hepatitis B virus and cofactor role of aflatoxin in hepatocarcinogenesis in Qidong, China. *Hepatology* 2002; **36**: 1214-1220
  - 20 **Luo RH**, Zhao ZX, Zhou XY, Gao ZL, Yao JL. Risk factors for primary liver carcinoma in Chinese population. *World J Gastroenterol* 2005; **11**: 4431-4434
  - 21 **Yu MW**, Chang HC, Chen PJ, Liu CJ, Liaw YF, Lin SM, Lee SD, Lin SC, Lin CL, Chen CJ. Increased risk for hepatitis B-related liver cirrhosis in relatives of patients with hepatocellular carcinoma in northern Taiwan. *Int J Epidemiol* 2002; **31**: 1008-1015
  - 22 **Yu MW**, Chang HC, Liaw YF, Lin SM, Lee SD, Liu CJ, Chen PJ, Hsiao TJ, Lee PH, Chen CJ. Familial risk of hepatocellular carcinoma among chronic hepatitis B carriers and their relatives. *J Natl Cancer Inst* 2000; **92**: 1159-1164
  - 23 **Mommeja-Marin H**, Mondou E, Blum MR, Rousseau F. Serum HBV DNA as a marker of efficacy during therapy for chronic HBV infection: analysis and review of the literature. *Hepatology* 2003; **37**: 1309-1319
  - 24 **Chen CJ**, Yang HI, Su J, Jen CL, You SL, Lu SN, Huang GT, Iloeje UH. Risk of hepatocellular carcinoma across a biological gradient of serum hepatitis B virus DNA level. *JAMA* 2006; **295**: 65-73
  - 25 **Ohata K**, Hamasaki K, Toriyama K, Ishikawa H, Nakao K, Eguchi K. High viral load is a risk factor for hepatocellular carcinoma in patients with chronic hepatitis B virus infection. *J Gastroenterol Hepatol* 2004; **19**: 670-675
  - 26 **Yang HI**, Lu SN, Liaw YF, You SL, Sun CA, Wang LY, Hsiao CK, Chen PJ, Chen DS, Chen CJ. Hepatitis B e antigen and the risk of hepatocellular carcinoma. *N Engl J Med* 2002; **347**: 168-174
  - 27 **Lai CL**, Yuen MF. The natural history and treatment of chronic hepatitis B: a critical evaluation of standard treatment criteria and end points. *Ann Intern Med* 2007; **147**: 58-61
  - 28 **Liaw YF**, Sung JJ, Chow WC, Farrell G, Lee CZ, Yuen H, Tanwandee T, Tao QM, Shue K, Keene ON, Dixon JS, Gray DF, Sabbat J. Lamivudine for patients with chronic hepatitis B and advanced liver disease. *N Engl J Med* 2004; **351**: 1521-1531
  - 29 **Yuen MF**, Seto WK, Chow DH, Tsui K, Wong DK, Ngai VW, Wong BC, Fung J, Yuen JC, Lai CL. Long-term lamivudine therapy reduces the risk of long-term complications of chronic hepatitis B infection even in patients without advanced disease. *Antivir Ther* 2007; **12**: 1295-1303
  - 30 **Liaw YF**. Prevention and surveillance of hepatitis B virus-related hepatocellular carcinoma. *Semin Liver Dis* 2005; **25** Suppl 1: 40-47

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RAPID COMMUNICATION

## Predictive value of MTT assay as an *in vitro* chemosensitivity testing for gastric cancer: One institution's experience

Bin Wu, Jin-Shui Zhu, Yi Zhang, Wei-Ming Shen, Qiang Zhang

Bin Wu, Yi Zhang, Wei-Ming Shen, Department of Pharmacy, Shanghai Sixth Hospital Affiliated to Shanghai Jiaotong University, Shanghai 200223, China

Jin-Shui Zhu, Qiang Zhang, Department of Gastroenterology, Shanghai Sixth Hospital Affiliated to Shanghai Jiaotong University, Shanghai 200223, China

**Author contributions:** Wu B and Zhang Q designed and performed the research; Zhu JS contributed to the statistical advices; Zhang Y supplied the MTT assay data; Shen WM offered the MTT assay advices.

**Correspondence to:** Dr. Qiang Zhang, MD, Department of Gastroenterology, Shanghai Sixth Hospital Affiliated to Shanghai Jiaotong University, Yishang Road 600, Shanghai 200223, China. [qzsztu@yahoo.cn](mailto:qzsztu@yahoo.cn)

Telephone: +86-21-64369181 Fax: +86-21-64701361

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### Abstract

**AIM:** To investigate the predictive clinical value of *in vitro* 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay for directing chemosensitivity in patients with gastric cancer.

**METHODS:** Results of a total of 353 consecutive patients with gastric cancer treated with MTT-directed chemotherapy or physician's empirical chemotherapy from July 1997 to April 2003 were reviewed and analyzed retrospectively.

**RESULTS:** The overall 5-year survival rate of MTT-sensitive group (MSG) and control group (CG) was 47.5% and 45.1%, respectively. The results of subgroup analysis with Cox proportional-hazards model were favorable for the MSG-sensitive group. However, no statistically significant difference in survival rate was observed between the two groups.

**CONCLUSION:** Individualized chemotherapy based on *in vitro* MTT assay is beneficial, but needs to be confirmed by further randomized controlled trials.

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**Key words:** Gastric cancer; Chemosensitivity testing; Chemotherapy; MTT assay; Survival rate

**Peer reviewer:** Toru Ishikawa, MD, Department of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan

Wu B, Zhu JS, Zhang Y, Shen WM, Zhang Q. Predictive value of MTT assay as an *in vitro* chemosensitivity testing for gastric cancer: One institution's experience. *World J Gastroenterol* 2008; 14(19): 3064-3068 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3064.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3064>

### INTRODUCTION

Gastric cancer ranks second of all cancers and is the leading cause of cancer-related deaths worldwide<sup>[1,2]</sup>. The incidence of gastric cancer worldwide is reported to be especially high in Asia, South America, and Eastern Europe<sup>[2-5]</sup>. Gastric cancer patients are treated in clinical practice with various therapies, such as chemotherapy and radiation, though further improvement and progress would be required. With the development of new anti-cancer drugs, such as taxanes, CPT-11, oxaliplatin, gefitinib and S-1, significant improvements in the efficacy of chemotherapies against gastric cancer have been achieved<sup>[4,6-8]</sup>. However, some patients still fail to respond to chemotherapy and finally die of the critical toxicity of intensive chemotherapy<sup>[9]</sup>. Thus, new therapies and technologies are desperately needed for the treatment of gastric cancer. Advances in this area would have a major impact on the outcome of a large number of patients with this disease. Hence, chemosensitivity assay has been developed to individualize chemotherapy for gastric cancer patients<sup>[10]</sup>. 3-(4,5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay is a rapid and quantitative colorimetric method for determination of cell viability by measuring the anticancer drug effectiveness on human tumor cells. Several studies on advanced gastric cancer using this approach revealed that *in vitro* sensitivities are associated with *in vivo* tumor responses<sup>[11-15]</sup>. However, most of these studies were small-scale trials (< 100 patients).

In this study, MTT assay was used to predict the efficacy of individualized assay-directed chemotherapy for Chinese gastric cancer patients, and to prove whether *in vitro* chemosensitivities are associated with *in vivo* tumor responses by survival analysis.

### MATERIALS AND METHODS

#### Patients

This was a retrospective study. The medical records of

patients registered for adjuvant chemotherapy from July 1997 to April 2003 were reviewed. The criteria for case inclusion were as follows: (1) a diagnosis of histologically or cytologically proven gastric cancer, (2) without prior chemotherapy or radiotherapy, (3) adequate blood counts (hemoglobin  $\geq 10$  g/L, WBC count of 3000/ $\mu$ L, and platelets of 100 000/ $\mu$ L), normal renal function (creatinine clearance  $\geq 60$  mL/min), and normal liver function (serum transaminase level less than double the normal upper limit). Patients with esophageal cancer, small cell carcinoma, lymphoma, and squamous cell carcinoma were excluded from the study. A total of 353 eligible records of patients were collected and analyzed. The patients were divided into MTT-sensitive group (MSG) and control group (CG). One hundred and fifty-seven patients in the MSG-sensitive group were treated by chemotherapy containing at least one sensitive drug based on the MTT assay results, and 196 patients received physician's empirical chemotherapy. The chemotherapeutic drugs used were cisplatin (CDDP), 5-fluorouracil (5-Fu), mitomycin (MMC), doxorubicin (DOX), paclitaxel (PAC) and docetaxel (DOC). The protocols of chemotherapy have been described elsewhere<sup>[6,16,17]</sup>.

### MTT assay

Fresh tumor tissue obtained from the surgically resected specimens was tested within 6 h. The tumor tissue was cut into pieces (smaller than 1 mm<sup>3</sup>) and passed through No. 100 and No. 200 stainless steel meshes respectively into a complete medium containing RPMI 1640 solution, 100  $\mu$ g/mL penicillin, and 100  $\mu$ g/mL streptomycin, and washed twice gently with the same solution. The viable cells were assessed using trypan blue exclusion method. Cell viability was measured by MTT assay to assess the chemosensitivity of tumor cells. Cell suspension was collected into sterile 96-well flat-bottomed microtiter plates ( $1 \times 10^5$  cells/per well) with or without chemotherapeutic agents. The drug and testing drug concentrations used were 25  $\mu$ g/mL cisplatin (CDDP), 100  $\mu$ g/mL 5-fluorouracil (5-Fu), 10  $\mu$ g/mL mitomycin (MMC), 4  $\mu$ g/mL doxorubicin (DOX), 100  $\mu$ g/mL paclitaxel (PAC) and 30  $\mu$ g/mL docetaxel (DOC). Each drug was tested in triplicate. The plates were then incubated at 37°C in a humidified atmosphere containing 50 mL/L CO<sub>2</sub> for 72 h. Microtiter wells containing tumor cells but no anticancer agents were used to control cell viability, in which the total number of tumor cells was equivalent to that in the test wells, and wells containing only a complete medium were used as blank controls for nonspecific dye reduction. After incubation, MTT solution was added to each well at a final concentration of 1 mg/mL per well and the plates were incubated at 37°C for another 4 h. Then the mixture containing the medium, the drug, and the unconverted MTT was removed. DMSO was added to each well to dissolve the formazan and absorbance was read at 550 nm using a spectrophotometric microplate reader (Labsystems, Finland). The inhibition rate of tumor cells for each drug with different concentrations was calculated following the formula: inhibition rate (%) =  $(1 - \text{OD}_{\text{drug exposure}} / \text{OD}_{\text{control}}) \times 100$ . The effective anticancer activity was regarded as

**Table 1** Baseline clinical and pathological characteristics of the patients *n* (%)

Characteristic	MSG ( <i>n</i> = 157)	CG ( <i>n</i> = 196)
Gender		
Male	104 (66.2)	137 (69.9)
Female	53 (33.8)	59 (30.1)
Age		
< 60 yr	87 (55.4)	110 (56.1)
60-69 yr	54 (34.4)	67 (34.2)
70-80 yr	16 (10.2)	19 (9.7)
Median year	58	62
Range year	29-80	31-78
Cancer stage, TNM classification		
I B	3 (1.9)	4 (2.0)
II	85 (54.1)	103 (52.6)
III	54 (34.4)	71 (36.2)
IV	15 (9.6)	18 (9.2)
Histologic type		
Differentiated	73 (46.5)	103 (52.6)
Undifferentiated	84 (53.5)	93 (47.4)

sensitive when the tumor inhibitory rate was greater than or equal to 70%.

### Toxicity

All patients who started treatment were considered assessable for toxicity. Toxicity was analyzed following the National Cancer Institute Common Toxicity Criteria (version 2.0).

### Statistical analysis

All statistical analyses were done using the SAS 6.12 statistical software (SAS Institute, Cary, NC). The clinical and pathological characteristics, including gender, age, cancer stage (TNM), and histological type (differentiated *versus* undifferentiated type), were evaluated by Mann-Whitney's *U*-test and the Kruskal-Wallis test. The overall probability was calculated using the Kaplan-Meier method for censored failure time data, and the statistical significance was analyzed by the log-rank test for comparison of survival rate between the two groups. The Cox proportional-hazards model was used to calculate the hazard ratios. *P* < 0.05 was considered statistically significant. All *P* values were two-tailed and unadjusted for potential multiple comparisons.

## RESULTS

### Patient characteristics

The clinical and pathological characteristics of the patients are outlined in Table 1. Between the MSG and CG arms, there was no significant difference in baseline clinical characteristics and pathological findings which were considered to be related to the prognosis of gastric cancer patients.

### Overall survival analysis

The overall 5-year survival rate of the patients was 47.5% and 45.1% in the MSG-sensitive group and CG group, respectively, with no statistical difference (Figure 1). The



Table 2 Severe adverse effects and toxicities (NCI-CTC version 2.0)

Toxicities	MSG ( <i>n</i> = 157)			CG ( <i>n</i> = 196)		
	Grade III (No.)	Grade IV (No.)	Grade III/IV (%)	Grade III (No.)	Grade IV (No.)	Grade III/IV (%)
Hematologic toxicity						
Leukopenia	2	2	2.5	4	1	2.6
Anemia	2	0	1.3	2	2	2.0
Thrombocytopenia	0	0	0.0	1	0	0.5
Non-hematologic toxicity						
Diarrhea	2	0	1.3	3	0	1.5
Stomatitis	1	0	0.6	0	0	0.0
Nausea	2	1	2.5	2	0	1.0
Vomiting	2	0	1.3	3	0	1.5
Anorexia	8	2	6.4	6	3	4.6
Fever	0	0	0.0	2	0	1.0
Rash	1	0	0.6	1	0	0.5
Elevated aminotransferase	2	0	1.3	3	1	2.0
Hyperbilirubinemia	1	0	0.6	2	0	1.0
Elevated creatinine	0	0	0.0	1	0	0.5

hazard ratio for deaths in the MSG-sensitive group, as compared with the CG group, was 0.92 [95% confidence interval (CI) = 0.69 to 1.23,  $P = 0.57$ ].

### Subgroup analysis

The overall survival rate of the patients was analyzed according to sex, age, cancer stage (TNM classification), and histologic type. The hazard ratio of deaths was favorable for the MSG-sensitive group (Figure 2). There were no significant interactions between the two groups and any of the variables studied.

### Adverse events and treatment compliance

Data on the 157 patients in the MSG-sensitive group and 196 patients in the CG group were analyzed for adverse events. The main emergent adverse toxicities (grades 3 and 4) related to treatment are listed in Table 2. The severe adverse events (defined according to NCI-CTC version 2.0), including hematologic and nonhematologic toxic effects, did not occur more frequently in the MSG-sensitive group than in the CG group.

## DISCUSSION

Conventional chemo-/radio-therapy for gastric cancer is limited to improve the treatment outcomes and quality of survival/life of human patients<sup>[18,19]</sup>. Physicians' empirical choice of chemotherapeutic regimen for gastric cancer is based on the data obtained from clinical trials<sup>[20]</sup>. However, even the same gastric cancer behaves so differently that the response rate of cancers to the chemotherapeutic agents varies. These variations are partly contributed to the failure of treatment of gastric cancer patients. The effectiveness of current chemotherapies for cancer is limited mainly due to its heterogeneity<sup>[21]</sup>. To overcome this problem, selecting a sensitive chemotherapeutic regimen *in vitro* for individual gastric cancer patients appears to be an attractive way. Chemosensitivity testing is an *ex vivo* means of determining the cytotoxic and/or cytostatic, or apoptosis-inducing effect of anticancer drugs. The most common *in vitro* assays include MTT and ATP-TCA

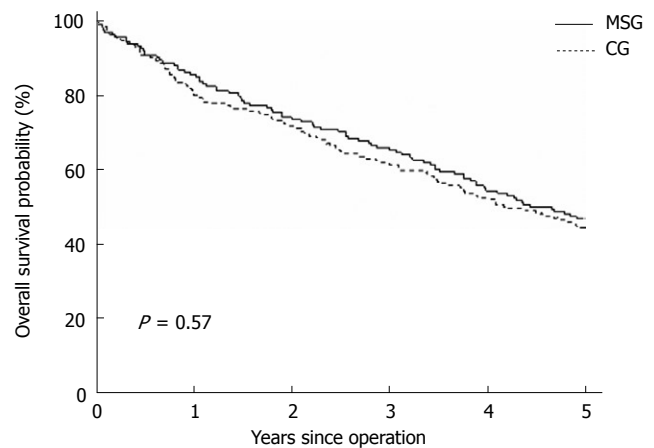
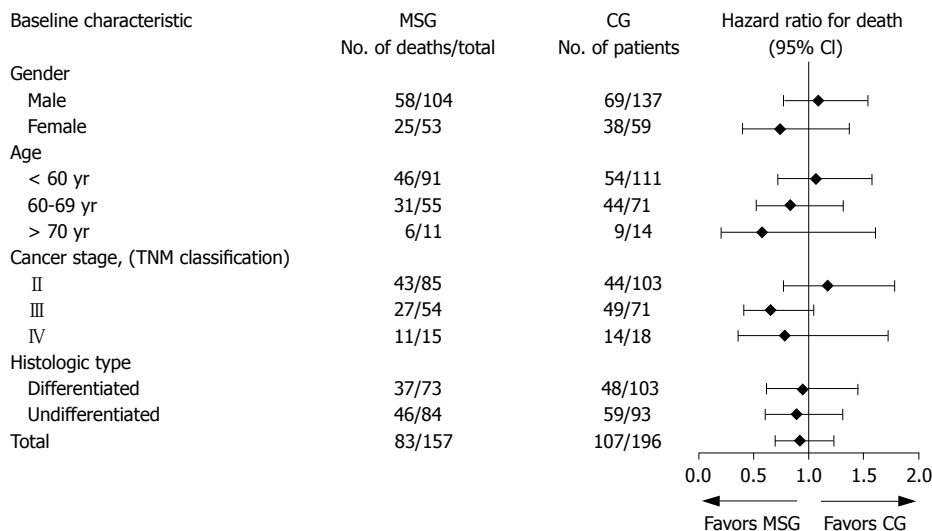


Figure 1 Kaplan-Meier curves of overall survival probabilities for gastric cancer patients, there was no significant difference between the two groups.

assays, *etc*<sup>[22]</sup>. These assays have been successfully used in the assay-guided chemotherapy for certain cancers, including breast, ovarian, melanoma and colorectal cancers<sup>[23-25]</sup>. MTT assay has been most widely used in different cancers, and is sensitive, accurate, and efficient in the *in vitro* evaluation of anticancer or immunological agents prior to preclinical and clinical testing. Some research groups have used MTT assay to guide individual adjuvant chemotherapy for gastric cancer<sup>[10]</sup>, showing that the therapy based on the chemosensitivity testing can improve the clinical outcomes of cancer patients. In the present study, based on the criteria for chemosensitivity *in vitro*, we predicted and evaluated the efficacy of chemotherapy for 353 gastric cancer patients according to the result of MTT assay. The overall survival rate of the patients, treated with chemotherapy regimen containing at least one sensitive agent, was higher in the MSG-sensitive group than in the CG group treated with the physicians' empirical therapy. The hazard ratio of most subgroups was favorable for the MSG-sensitive group as demonstrated in Cox proportional-hazards mode. However, no significant difference between the two groups was observed. These results indicate that MTT



**Figure 2** Hazard ratios of deaths for the baseline characteristics among gastric cancer patients. NS: No significant difference.

assay can lead to additional clinical outcomes as compared with physicians' empirical therapy. The real benefit of MTT assay for chemosensitivity testing is to predict which agent is useful or not useful. Although the results of this retrospective study are not all concordant with previous studies<sup>[11,12,22,26]</sup>, and do not definitively support the clinical values of MTT assay in detecting chemosensitivity to the adjuvant chemotherapy for patients with gastric cancer, they support chemosensitivity testing in patients with gastric cancer. Since the frequency of toxicity in patients is not reported in previous studies<sup>[11,12,26]</sup>, we compared the frequency of severe toxicity of grades 3 and 4 between the two groups, showing that chemotherapy regimen based on MTT assay could not reduce its the adverse effect and toxicity.

At present, although some studies have shown a potential clinical benefit of chemotherapy for patients with drug-sensitive cancer<sup>[22,27]</sup>, chemosensitivity testing has not been widely accepted by physicians. Meanwhile, prediction of chemosensitivity in clinical practice is a challenge because *in vitro* chemosensitivity testing systems have not considered the pharmacokinetic and pharmacodynamic variables affecting drug action *in vivo*. Because of varied pharmacogenetic make-ups of cancer patients, leading to interpatient variations in drug half-life, volume of distribution, types of metabolites formed, and route of elimination, dependence on *in vitro* and *in vivo* results is often not a straightforward process<sup>[28]</sup>. Ultimately, individualized chemotherapy based on cellular and genetic characteristics of cancer patients may be on the horizon<sup>[29-31]</sup>. The potential clinical benefits of individualized chemotherapy based on chemosensitivity testing need to be confirmed by further randomized controlled trials in comparison with standard chemotherapy.

information to help physicians choose sensitive chemotherapeutic agents for eliminating potentially ineffective agents used in chemotherapeutic regimens for each cancer patients.

### Research frontiers

At present, several new chemosensitivity assays, such as histoculture drug response assay (HDRA), collagen gel-droplet-embedded culture drug sensitivity test (CD-DST) and fluorometric microculture cytotoxicity assay (FMCA), are used in selection of an appropriate chemotherapeutic drug, showing the predictive value of chemotherapy for cancer patients.

### Innovations and breakthroughs

There is no evidence for the clinical benefits of MTT chemosensitivity assay. The present study evaluated the clinical usefulness of MTT chemosensitivity assay in gastric cancer patients, and showed no significant differences in clinical outcomes between the MTT-sensitive group (MSG) and the control group (CG), indicating that the potential value of MTT assay for patients with gastric cancer is limited.

### Applications

Although some studies have shown a potential clinical benefit for patients with drug-sensitive cancer, MTT assay of chemosensitivity is not widely accepted by physicians because there is no sufficient evidence obtained in the clinical setting. The potential clinical benefits of individualized chemotherapy based on chemosensitivity assay for gastric cancer patients need to be confirmed by further randomized controlled trials in comparison with standard chemotherapy.

### Terminology

MTT assay is a laboratory test and a standard colorimetric assay for measuring cellular proliferation. Yellow MTT is reduced to purple formazan in the mitochondria of living cells. A solution (usually dimethyl sulfoxide) is added to dissolve the insoluble purple formazan products into a colored solution. The absorbance of this colored solution can be quantified at a certain wavelength with a spectrophotometer.

### Peer review

This is an interesting report on the predictive value of MTT assay as an *in vitro* chemosensitivity testing for gastric cancer patients. Individualized chemotherapy based on *in vitro* MTT assay has clinical benefit, but needs to be confirmed by further randomized controlled trials.

## COMMENTS

### Background

Since cancer patients with histologically similar tumors respond differently to standard drug treatment, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) chemosensitivity assay is performed to provide predictive

## REFERENCES

- 1 Yang L. Incidence and mortality of gastric cancer in China. *World J Gastroenterol* 2006; **12**: 17-20
- 2 Alberts SR, Cervantes A, van de Velde CJ. Gastric cancer: epidemiology, pathology and treatment. *Ann Oncol* 2003; **14** Suppl 2: ii31-ii36

- 3 **Plummer M**, Franceschi S, Munoz N. Epidemiology of gastric cancer. *IARC Sci Publ* 2004; 311-326
- 4 **Wainess RM**, Dimick JB, Upchurch GR Jr, Cowan JA, Mulholland MW. Epidemiology of surgically treated gastric cancer in the United States, 1988-2000. *J Gastrointest Surg* 2003; 7: 879-883
- 5 **Goh KL**. Changing trends in gastrointestinal disease in the Asia-Pacific region. *J Dig Dis* 2007; 8: 179-185
- 6 **Schipper DL**, Wagener DJ. Chemotherapy of gastric cancer. *Anticancer Drugs* 1996; 7: 137-149
- 7 **Jackson C**, Mochlinski K, Cunningham D. Therapeutic options in gastric cancer: neoadjuvant chemotherapy vs postoperative chemoradiotherapy. *Oncology (Williston Park)* 2007; 21: 1084-1087; discussion 1090, 1096-1098, 1101
- 8 **Macdonald JS**. Gastric cancer--new therapeutic options. *N Engl J Med* 2006; 355: 76-77
- 9 **Zhang D**, Fan D. Multidrug resistance in gastric cancer: recent research advances and ongoing therapeutic challenges. *Expert Rev Anticancer Ther* 2007; 7: 1369-1378
- 10 **Kim R**, Emi M, Tanabe K, Uchida Y, Toge T. Chemosensitivity testing for gastrointestinal cancer: survival benefit potential and limitations. *Anticancer Drugs* 2003; 14: 715-723
- 11 **Nakamura R**, Saikawa Y, Kubota T, Kumagai A, Kiyota T, Ohashi M, Yoshida M, Otani Y, Kumai K, Kitajima M. Role of the MTT chemosensitivity test in the prognosis of gastric cancer patients after postoperative adjuvant chemotherapy. *Anticancer Res* 2006; 26: 1433-1437
- 12 **Iwahashi M**, Nakamori M, Nakamura M, Noguchi K, Ueda K, Nakatani Y, Ojima T, Ishida K, Naka T, Yamaue H. Individualized adjuvant chemotherapy guided by chemosensitivity test sequential to extended surgery for advanced gastric cancer. *Anticancer Res* 2005; 25: 3453-3459
- 13 **Mitsuhashi Y**, Inaba M, Sugiyama Y, Kobayashi T. In vitro measurement of chemosensitivity of human small cell lung and gastric cancer cell lines toward cell cycle phase-nonspecific agents under the clinically equivalent area under the curve. *Cancer* 1992; 70: 2540-2546
- 14 **Kurihara N**, Kubota T, Furukawa T, Watanabe M, Otani Y, Kumai K, Kitajima M. Chemosensitivity testing of primary tumor cells from gastric cancer patients with liver metastasis can identify effective antitumor drugs. *Anticancer Res* 1999; 19: 5155-5158
- 15 **Noguchi K**, Iwahashi M, Tani M, Nakamura M, Nakamori M, Nakatani Y, Ueda K, Ishida K, Naka T, Ojima T, Hotta T, Mizobata S, Yamaue H. Evaluation of chemosensitivity testing with highly purified tumor cells in 435 patients with gastric carcinoma using an MTT assay. *Anticancer Res* 2005; 25: 931-937
- 16 **Sastre J**, Garcia-Saenz JA, Diaz-Rubio E. Chemotherapy for gastric cancer. *World J Gastroenterol* 2006; 12: 204-213
- 17 **Furue H**. Chemotherapy for gastric cancer in Japan. *Gan To Kagaku Ryoho* 1997; 24 Suppl 1: 120-125
- 18 **Roukos DH**. Current status and future perspectives in gastric cancer management. *Cancer Treat Rev* 2000; 26: 243-255
- 19 **Cunningham SC**, Schulick RD. Palliative management of gastric cancer. *Surg Oncol* 2007; 16: 267-275
- 20 **Roukos DH**, Kappas AM. Perspectives in the treatment of gastric cancer. *Nat Clin Pract Oncol* 2005; 2: 98-107
- 21 **Mercer SJ**, Somers SS, Knight LA, Whitehouse PA, Sharma S, Di Nicolantonio F, Glaysher S, Toh S, Cree IA. Heterogeneity of chemosensitivity of esophageal and gastric carcinoma. *Anticancer Drugs* 2003; 14: 397-403
- 22 **Blumenthal RD**, Goldenberg DM. Methods and goals for the use of in vitro and in vivo chemosensitivity testing. *Mol Biotechnol* 2007; 35: 185-197
- 23 **Taylor CG**, Sargent JM, Elgie AW, Williamson CJ, Lewandowicz GM, Chappatte O, Hill JG. Chemosensitivity testing predicts survival in ovarian cancer. *Eur J Gynaecol Oncol* 2001; 22: 278-282
- 24 **Ugurel S**, Tilgen W, Reinhold U. Chemosensitivity testing in malignant melanoma. *Recent Results Cancer Res* 2003; 161: 81-92
- 25 **Xu JM**, Song ST, Tang ZM, Jiang ZF, Liu XQ, Zhou L, Zhang J, Liu XW. Predictive chemotherapy of advanced breast cancer directed by MTT assay in vitro. *Breast Cancer Res Treat* 1999; 53: 77-85
- 26 **Kubota T**, Egawa T, Otani Y, Furukawa T, Saikawa Y, Yoshida M, Watanabe M, Kumai K, Kitajima M. Cancer chemotherapy chemosensitivity testing is useful in evaluating the appropriate adjuvant cancer chemotherapy for stages III/IV gastric cancers without peritoneal dissemination. *Anticancer Res* 2003; 23: 583-587
- 27 **Hwu P**, Bedikian AY, Grimm EA. Challenges of chemosensitivity testing. *Clin Cancer Res* 2006; 12: 5258-5259
- 28 **Blumenthal RD**. An overview of chemosensitivity testing. *Methods Mol Med* 2005; 110: 3-18
- 29 **Idbaih A**, Omuro A, Ducray F, Hoang-Xuan K. Molecular genetic markers as predictors of response to chemotherapy in gliomas. *Curr Opin Oncol* 2007; 19: 606-611
- 30 **Park DJ**, Lenz HJ. Determinants of chemosensitivity in gastric cancer. *Curr Opin Pharmacol* 2006; 6: 337-344
- 31 **Covell DG**. Connecting chemosensitivity, gene expression and disease. *Trends Pharmacol Sci* 2008; 29: 1-5

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## Significance of Bcl-xL in human colon carcinoma

You-Li Zhang, Li-Qun Pang, Ying Wu, Xiao-Yan Wang, Chong-Qiang Wang, Yu Fan

You-Li Zhang, Ying Wu, Xiao-Yan Wang, Department of Gastroenterology, Affiliated Hospital of Jiangsu University, Zhenjiang 212001, Jiangsu Province, China

Li-Qun Pang, Department of General Surgery, Affiliated Hospital of Jiangsu University, Zhenjiang 212001, Jiangsu Province, China  
Chong-Qiang Wang, Yu Fan, Cancer Institute, Department of Chemotherapy, Affiliated People's Hospital of Jiangsu University, Zhenjiang 212002, Jiangsu Province, China

**Author contributions:** Zhang YL and Fan Y contributed equally to this work; Fan Y designed this work; Zhang YL performed this work and wrote the paper; Pang LQ and Wu Y performed the research; Wang XY and Wang CQ analyzed the data.

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**Correspondence to:** Yu Fan, Department of Chemotherapy, Affiliated People's Hospital of Jiangsu University, Zhenjiang 212002, Jiangsu Province, China. [yufanzh99@sina.com](mailto:yufanzh99@sina.com)

**Telephone:** +86-511-85797359 **Fax:** +86-511-85026387

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### Abstract

**AIM:** To investigate the clinical significance of Bcl-xL gene in the pathogenesis of human colon carcinoma.

**METHODS:** Fifty-six pair tissue samples from patients with colon cancer were collected, and protein level of the Bcl-xL gene was measured by immunohistochemistry method. The correlation of Bcl-xL expression with clinical index was evaluated. After human colon cancer cell line HT29 was transfected with Bcl-xL small interfering RNA (siRNA), the anchorage-independent growth of cancer cells was detected by colony formation in soft agar and invasion ability of cancer cells was determined by a transwell model.

**RESULTS:** The Bcl-xL expression was higher in cancerous tissue samples than in normal tissue samples ( $38.78 \pm 11.36$  vs  $0.89 \pm 0.35$ ,  $P < 0.001$ ), and was associated with the pathological grade, lymphnode metastasis and Duke's stage of colorectal carcinoma. Transfection with Bcl-xL siRNA inhibited the colony formation and invasion ability of human colon cancer cell line HT29 *in vitro*.

**CONCLUSION:** Bcl-xL gene plays an important role in carcinogenesis of human colorectal carcinoma and is associated with malignant biological behaviors of human colorectal carcinoma.

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**Key words:** Colorectal carcinoma; Bcl-xL; Clinical significance

**Peer reviewers:** Amedeo Columbano, Dipartimento di Tossicologia, Sezione di Oncologia e Patologia Molecolare, Via Porcell 4, 09124 Cagliari, Italy; Peter L Lakatos, MD, PhD, Assistant Professor, 1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

Zhang YL, Pang LQ, Wu Y, Wang XY, Wang CQ, Fan Y. Significance of Bcl-xL in human colon carcinoma. *World J Gastroenterol* 2008; 14(19): 3069-3073 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3069.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3069>

### INTRODUCTION

Colorectal cancer is the third most common malignant neoplasm worldwide<sup>[1]</sup> and the second leading cause of cancer-related death<sup>[2]</sup>. Despite recent advances in diagnostic and therapeutic measures, the prognosis of colorectal cancer patients with distant metastasis still remains poor. Enhanced understanding of the signaling mechanisms that regulate metastasis of colon cancer may provide important insights into more effective therapeutic strategies.

Cells harboring multiple genetic alterations are normally eliminated by apoptosis. Diminished apoptosis plays a critical role in tumor initiation, invasion, metastasis, progression, and drug resistance. Results of numerous scientific and clinical studies link altered expression of apoptosis-regulatory proteins to the development of a lot of cancer cells. Among them, the Bcl-2 family of genes, which share sequence homology domains, plays a key role in the regulation of apoptotic cell death induced by a wide variety of therapeutic stimuli<sup>[3]</sup>. These genes can form homodimers and/or heterodimers that modulate one another's function, whereby their relative concentrations function as a rheostat for the apoptotic program<sup>[4]</sup>. Of them, Bcl-xL gene has been well characterized as a potential gene involved in the apoptotic signal pathway.

Bcl-xL, a mitochondrial membrane protein, promotes cell survival by regulating the electrical and osmotic homeostasis of mitochondria in response to a variety of stimuli<sup>[5,6]</sup>. Over-expression of Bcl-xL is reported to confer a multidrug resistance phenotype<sup>[7,8]</sup>. Moreover, inhibition of Bcl-xL expression by some ways results in an altered ratio of BAX to Bcl-xL and subsequent mitochondria-mediated cell death<sup>[9]</sup>. Thus, Bcl-xL might serve as an ideal molecular target of anticancer therapy

However, previous studies about Bcl-xL gene have mainly focused on the regulation of apoptosis and drug resistance. There is little information about the linkage of Bcl-xL with invasion in cancer cells. Increasing data show



that Bcl-xL over-expression might be related to invasion and metastasis of some solid tumors, such as breast cancer<sup>[10,11]</sup>, hepatocellular carcinoma<sup>[12]</sup>, ovarian cancer<sup>[13]</sup>, glioma<sup>[14]</sup>, and lung carcinoma<sup>[15]</sup>. We have found in previous works that human colon cancer cells transfected with signal transducer and activator of transcription 3 (STAT3) small interfering RNA (siRNA) can inhibit the invasion ability of cancer cells. Meanwhile, expression of Bcl-xL protein is also markedly down-regulated in transfected cancer cells<sup>[16]</sup>. However, the possible role of Bcl-xL in invasion of human colon cancer is not clear.

In the present study, we investigated the linkage of Bcl-xL with the invasion of human colon cancer *in vivo* and *in vitro*.

## MATERIALS AND METHODS

### Tissue samples

A total of 56 paired colon cancer tissue and distant normal colon tissue samples were obtained from 56 patients undergone surgical operation. Tumor histotype and grade of differentiation were defined according to the WHO criteria. The clinical and pathological stages were defined according to Duke's staging. These patients did not receive any chemotherapy or radiotherapy before operation. This study was approved by the Medical Ethical Committee of Affiliated Hospital of Jiangsu University, and all patients provided their written informed consent to participate in the study. All the specimens were fixed in 10% neutral-buffered formalin, dehydrated in ascending series of ethanol and routinely embedded in paraplast. Sections were cut at 4  $\mu$ m, stained with hematoxylin and eosin for histopathological and immunohistochemical evaluation. The clinicopathological parameters are summarized in Table 1.

### Immunohistochemical analysis and quantitative evaluation

All the tissue sections were deparaffinized, rehydrated and incubated in a citrate buffer (0.01 mol/L, pH 6.0) for 1 min at 121°C. The endogenous peroxidase activity was blocked by covering the sections with 3% H<sub>2</sub>O<sub>2</sub>/methanol for 15 min. The sections were then incubated in a 1:100 dilution of goat antihuman Bcl-xL IgG at 4°C overnight. After washed with PBS containing 0.05% Tween, the tissue sections were incubated in a 1:50 dilution of biotinylated donkey anti-goat IgG (Santa Cruz) for 30 min. The SABC reagents were used to amplify the immunoreactivity that was detected using 3'-diaminobenzidine according to the manufacturer's instructions. The sections were counterstained with hematoxylin. The positive unit (PU) represents the relative concentration of positive staining according to previous data<sup>[17]</sup>. Each section was observed randomly at five areas and the mean PU was assembled and calculated.

### Sequence of Bcl-xL siRNAs

The anti-sense sequence of siRNA (5'-CTCTGATATGCTGTCCCTG-3') corresponding to Bcl-xL mRNA with dTdT on 3'-overhangs was designed and chemically

Table 1 Relationship between Bcl-xL expression and clinical parameter in 56 cases of colorectal carcinoma (mean  $\pm$  SD)

Characteristic	n	Bcl-xL PU	P
Sex			> 0.05
Male	30	39.22 $\pm$ 11.35	
Female	26	37.36 $\pm$ 12.18	
Age (yr)			> 0.05
$\leq$ 55	25	39.89 $\pm$ 15.78	
> 55	31	38.66 $\pm$ 12.56	
Tumor differentiation			< 0.05
Well	12	31.58 $\pm$ 12.69	
Moderate	19	39.77 $\pm$ 16.55	
Poor	25	53.95 $\pm$ 17.89	
Lymph node metastasis			< 0.05
Negative	27	32.19 $\pm$ 13.35	
Positive	29	56.36 $\pm$ 11.95	
Duke's staging			< 0.05
A + B	28	31.55 $\pm$ 12.39	
C + D	28	58.78 $\pm$ 11.68	

synthesized according to the recommendation of the manufacturer (Dharmacon Research, USA). The scrambled siRNA served as a control, and its sequences are 5'-UUCUCCGAACGUGUCACGUTdTdT-3' and 5'-ACGUGACACGUUCGGAGAATdTdT-3'.

### Cell culture and Bcl-xL siRNA transfection

Human colon cancer cell line HT29 (Institute of Cell Biology, Shanghai, China) was cultured in RPMI 1640 (Invitrogen, Inc.) supplemented with 10% fetal bovine serum (FBS) in an atmosphere containing 50 mL/L CO<sub>2</sub> at 37°C. siRNA was transfected with a commercial reagent, oligofectamine (Invitrogen, USA) in 6-well plates following its manufacturer's instructions. Briefly, On the day before transfection, confluent layers of cells were trypsinized, counted and re-suspended. Cells ( $1 \times 10^5$ ) were plated into each well of the 6-well plates, so that they could become about 70% confluence next day at the time of transfection. Oligofectamine was diluted in serum-free RPMI 1640 and mixed with siRNA at a 1:2 ratio (4  $\mu$ L of 20  $\mu$ mol/L of siRNA formulated with 8  $\mu$ L of oligofectamine). The cells were then incubated for other 48 h. The number of cells was determined using a hemocytometer before subsequent assays.

### RNA isolation and complementary DNA synthesis

Total cellular RNA was isolated from cancer cell lines using Trizol. Final RNA pellets were dissolved in 20  $\mu$ L of diethyl pyrocarbonate-treated water. RNA yield was determined by spectroscopy. For complementary DNA (cDNA) synthesis, 2  $\mu$ g of total RNA was transcribed with cDNA transcription reagents using 0.2  $\mu$ g of the oligo(dT)18 primer for subsequent quantitative, real-time polymerase chain reaction (RT-PCR).

### Real time transcription polymerase chain reaction (RT-PCR)

Real-time RT-PCR analyses were performed on an ABI Prism 7700 sequence detection system (Perkin-Elmer Applied Biosystems, Foster City, CA). For Bcl-xL amplification, primers with the sequences 5'-TCCTTGTCTACGCTTTCCACG-3' and 5'-GGTCGCATTTGTGGCCTTT-3' were

used in combination with a sequence 5'-ACAGTGCCC CGCCGAAGGAGA-3'. Primers, Taqman and TaqMan probes were designed by the Primer Express™ 1.0 (Applied Biosystems) software and the probes were labeled at 5' end with the reporter dye molecule FAM (6-carboxy-fluorescein) and at 3' end with the quencher dye molecule TAMARA (6-carboxytetramethyl-rhodamine). Real-time PCR was conducted in a total volume of 50 µL with 1 × TaqMan Master Mix (Applied Biosystems) and primers. Thermal cycle parameters included one cycle at 95°C for 3 min, and 45 cycles involving denaturation at 95°C for 30 s, annealing at 52°C for 45 s, extension at 72°C for 45 s, followed by a final extension at 72°C for 10 min. The relative amount of each cDNA in each sample was calculated by dividing the CT value with the corresponding value of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All reactions were performed in triplicate.

#### **Western blotting analysis for Bcl-xL protein**

HT29 cells were harvested and lysed in a buffer containing 10 mmol/L Tris-HCl (pH 8.0), 150 mmol/L NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mmol/L EDTA (pH 8.0), 2 mmol/L phenylmethylsulfonyl fluoride, 2 mg/L aprotinin, 2 mg/L leupeptin, and 1 mmol/L Na<sub>3</sub>VO<sub>4</sub>. For Western blotting analysis, 30 µg of total extracted proteins was applied per lane before SDS-PAGE. Following transfer to nitrocellulose membranes, protein expression levels were detected using anti-Bcl-xL (Alpha Diagnostics International, TX). The expression of β-actin (Sigma-Aldrich, MO) was used as a normalization control for protein loading.

#### **Anchorage-independent growth assay**

For the anchorage-independent growth experiments, HT29 cells ( $8 \times 10^3$  cells/well) were seeded in 0.3% Difco Bactoagar (Difco, MI) supplemented with a complete culture medium. This suspension was layered over 0.5 mL of 0.8% agar-medium base layer in 24 multiwell cluster dishes (Becton Dickinson, Italy). After 15 d, the colonies were stained with nitroblue tetrazolium, and colonies larger than 50 µm were acquired with a micro-Scopeman camera system (Moritex Europe Ltd, Italy) and analyzed with Image-Pro Plus (Media Cybernetics, MD) computer program.

#### **Cell invasion assay**

Transwell invasion assays were performed using HT29 cells cultured in 12-well plates containing either 8 µm pore matrigel-coated inserts according to the manufacturer's instructions (Becton Dickinson, Bedford, MA). The membranes were rehydrated with warm serum-free (SF) Dulbecco's modified Eagle's medium (DMEM) (1.0 mL/chamber) for 2 h. The upper chamber was filled with  $1 \times 10^5$  cells in L-15 medium containing 5% FBS. The lower chamber was filled with L-15 medium containing 25% FBS as a chemo-attractant. After the chambers were incubated for 24 h at 37°C in an atmosphere containing 50 mL/L CO<sub>2</sub>, non-invading cells were removed from the upper surface of the membrane by scrubbing, and

invading cells on the lower surface of the membrane were fixed and stained with HE. The number of cells penetrating the filter was counted by a technician blinded to the experimental settings in four microscopic fields of each filter, under  $\times 20$  magnification. The percentage of invasion was expressed as the ratio of the mean cell number from the invasion chamber to the mean cell number from the control chamber according to the manufacturer's recommendation.

#### **Statistical analysis**

All analyses were performed with *t* test and ANOVA using SPSS 11.5 software (Statistical Package for Social Science). *P* < 0.05 was considered statistically significant.

## **RESULTS**

#### **Expression of Bcl-xL PU in human colon cancerous and normal tissue samples**

Bcl-xL expression was rarely expressed in normal large intestinal mucosa. However, Bcl-xL was mainly expressed in cytoplasm of the para-cancerous or cancer cells. The nuclei were stained brownish yellow, located sporadically or in the form of sheets. Quantitative immunohistochemistry analysis is summarized in Table 1. Bcl-xL PU was significantly higher in cancerous tissue samples than in normal tissue samples ( $38.78 \pm 11.36$  vs  $0.89 \pm 0.35$ , *P* < 0.001).

#### **Relationship between Bcl-xL PU and clinicopathological parameters**

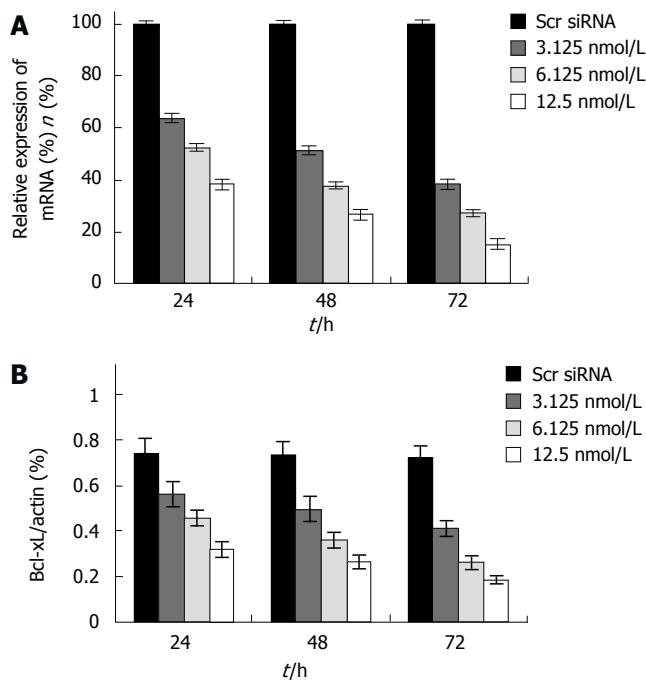
Correlation of Bcl-xL expression with clinicopathological parameters was evaluated. Bcl-xL PU, positive lymph nodes and Duke's C/D stage were higher in cancerous tissue samples with low differentiation than in cancerous tissue samples with high differentiation (*P* < 0.05, Table 1). However, Bcl-xL expression was not correlated with sex, age of the patients.

#### **Suppression of Bcl-xL by siRNA**

To further clarify the role of Bcl-xL gene, siRNA was used to knockdown the Bcl-xL expression in human colon cancer cells. Bcl-xL siRNA was transfected into the colon cancer cell line HT29. The ability of siRNA to down-regulate Bcl-xL expression was quantified by real time RT-PCR analysis and Western blot assay, respectively. siRNA significantly reduced the Bcl-xL mRNA and protein level in a dose- and time- dependent manner (Figure 1). However, the control scrambled siRNA treatment had no effect on Bcl-xL expression, thus supporting the specificity of Bcl-xL siRNA.

#### **Bcl-xL siRNA inhibited anchorage-independent growth of human colon cancer cells**

Next we evaluated the biological effects of Bcl-xL suppression on human colon cancer HT29 cells using several different types of assays. Colony formation in soft agar is a property closely associated with malignancy. Treatment with Bcl-xL siRNA significantly inhibited the anchorage-independent growth of human colon cancer cells in a dose-dependent manner (Figure 2A).



**Figure 1** Effects of siRNA on Bcl-xL expression in human colon cancer HT29 cells. **A:** Bcl-xL mRNA level; **B:** Bcl-xL protein level.

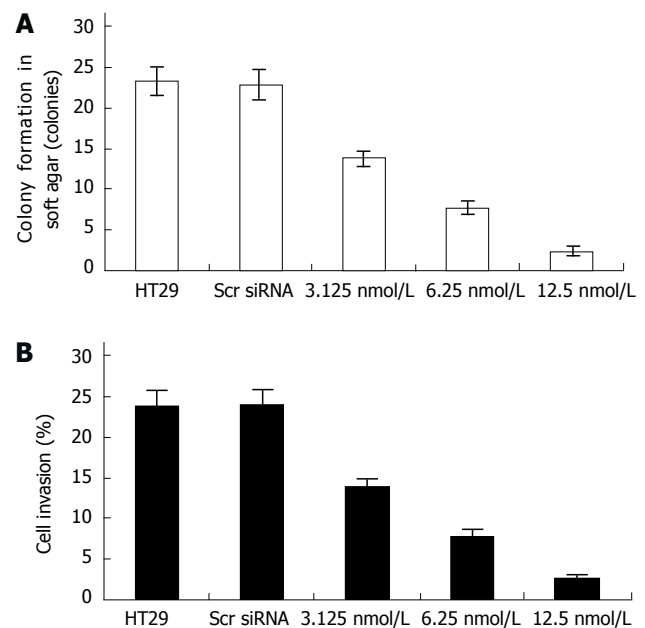
### Down-regulation of Bcl-xL decreased the ability of human colon cancer cells to grow in vitro

Given the known role of Bcl-xL siRNA in down-regulation of anchorage-independent HT29 cell growth, we attempted further to evaluate whether the Bcl-xL gene contributes to cell invasion of colon cancer cells. Cell invasion studies were performed using the matrigel matrix assays. The results showed that Bcl-xL siRNA treatment resulted in a dramatic low level of invasion potential of HT29 cells (Figure 2B), but not scrambled siRNA treatment.

## DISCUSSION

The Bcl-2 family is characterized by the presence of Bcl-2 homology domains and falls into two main groups: anti-apoptotic proteins, such as Bcl-2, Bcl-xL, Bcl-w, Mcl-1, A1, and proapoptotic proteins, such as Bax, Bak, Bad, Bid, and Bcl-xS<sup>[6]</sup>. The Bcl-x gene encodes two proteins, a long form (Bcl-xL) and a short form (Bcl-xS), through an alternative splicing mechanism. Bcl-xL, displaying remarkable amino acids and an overall structural homology to Bcl-2, can effectively block apoptosis, whereas Bcl-xS, lacking 63 amino acids in Bcl-xL, is a dominant inhibitor of Bcl-2 activity and thereby acts as a proapoptotic factor<sup>[9]</sup>.

Although there is evidence of cell apoptosis and Bcl-xL gene, the relationship between Bcl-xL and invasion of malignant tumors remains unclear. It was reported that Bcl-xL is related to the invasion and metastasis of some solid tumors. Zhang *et al.*<sup>[18]</sup> and Takada *et al.*<sup>[19]</sup> have reported the inhibition of invasion of cancer cells after treated with the Bcl-xL gene. Our previous study showed that STAT3 siRNA transfection inhibits the invasion ability of human colon cancer cell line HT29 and that the Bcl-xL protein is significantly inhibited in transfected cancer



**Figure 2** Effects of Bcl-xL siRNA on anchorage-independent growth (**A**) and invasion ability (**B**) of human colon cancer HT29 cell.

cells<sup>[16]</sup>, suggesting that Bcl-xL contributes to the invasion of human colon cancer cells. To verify it, we studied the relationship between Bcl-xL and invasion of human colon cancer *in vivo* and *in vitro*.

The expression of Bcl-xL protein in human colon cancer was determined by immunohistochemistry assay, showing that Bcl-xL protein was over-expressed in colon cancer tissue samples compared to normal tissue samples ( $P < 0.001$ ). Meanwhile, Bcl-xL expression had no significant correlation with sex and age of the patients, but was greatly correlated with differentiation stage, lymph node metastasis, and Duke's stage of colorectal carcinoma ( $P < 0.05$ ), indicating that Bcl-xL over-expression is related to the development and invasion of human colon cancer.

In order to further investigate the relationship between Bcl-xL and invasion of human colon cancer cells, we studied the effects of Bcl-xL down-regulated by siRNA on the invasion ability of human colon cancer cells. siRNA is a short oligonucleotide consisting of 21-23 nucleotides that can be used *in vitro* to induce sequence specific gene silencing of mammalian cells<sup>[20]</sup>. To elucidate the role of Bcl-xL gene in human colon cancer, siRNA was used to knockdown the Bcl-xL expression in human colon cancer cell line HT29. Real time RT-PCR and Western blot analysis showed that the expression of Bcl-xL in HT29 cancer cells transfected with siRNA was significantly reduced in a dose- and time-dependent manner. In addition, transfection of human colon cancer HT29 cells with Bcl-xL siRNA decreased the invasion ability and anchorage-independent growth of human colon cancer cells. The data *in vitro* suggest that the Bcl-xL gene plays an important role in regulating the invasion of human colon cancer cell.

In conclusion, the Bcl-xL gene is relevant to the invasion and progression of human colon cancer, and can be used in evaluating the carcinogenesis of human colon cancer. However, the precise mechanism of Bcl-xL underlying the

carcinogenesis of human colon cancer is still unclear, and further study is needed.

## COMMENTS

### Background

Despite recent advances in diagnostic and therapeutic measures, the prognosis of colorectal cancer patients with distant metastasis still remains poor. Enhanced understanding of the signaling mechanism underlying metastasis of colon cancer may provide important insights into more effective therapeutic strategies.

### Research frontiers

The results of this study indicate that the Bcl-xL gene plays an important role in the carcinogenesis of human colorectal carcinoma and is associated with the malignant biological behaviors of colorectal carcinoma.

### Innovations and breakthroughs

The results of the present study suggest that the Bcl-xL gene is relevant to the invasion and progression of human colon cancer and can be used in evaluating the carcinogenesis of human colon cancer.

### Applications

The paper helps to clarify the mechanism underlying the invasion and metastasis of colon cancer and contributes to the choice of therapeutic strategies.

### Peer review

This interesting article indicates that the Bcl-xL gene is relevant to the invasion and progression of human colon cancer, and might be used in evaluating the carcinogenesis of human colon cancer.

## REFERENCES

- 1 Shike M, Winawer SJ, Greenwald PH, Bloch A, Hill MJ, Swaroop SV. Primary prevention of colorectal cancer. The WHO Collaborating Centre for the Prevention of Colorectal Cancer. *Bull World Health Organ* 1990; **68**: 377-385
- 2 Winawer SJ, Fletcher RH, Miller L, Godlee F, Stolar MH, Mulrow CD, Woolf SH, Glick SN, Ganiats TG, Bond JH, Rosen L, Zapka JG, Olsen SJ, Giardiello FM, Sisk JE, Van Antwerp R, Brown-Davis C, Marciniak DA, Mayer RJ. Colorectal cancer screening: clinical guidelines and rationale. *Gastroenterology* 1997; **112**: 594-642
- 3 Nunez G, Clarke MF. The Bcl-2 family of proteins: regulators of cell death and survival. *Trends Cell Biol* 1994; **4**: 399-403
- 4 Chao DT, Korsmeyer SJ. BCL-2 family: regulators of cell death. *Annu Rev Immunol* 1998; **16**: 395-419
- 5 Vander Heiden MG, Chandel NS, Williamson EK, Schumacker PT, Thompson CB. Bcl-xL regulates the membrane potential and volume homeostasis of mitochondria. *Cell* 1997; **91**: 627-637
- 6 Gottlieb E, Vander Heiden MG, Thompson CB. Bcl-x(L) prevents the initial decrease in mitochondrial membrane potential and subsequent reactive oxygen species production during tumor necrosis factor alpha-induced apoptosis. *Mol Cell Biol* 2000; **20**: 5680-5689
- 7 Minn AJ, Rudin CM, Boise LH, Thompson CB. Expression of bcl-xL can confer a multidrug resistance phenotype. *Blood* 1995; **86**: 1903-1910
- 8 Kharbanda S, Pandey P, Schofield L, Israels S, Roncinske R, Yoshida K, Bharti A, Yuan ZM, Saxena S, Weichselbaum R, Nalin C, Kufe D. Role for Bcl-xL as an inhibitor of cytosolic cytochrome C accumulation in DNA damage-induced apoptosis. *Proc Natl Acad Sci USA* 1997; **94**: 6939-6942
- 9 Zhang L, Yu J, Park BH, Kinzler KW, Vogelstein B. Role of BAX in the apoptotic response to anticancer agents. *Science* 2000; **290**: 989-992
- 10 Fernández Y, España L, Mañas S, Fabra A, Sierra A. Bcl-xL promotes metastasis of breast cancer cells by induction of cytokines resistance. *Cell Death Differ* 2000; **7**: 350-359
- 11 España L, Fernández Y, Rubio N, Torregrosa A, Blanco J, Sierra A. Overexpression of Bcl-xL in human breast cancer cells enhances organ-selective lymph node metastasis. *Breast Cancer Res Treat* 2004; **87**: 33-44
- 12 Watanabe J, Kushihata F, Honda K, Sugita A, Tateishi N, Mominoki K, Matsuda S, Kobayashi N. Prognostic significance of Bcl-xL in human hepatocellular carcinoma. *Surgery* 2004; **135**: 604-612
- 13 Frankel A, Rosen K, Filmus J, Kerbel RS. Induction of anoikis and suppression of human ovarian tumor growth in vivo by down-regulation of Bcl-X(L). *Cancer Res* 2001; **61**: 4837-4841
- 14 Weiler M, Bahr O, Hohlweg U, Naumann U, Rieger J, Huang H, Tabatabai G, Krell HW, Ohgaki H, Weller M, Wick W. BCL-xL: time-dependent dissociation between modulation of apoptosis and invasiveness in human malignant glioma cells. *Cell Death Differ* 2006; **13**: 1156-1169
- 15 Sánchez-Ceja SG, Reyes-Maldonado E, Vázquez-Manríquez ME, López-Luna JJ, Belmont A, Gutiérrez-Castellanos S. Differential expression of STAT5 and Bcl-xL, and high expression of Neu and STAT3 in non-small-cell lung carcinoma. *Lung Cancer* 2006; **54**: 163-168
- 16 Fan Y, Zhang YL, Wu Y, Zhang W, Wang YH, Cheng ZM, Li H. Inhibition of signal transducer and activator of transcription 3 expression by RNA interference suppresses invasion through inducing anoikis in human colon cancer cells. *World J Gastroenterol* 2008; **14**: 428-434
- 17 Tan HY, Liu J, Wu SM, Luo HS. Expression of a novel apoptosis inhibitor-survivin in colorectal carcinoma. *World J Gastroenterol* 2005; **11**: 4689-4692
- 18 Zhang X, Xu Q, Saiki I. Quercetin inhibits the invasion and mobility of murine melanoma B16-BL6 cells through inducing apoptosis via decreasing Bcl-2 expression. *Clin Exp Metastasis* 2000; **18**: 415-421
- 19 Takada Y, Kobayashi Y, Aggarwal BB. Evodiamine abolishes constitutive and inducible NF-kappaB activation by inhibiting IkappaBalpha kinase activation, thereby suppressing NF-kappaB-regulated antiapoptotic and metastatic gene expression, up-regulating apoptosis, and inhibiting invasion. *J Biol Chem* 2005; **280**: 17203-17212
- 20 Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* 2001; **411**: 494-498

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RAPID COMMUNICATION

## Detection of *RASSF1A* promoter hypermethylation in serum from gastric and colorectal adenocarcinoma patients

Yu-Cai Wang, Zheng-Hong Yu, Chang Liu, Li-Zhi Xu, Wen Yu, Jia Lu, Ren-Min Zhu, Guo-Li Li, Xin-Yi Xia, Xiao-Wei Wei, Hong-Zan Ji, Heng Lu, Yong Gao, Wei-Min Gao, Long-Bang Chen

Yu-Cai Wang, Zheng-Hong Yu, Chang Liu, Long-Bang Chen, Department of Medical Oncology, Jinling Hospital, Nanjing 210002, Jiangsu Province, China

Yu-Cai Wang, Li-Zhi Xu, Wen Yu, Xiao-Wei Wei, Long-Bang Chen, Medical School of Nanjing University, Nanjing 210093, Jiangsu Province, China

Yu-Cai Wang, Department of Experimental Radiation Oncology, The University of Texas M.D. Anderson Cancer Center, The University of Texas Graduate School of Biomedical Sciences at Houston, Houston TX 77030, United States

Jia Lu, Department of Molecular Pathology, The University of Texas M. D. Anderson Cancer Center, Houston TX 77030, United States

Ren-Min Zhu, Hong-Zan Ji, Heng Lu, Department of Gastroenterology, Jinling Hospital, Nanjing 210002, Jiangsu Province, China

Guo-Li Li, Xiao-Wei Wei, Yong Gao, Institute of General Surgery, Jinling Hospital, Nanjing 210002, Jiangsu Province, China

Xin-Yi Xia, Institute of Laboratory Medicine, Jinling Hospital, Nanjing 210002, Jiangsu Province, China

Wei-Min Gao, Department of Environmental Toxicology, The Institute of Environmental and Human Health, Texas Tech University, Lubbock TX 79409, United States

**Author contributions:** Wang YC and Yu ZH contributed equally to this work; Wang YC, Yu ZH, Gao WM and Chen LB designed the research; Wang YC, Liu C, Xu LZ, Yu W, Zhu RM, Li GL, Xia XY, Wei XW, Ji HZ, Lu H and Gao Y performed the research; Wang YC and Lu J analyzed the data; Wang YC, Yu ZH, Lu J and Chen LB wrote the paper.

**Correspondence to:** Dr. Long-Bang Chen, Department of Medical Oncology, Jinling Hospital, 305 East Zhongshan Road, Nanjing 210002, Jiangsu Province, China. [chenlongbang@yeah.net](mailto:chenlongbang@yeah.net)

Telephone: +86-25-80860123 Fax: +86-25-84824051

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### Abstract

**AIM:** To evaluate the diagnostic role of serum *RASSF1A* promoter hypermethylation in gastric and colorectal adenocarcinoma.

**METHODS:** Methylation-specific polymerase chain reaction (MSPCR) was used to examine the promoter methylation status of the serum *RASSF1A* gene in 47 gastric adenocarcinoma patients, 45 colorectal adenocarcinoma patients, 60 patients with benign gastrointestinal disease (30 with benign gastric disease and 30 with benign colorectal disease), and 30 healthy donor controls. A

paired study of *RASSF1A* promoter methylation status in primary tumor, adjacent normal tissue, and postoperative serum were conducted in 25 gastric and colorectal adenocarcinoma patients who later were underwent surgical therapy.

**RESULTS:** The frequencies of detection of serum *RASSF1A* promoter hypermethylation in gastric (34.0%) and colorectal (28.9%) adenocarcinoma patients were significantly higher than those in patients with benign gastric (3.3%) or colorectal (6.7%) disease or in healthy donors (0%) ( $P < 0.01$ ). The methylation status of *RASSF1A* promoter in serum samples was consistent with that in paired primary tumors, and the MSPCR results for *RASSF1A* promoter methylation status in paired preoperative samples were consistent with those in postoperative serum samples. The serum *RASSF1A* promoter hypermethylation did not correlate with patient sex, age, tumor differentiation grade, surgical therapy, or serum carcinoembryonic antigen level. Although the serum *RASSF1A* promoter hypermethylation frequency tended to be higher in patients with distant metastases, there was no correlation between methylation status and metastasis.

**CONCLUSION:** Aberrant CpG island methylation within the promoter region of *RASSF1A* is a promising biomarker for gastric and colorectal cancer.

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**Key words:** Gastric cancer; Colorectal cancer; Gene methylation; *RASSF1A*

**Peer reviewers:** Yoshiharu Motoo, MD, PhD, FACP, FACG, Professor and Chairman, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan; Qin Su, Professor, Department of Pathology, Cancer Hospital and Cancer Institute, Chinese Academy of Medical Sciences and Peking Medical College, PO Box 2258, Beijing 100021, China

Wang YC, Yu ZH, Liu C, Xu LZ, Yu W, Lu J, Zhu RM, Li GL, Xia XY, Wei XW, Ji HZ, Lu H, Gao Y, Gao WM, Chen LB. Detection of *RASSF1A* promoter hypermethylation in serum from gastric and colorectal adenocarcinoma patients. *World J Gastroenterol* 2008; 14(19): 3074-3080 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3074.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3074>

## INTRODUCTION

Gastric and colorectal cancers are two of the most common causes of cancer-related death worldwide. Development of efficient diagnostic methods to enable their early detection plays an essential role in increasing the survival rate of patients with these diseases. Although endoscopy is considered the most sensitive screening tool for gastric and colorectal cancers, its use is limited due to its considerable cost and risk, and patients' lack of acceptance of the invasive procedure. Therefore, reliable noninvasive test, preferably blood test, for screening and diagnostic purposes are obviously needed.

Conventional tumor markers in serum, such as carcinoembryonic antigen (CEA), are generally insensitive for screening purposes<sup>[1]</sup>. Consequently, novel serum biomarkers are clearly needed for the early detection of gastric and colorectal cancers.

Aberrant DNA methylation, a feature of many human cancers, frequently occurs as an early event in tumorigenesis and is characterized by general hypomethylation and regional hypermethylation<sup>[2]</sup>. The hypermethylation of CpG islands within the promoter and/or upstream exon regions is an important epigenetic mechanism underlying the inactivation of tumor-suppressor genes (TSGs)<sup>[3]</sup>. It was reported that quite a few TSGs, including the *Ras association domain family 1A* (*RASSF1A*) gene, are epigenetically silenced by aberrant promoter hypermethylation in gastric and colorectal cancer<sup>[4-10]</sup>. *RASSF1A* is a newly identified candidate TSG located in the 3p21.3 region<sup>[11]</sup>, and promoter hypermethylation of *RASSF1A*, which is its most common inactivation mechanism, has been observed in many human solid tumors, including gastric and colorectal cancers<sup>[11-17]</sup>.

It has been long known that the serum level of free DNA is increased in cancer patients, which is believed to be released from cancer cells<sup>[18,19]</sup>. It was reported that genetic and epigenetic alterations in serum DNA (such as point mutation, gene amplification, loss of heterozygosity, microsatellite instability, and aberrant methylation) are identical to those found in primary human cancers<sup>[20-24]</sup>. Because the promoter methylation status of TSGs in primary tumors and matched serum samples was consistent with each other<sup>[4,25,26]</sup>, promoter hypermethylation of TSGs in serum DNA may become a promising biomarker for gastric and colorectal cancers.

In the present study, we attempted to identify the *RASSF1A* promoter methylation status both in serum DNA and in available paired tumor genomic DNA from patients with gastric and colorectal adenocarcinomas by using methylation-specific polymerase chain reaction (MSPCR). We also analyzed the correlation between serum *RASSF1A* gene promoter hypermethylation and patients' clinicopathologic parameters to further evaluate the clinical significance of this molecular change.

## MATERIALS AND METHODS

### Study population

This study included 47 gastric adenocarcinoma patients and 45 colorectal adenocarcinoma patients diagnosed at

Table 1 Clinicopathologic characteristics of patients with gastric and colorectal adenocarcinoma

Characteristics		Patients (n)	
		Gastric cancer	Colorectal cancer
Sex	Male	29	24
	Female	18	21
Age (yr)	≤ 60	21	31
	> 60	26	14
Differentiation grade	G1/Broders' I	2	4
	G2/Broders' II	23	34
	G3/Broders' III & IV	22	7
Stage	TNM I /Duke's A	4	5
	TNM II /Duke's B	15	16
	TNM III /Duke's C	16	14
	TNM IV /Duke's D	12	10

Departments of General Surgery, Gastroenterology, and Medical Oncology of Jinling Hospital (Nanjing, China) between August 1, 2006 and November 30, 2007. All diagnoses were based on pathologic evidence, and only patients with adenocarcinoma, the most common histologic type of gastric and colorectal cancer, were included. The clinicopathologic characteristics of these patients are summarized in Table 1.

The control population consisted of 60 patients with benign gastrointestinal diseases (30 with benign gastric disease and 30 with benign colorectal disease, such as chronic gastritis, gastric ulcer, benign polyp, nonmalignant adenoma, and ulcerative colitis; data not shown) and 30 healthy donors.

Gastric adenocarcinoma was staged according to the sixth edition of the TNM staging system<sup>[27]</sup>, and colorectal adenocarcinoma was staged according to the Duke's staging system. Gastric and colorectal adenocarcinoma differentiation was graded according to the World Health Organization grading system and the Broders' grading system, respectively.

Our study was approved by the ethical committee of the hospital and informed consent was obtained from all patients.

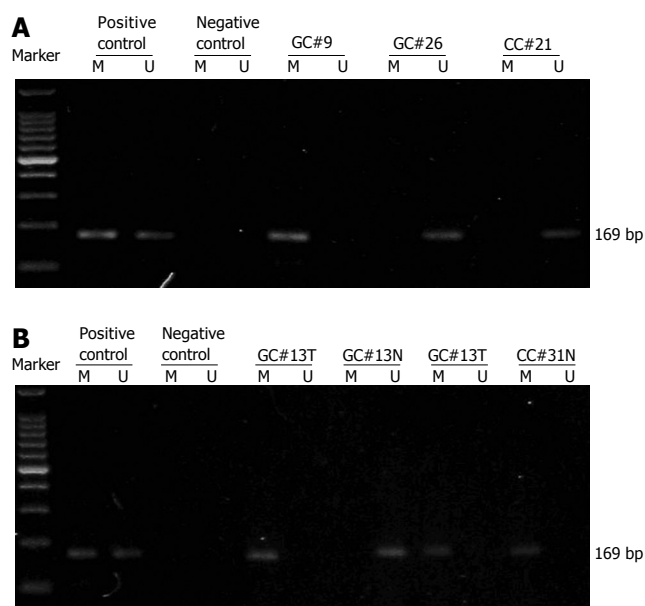
### Sample collection

Five mL of peripheral venous blood was collected from each patient 1 day after the patients were admitted to our hospital. At this time, the patients did not start their treatment. Any previous treatment (surgery and/or chemotherapy), if given, was discontinued at least 4 wk earlier. Fresh tumor tissue and paired adjacent normal tissue were obtained from 16 gastric and 9 colorectal adenocarcinoma patients who later were underwent to surgical therapy in Jinling Hospital. An additional 5 mL peripheral venous blood was collected from these 25 patients 4 wk after surgery for a comparative study. All blood samples were kept in tubes containing clot activator at 4°C for 2 h, and samples were centrifuged at 3000 r/min for 10 min to isolate sera. Thirty serum samples from healthy donors were obtained from the Blood Center of Jinling Hospital as normal controls. All serum and tissue samples were stored at -80°C until use.

Table 2 Sequences of the primers used in MSP

Primer	Sequence (5'-3')	Amplicon location <sup>1</sup>	Annealing temperature	Product size (bp)
MF	GGGTTTTCGAGAGCGCG	17882-18050	64°C	169
MR	GCTAACAAACGCGAACCG			
UF	GGTTTGTGAGAGTGTTTAG	17883-18051	59°C	169
UR	CACTAACAAACACAAACAAAC			

<sup>1</sup>GenBank accession number of *RASSF1A* is AC002481. F: Forward; R: Reverse; M: Methylated; U: Unmethylated.



**Figure 1** Representative results showing *RASSF1A* promoter methylation status identified by MSPCR in gastric and adenocarcinoma patients. Identification of *RASSF1A* promoter methylation status in serum samples from gastric and colorectal adenocarcinoma patients (A) and in paired tumor and adjacent normal tissue from gastric and colorectal adenocarcinoma patients (B). A 100-bp DNA ladder marker (TaKaRa, Shiga, Japan) was used. Lanes M and U indicate the amplified products with primers recognizing specific methylated and unmethylated sequences, respectively. GC: Gastric adenocarcinoma; CC: Colorectal adenocarcinoma; T: Tumor tissue; N: Paired adjacent normal tissue.

### DNA extraction and bisulfite treatment

Serum DNA, extracted with the QIAamp blood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, was stored at -80°C until use. Genomic DNA isolated from tissue samples was prepared using standard phenol/chloroform extraction protocols.

The extracted DNA was modified according to Herman *et al*<sup>[28]</sup> with minor modifications, to convert all unmethylated cytosines to uracils. Briefly, 1 µg of genomic DNA, or serum DNA extracted from 5 mL blood plus 1 µg of salmon sperm carrier DNA (Sigma, St. Louis, MO, USA), in a total volume of 50 µL, were denatured by NaOH (0.3 mol/L final concentration) at 40°C for 15 min. After 30 µL of freshly prepared 10 mmol/L hydroquinone (Sigma) and 520 µL of freshly prepared 3 mol/L sodium bisulfite (Sigma) at pH 5.0 were added, the samples were incubated under mineral oil at 55°C in darkness for 14 h. The modified DNA was purified using the Wizard DNA clean-up system (Promega, Madison, WI, USA), following its manufacturer's protocol. Modification was completed

by NaOH (0.3 mol/L final concentration) treatment for 10 min at room temperature, followed by ethanol precipitation. The modified DNA was resuspended in sterile deionized water (100 µL for genomic DNA and 25 µL for serum DNA) and used immediately or stored at -80°C.

### MSPCR

Two sets of primers, described elsewhere<sup>[29]</sup>, were used to discriminate between methylated and unmethylated alleles (Table 2). The PCR system has been described previously<sup>[30]</sup>. Briefly, the PCR mixture containing 2.5 µL of 10 × reaction buffer (100 mmol/L Tris-HCl (pH 8.3), 500 mmol/L KCl, 15 mmol/L MgCl<sub>2</sub>), 10 µL of modified DNA, 15 pmol of each primer (Shenry Biocolor, Shanghai, China), 2 µL of deoxynucleotide triphosphates (200 µmol/L each, final concentration), and 1 U TaKaRa Taq™ polymerase (Hot Start Version, TaKaRa, Shiga, Japan) was adjusted by H<sub>2</sub>O to a final volume of 25 µL. The cycling conditions consisted of an incubation period at 95°C for 15 min, 40 cycles of denaturation at 94°C for 30 s, annealing at 64°C or at 59°C for 50 s (Table 2), extension at 72°C for 30 s, and a final extension at 72°C for 10 min. PCR products were separated in 2% agarose gel and visualized under UV illumination.

Lymphocyte DNA, original or methylated *in vitro* by excessive CpG (Sss I) methylase (New England Biolabs, Beverly, MA, USA), was used as an unmethylated and methylated DNA positive control, respectively (Figure 1A). Water blank was used as a negative control.

### Statistical analysis

We analyzed the correlation between methylation status of serum *RASSF1A* promoter and clinicopathologic parameters. Chi-square test or Fisher's exact test was conducted to examine the association of two categorical variables using SAS software (SAS Institute, Cary, NC, USA). All statistical tests were two-sided, and *P* < 0.05 was considered statistically significant.

## RESULTS

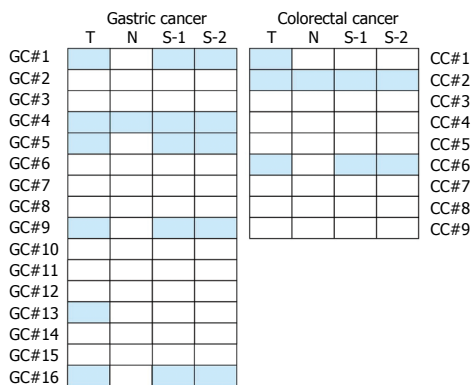
### Serum *RASSF1A* promoter hypermethylation profile in gastric and colorectal adenocarcinoma patients

First we analyzed the methylation status of CpG islands within the *RASSF1A* promoter in serum DNA from 47 gastric adenocarcinoma patients, 45 colorectal adenocarcinoma patients, 60 benign gastrointestinal disease patients (30 with benign gastric disease and 30 with benign colorectal disease), and 30 healthy donors. Hypermethylation of the *RASSF1A* promoter was detected in 16 gastric adeno-

**Table 3** Correlation between serum *RASSF1A* gene promoter methylation status and clinicopathologic parameters in gastric and colorectal adenocarcinoma patients

Clinicopathologic parameters		Gastric cancer			Colorectal cancer		
		<i>RASSF1A</i> promoter status		<i>P</i> value	<i>RASSF1A</i> promoter status		<i>P</i> value
		M	U		M	U	
Sex	Male	9	20	0.5807 <sup>1</sup>	7	17	0.9649 <sup>1</sup>
	Female	7	11		6	15	
Age (yr)	≤ 60	8	13	0.5982 <sup>1</sup>	8	23	0.5024 <sup>2</sup>
	> 60	8	18		5	9	
Differentiation grade	G1/Broders' I	0	2	0.2280 <sup>1</sup>	1	3	0.9830 <sup>1</sup>
	G2/Broders' II	6	17		10	24	
	G3/Broders' III & IV	10	12		2	5	
Surgical resection	Yes	7	20	0.2203 <sup>1</sup>	5	10	0.7325 <sup>2</sup>
	No	9	11		8	22	
Distant metastasis	Yes	7	5	0.0746 <sup>2</sup>	5	5	0.1237 <sup>2</sup>
	No	9	26		8	27	
Serum CEA	Elevated	7	7	0.2365 <sup>2</sup>	6	7	0.1232 <sup>2</sup>
	Normal	3	10		3	14	

<sup>1</sup>Chi-square test; <sup>2</sup>Fisher's exact test. CEA: Carcinoembryonic antigen; M: Methylated; U: Unmethylated.



**Figure 2** Comparison of *RASSF1A* promoter methylation status in tissue and serum samples. For each patient, the *RASSF1A* promoter methylation status was analyzed in tumor tissue (T), adjacent normal tissue (N), preoperative serum (S-1), and postoperative serum collected 4 wk after surgery (S-2). Solid boxes indicate methylation, blank ones indicate unmethylation of *RASSF1A* promoter. GC: Gastric adenocarcinoma; CC: Colorectal adenocarcinoma.

carcinoma patients, 13 colorectal adenocarcinoma patients, 1 benign gastric disease patient (chronic fundal gastritis), and 2 benign colorectal disease patients (both colon adenomas). The representative agarose gel electrophoresis results are shown in Figure 1A. The frequencies of detection of serum *RASSF1A* promoter hypermethylation in gastric (34.0%) and colorectal (28.9%) adenocarcinoma patients were significantly higher than those in benign gastric disease patients (3.3%), benign colorectal disease patients (6.7%) and healthy donors (0%), respectively ( $P < 0.01$ ).

#### ***RASSF1A* promoter hypermethylation profile in paired tissue and serum samples from gastric and colorectal adenocarcinoma patients**

Next we compared the *RASSF1A* promoter methylation status in paired tissue and serum samples from 16 gastric and 9 colorectal adenocarcinoma patients who later were underwent to surgical resection in Jinling Hospital. For each patient, the *RASSF1A* promoter methylation status

was analyzed in tumor tissue, adjacent normal tissue, preoperative serum, and postoperative serum collected 4 wk after surgery. The representative agarose gel electrophoresis results and the paired MSPCR results are shown in Figure 1B and Figure 2, respectively. In seven patients, the *RASSF1A* promoter hypermethylation was detected both in cancer tissue samples and in serum samples. In two patients, the hypermethylated *RASSF1A* promoter was present in tumor tissue samples but not in paired serum samples. The *RASSF1A* promoter hypermethylation was never detected in serum samples if it was not present in tumor tissue. In addition, the preoperative and postoperative serum *RASSF1A* promoter methylation status remained unchanged in all patients.

#### ***Correlation between serum RASSF1A promoter hypermethylation and clinicopathologic parameters in patients with gastric and colorectal adenocarcinoma***

We further analyzed the relationship between serum *RASSF1A* promoter methylation status and clinicopathologic features in gastric and colorectal adenocarcinoma patients. The results are listed in Table 3. As indicated in the table, there was no correlation between *RASSF1A* promoter methylation status and patients' sex, age, tumor differentiation grade, or serum CEA levels. No difference in serum *RASSF1A* promoter hypermethylation frequencies was detected between postoperative patients and those whose tumor was not resected. Although the serum *RASSF1A* promoter hypermethylation frequency tended to be higher in patients with distant metastases, no correlation between methylation status and metastasis was found.

## **DISCUSSION**

*RASSF1A* protein is actively involved in microtubule regulation, genomic stability maintenance, cell-cycle regulation, apoptosis modulation, cell motility and invasion control<sup>[31-39]</sup>. The frequent inactivation of TSG *RASSF1A* due to aberrant promoter methylation has been reported in various tumor types<sup>[13]</sup>, suggesting that it plays a pivotal



role in human cancer development. It was reported that *RASSF1A* is inactivated by promoter hypermethylation in gastric and colorectal cancer, but the frequencies of aberrant *RASSF1A* methylation vary widely<sup>[8,9,14-16,40,41]</sup>. In addition, serum promoter methylation of *RASSF1A* in gastric and colorectal cancer has not been extensively studied, and few comparative studies using both primary tumor and serum samples are available. To our knowledge, there is only one related study with a limited sample size<sup>[10]</sup>. In the present study, we identified the *RASSF1A* promoter methylation status both in serum DNA and in available paired tumor genomic DNA from patients with gastric and colorectal adenocarcinoma, showing that serum *RASSF1A* promoter hypermethylation is a potential biomarker for gastric and colorectal cancer diagnosis.

In the present study, serum *RASSF1A* promoter hypermethylation was detected in 34.0% of patients with gastric adenocarcinoma and in 28.9% of those with colorectal adenocarcinoma. The frequencies were slightly higher than those reported by Tan *et al*<sup>[10]</sup> (25% in gastric cancer and 24% in colorectal cancer, respectively). The serum *RASSF1A* promoter hypermethylation frequencies in gastric and colorectal adenocarcinoma patients were significantly higher than those in patients with benign gastric or colorectal disease or in healthy donors ( $P < 0.01$ ). The sensitivity of serum *RASSF1A* promoter hypermethylation in detecting gastric and colorectal cancer is relatively low. Perhaps a simultaneous analysis of the methylation status of a panel of TSGs would be more sensitive in detecting gastric and colorectal cancer. On the other hand, the specificity of serum *RASSF1A* promoter hypermethylation was very high (approximate 98.3%). Since clinical tests with a high specificity are usually useful in confirming the diagnosis, serum *RASSF1A* promoter methylation status is a potential marker for the diagnosis of gastric and colorectal cancer.

We also compared the *RASSF1A* promoter methylation status in paired tissue and serum samples from 25 gastric and colorectal adenocarcinoma patients. For the seven patients with hypermethylated *RASSF1A* promoter detected in their serum samples, *RASSF1A* promoter hypermethylation was also present in the primary tumor, which supports the presumption that circulating DNA in peripheral blood of cancer patients reflects the epigenetic change in the primary tumor. In two patients, however, hypermethylated *RASSF1A* promoter could be detected in the primary tumor samples but not in the paired serum samples, suggesting that not all cancer patients have detectable tumor-originating DNA in their peripheral blood.

*RASSF1A* promoter hypermethylation was detected in adjacent normal tissue from 2 patients, which can be explained by the invisible invasion of the primary tumor to the adjacent tissue. Another possible reason is the presence of aberrant promoter methylation of TSGs in precancerous lesions adjacent to the primary tumor. Lee *et al*<sup>[9]</sup> reported that *RASSF1A* promoter hypermethylation occurs in 2.1% of colorectal adenomas, and Derks *et al*<sup>[42]</sup> found that aberrant *RASSF1A* promoter methylation is present in 19.1% of non-progressed adenomas and in 24.4% of progressive adenomas. In our study, we also detected methylated *RASSF1A* promoter in the serum from one patient with chronic fundal gastritis and two patients with colon adenoma, believed to be precancerous lesions in gastric and colon cancer, respectively.

These findings suggest that aberrant promoter hypermethylation of *RASSF1A* might be an early event in the development of gastric and colorectal cancer. Therefore, identification of serum *RASSF1A* promoter methylation status may contribute to the early diagnosis of gastric and colorectal cancer.

In the present study, no association was observed between *RASSF1A* promoter methylation status and patients' sex, age, tumor differentiation grade, distal metastasis, or surgical therapy. We also compared the methylation status of *RASSF1A* promoter in preoperative and postoperative serum samples from patients who were underwent to surgical therapy in our hospital, and the status remained unchanged in all patients. Theoretically, when the primary tumor is resected, tumor-specific methylated DNA would decrease considerably in peripheral blood. However, this does not seem to be the case. Fiegl *et al*<sup>[43]</sup> monitored the serum *RASSF1A* promoter methylation status in 148 breast cancer patients for up to 1 year after surgery, and only 21 patients showed positive to negative transition in MSPCR analysis of serum *RASSF1A* promoter. A possible source of persistently present methylated copy after surgery is the micrometastases that may present before surgery.

We investigated whether serum *RASSF1A* promoter hypermethylation is correlated with elevated serum CEA levels and found that there is no correlation between them. Koike *et al*<sup>[44]</sup> reported that the detection rate of TSG (*p16*, *E-cadherin*, and *RARβ*) hypermethylation is higher than that of conventional tumor marker (CEA and CA19-9) abnormalities in the serum from gastric cancer patients, and that there is no correlation between them. Since serum CEA and TSG hypermethylation are not correlated, a combinational analysis of serum *RASSF1A* promoter methylation status and serum CEA level may be useful in the diagnosis of gastric and colorectal cancer.

In conclusion, serum *RASSF1A* promoter hypermethylation is common in gastric and colorectal adenocarcinoma and aberrant CpG island methylation within the promoter region of *RASSF1A* is a promising biomarker for such cancers.

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## COMMENTS

### Background

*RASSF1A* inactivation by promoter hypermethylation in gastric and colorectal cancer has been reported. However, serum promoter methylation of *RASSF1A* in gastric and colorectal cancer has not been extensively studied. Particularly, comparative studies using both primary tumor and serum samples are indicated can evaluate the diagnostic role of serum *RASSF1A* promoter hypermethylation in gastric and colorectal cancer.

### Research frontiers

Circulating nucleotide acid is a hotspot in the early diagnosis of cancer. Characterization of molecular changes in serum DNA reflecting the genetic and

epigenetic alterations in primary tumor would provide an alternative approach to the early detection of cancer.

### Innovations and breakthroughs

This is the first comprehensive study on *RASSF1A* promoter hypermethylation status both in tumor and normal tissue samples and in pre- and post serum samples from gastric and colorectal cancer patients. Our results indicate that aberrant hypermethylation of *RASSF1A* promoter is a promising serum biomarker for gastric and colorectal cancer diagnosis.

### Applications

A combined study on promoter hypermethylation of a panel of relevant tumor suppressor genes in serum samples may have a bright future in the early diagnosis of gastric and colorectal cancer.

### Terminology

In DNA, methylation is the addition of a methyl group to a cytosine residue to convert it to 5-methylcytosine. DNA methylation is the main epigenetic modification in humans, and changes in methylation patterns play an important role in tumorigenesis. In particular, hypermethylation of normally unmethylated CpG islands in the promoter region of tumor suppressor genes correlates with their loss of expression and may confer growth advantages to those cells that favor cancer development.

### Peer review

This paper is very interesting. The study is well designed. The authors evaluated the role of serum *RASSF1A* promoter hypermethylation in diagnosing gastric and colorectal adenocarcinoma, showing that aberrant CpG island methylation within the promoter region of *RASSF1A* is a promising biomarker for gastric and colorectal cancer.

## REFERENCES

- 1 Macdonald JS. Carcinoembryonic antigen screening: pros and cons. *Semin Oncol* 1999; **26**: 556-560
- 2 Jones PA, Baylin SB. The fundamental role of epigenetic events in cancer. *Nat Rev Genet* 2002; **3**: 415-428
- 3 Baylin SB, Herman JG, Graff JR, Vertino PM, Issa JP. Alterations in DNA methylation: a fundamental aspect of neoplasia. *Adv Cancer Res* 1998; **72**: 141-196
- 4 Lee TL, Leung WK, Chan MW, Ng EK, Tong JH, Lo KW, Chung SC, Sung JJ, To KF. Detection of gene promoter hypermethylation in the tumor and serum of patients with gastric carcinoma. *Clin Cancer Res* 2002; **8**: 1761-1766
- 5 Kim H, Kim YH, Kim SE, Kim NG, Noh SH, Kim H. Concerted promoter hypermethylation of hMLH1, p16INK4A, and E-cadherin in gastric carcinomas with microsatellite instability. *J Pathol* 2003; **200**: 23-31
- 6 Tamura G. Alterations of tumor suppressor and tumor-related genes in the development and progression of gastric cancer. *World J Gastroenterol* 2006; **12**: 192-198
- 7 Zhao YF, Zhang YG, Tian XX, Juan Du, Jie Zheng. Aberrant methylation of multiple genes in gastric carcinomas. *Int J Surg Pathol* 2007; **15**: 242-251
- 8 Xu XL, Yu J, Zhang HY, Sun MH, Gu J, Du X, Shi DR, Wang P, Yang ZH, Zhu JD. Methylation profile of the promoter CpG islands of 31 genes that may contribute to colorectal carcinogenesis. *World J Gastroenterol* 2004; **10**: 3441-3454
- 9 Lee S, Hwang KS, Lee HJ, Kim JS, Kang GH. Aberrant CpG island hypermethylation of multiple genes in colorectal neoplasia. *Lab Invest* 2004; **84**: 884-893
- 10 Tan SH, Ida H, Lau QC, Goh BC, Chieng WS, Loh M, Ito Y. Detection of promoter hypermethylation in serum samples of cancer patients by methylation-specific polymerase chain reaction for tumour suppressor genes including RUNX3. *Oncol Rep* 2007; **18**: 1225-1230
- 11 Dammann R, Li C, Yoon JH, Chin PL, Bates S, Pfeifer GP. Epigenetic inactivation of a RAS association domain family protein from the lung tumour suppressor locus 3p21.3. *Nat Genet* 2000; **25**: 315-319
- 12 Pfeifer GP, Dammann R. Methylation of the tumor suppressor gene *RASSF1A* in human tumors. *Biochemistry (Mosc)* 2005; **70**: 576-583
- 13 Dammann R, Schagdarsurengin U, Seidel C, Strunnikova M, Rastetter M, Baier K, Pfeifer GP. The tumor suppressor *RASSF1A* in human carcinogenesis: an update. *Histol Histopathol* 2005; **20**: 645-663
- 14 Byun DS, Lee MG, Chae KS, Ryu BG, Chi SG. Frequent epigenetic inactivation of *RASSF1A* by aberrant promoter hypermethylation in human gastric adenocarcinoma. *Cancer Res* 2001; **61**: 7034-7038
- 15 Wagner KJ, Cooper WN, Grundy RG, Caldwell G, Jones C, Wadey RB, Morton D, Schofield PN, Reik W, Latif F, Maher ER. Frequent *RASSF1A* tumour suppressor gene promoter methylation in Wilms' tumour and colorectal cancer. *Oncogene* 2002; **21**: 7277-7282
- 16 van Engeland M, Roemen GM, Brink M, Pachen MM, Weijnenberg MP, de Bruine AP, Arends JW, van den Brandt PA, de Goeij AF, Herman JG. K-ras mutations and *RASSF1A* promoter methylation in colorectal cancer. *Oncogene* 2002; **21**: 3792-3795
- 17 Oliveira C, Velho S, Domingo E, Preto A, Hofstra RM, Hamelin R, Yamamoto H, Seruca R, Schwartz S Jr. Concomitant *RASSF1A* hypermethylation and *KRAS*/*BRAF* mutations occur preferentially in MSI sporadic colorectal cancer. *Oncogene* 2005; **24**: 7630-7634
- 18 Leon SA, Shapiro B, Sklaroff DM, Yaros MJ. Free DNA in the serum of cancer patients and the effect of therapy. *Cancer Res* 1977; **37**: 646-650
- 19 Stroun M, Anker P, Maurice P, Lyautey J, Lederrey C, Beljanski M. Neoplastic characteristics of the DNA found in the plasma of cancer patients. *Oncology* 1989; **46**: 318-322
- 20 Camps C, Sirera R, Bremnes R, Blasco A, Sancho E, Bayo P, Safont MJ, Sanchez JJ, Taron M, Rosell R. Is there a prognostic role of K-ras point mutations in the serum of patients with advanced non-small cell lung cancer? *Lung Cancer* 2005; **50**: 339-346
- 21 Gotoh T, Hosoi H, Iehara T, Kuwahara Y, Osone S, Tsuchiya K, Ohira M, Nakagawara A, Kuroda H, Sugimoto T. Prediction of MYCN amplification in neuroblastoma using serum DNA and real-time quantitative polymerase chain reaction. *J Clin Oncol* 2005; **23**: 5205-5210
- 22 Cuda G, Gallelli A, Nistico A, Tassone P, Barbieri V, Tagliaferri PS, Costanzo FS, Tranfa CM, Venuta S. Detection of microsatellite instability and loss of heterozygosity in serum DNA of small and non-small cell lung cancer patients: a tool for early diagnosis? *Lung Cancer* 2000; **30**: 211-214
- 23 Nawroz-Danish H, Eisenberger CF, Yoo GH, Wu L, Koch W, Black C, Ensley JF, Wei WZ, Sidransky D. Microsatellite analysis of serum DNA in patients with head and neck cancer. *Int J Cancer* 2004; **111**: 96-100
- 24 Fujiwara K, Fujimoto N, Tabata M, Nishii K, Matsuo K, Hotta K, Kozuki T, Aoe M, Kiura K, Ueoka H, Tanimoto M. Identification of epigenetic aberrant promoter methylation in serum DNA is useful for early detection of lung cancer. *Clin Cancer Res* 2005; **11**: 1219-1225
- 25 Ramirez JL, Sarries C, de Castro PL, Roig B, Queralt C, Escuin D, de Aguirre I, Sanchez JM, Manzano JL, Margeli M, Sanchez JJ, Astudillo J, Taron M, Rosell R. Methylation patterns and K-ras mutations in tumor and paired serum of resected non-small-cell lung cancer patients. *Cancer Lett* 2003; **193**: 207-216
- 26 Yamaguchi S, Asao T, Nakamura J, Ide M, Kuwano H. High frequency of DAP-kinase gene promoter methylation in colorectal cancer specimens and its identification in serum. *Cancer Lett* 2003; **194**: 99-105
- 27 Sobin LH, Wittekind C. TNM Classification of Malignant Tumours, 6th Edition. New York: Wiley-Liss, 2002
- 28 Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996; **93**: 9821-9826
- 29 Burbee DG, Forgacs E, Zochbauer-Muller S, Shivakumar L, Fong K, Gao B, Randle D, Kondo M, Virmani A, Bader S, Sekido Y, Latif F, Milchgrub S, Toyooka S, Gazdar AF, Lerman MI, Zabarovsky E, White M, Minna JD. Epigenetic inactivation of

- RASSF1A in lung and breast cancers and malignant phenotype suppression. *J Natl Cancer Inst* 2001; **93**: 691-699
- 30 **Wang Y**, Yu Z, Wang T, Zhang J, Hong L, Chen L. Identification of epigenetic aberrant promoter methylation of RASSF1A in serum DNA and its clinicopathological significance in lung cancer. *Lung Cancer* 2007; **56**: 289-294
- 31 **Donninger H**, Vos MD, Clark GJ. The RASSF1A tumor suppressor. *J Cell Sci* 2007; **120**: 3163-3172
- 32 **Liu L**, Tommasi S, Lee DH, Dammann R, Pfeifer GP. Control of microtubule stability by the RASSF1A tumor suppressor. *Oncogene* 2003; **22**: 8125-8136
- 33 **Vos MD**, Martinez A, Elam C, Dallol A, Taylor BJ, Latif F, Clark GJ. A role for the RASSF1A tumor suppressor in the regulation of tubulin polymerization and genomic stability. *Cancer Res* 2004; **64**: 4244-4250
- 34 **Shivakumar L**, Minna J, Sakamaki T, Pestell R, White MA. The RASSF1A tumor suppressor blocks cell cycle progression and inhibits cyclin D1 accumulation. *Mol Cell Biol* 2002; **22**: 4309-4318
- 35 **Whang YM**, Kim YH, Kim JS, Yoo YD. RASSF1A suppresses the c-Jun-NH2-kinase pathway and inhibits cell cycle progression. *Cancer Res* 2005; **65**: 3682-3690
- 36 **Vos MD**, Ellis CA, Bell A, Birrer MJ, Clark GJ. Ras uses the novel tumor suppressor RASSF1 as an effector to mediate apoptosis. *J Biol Chem* 2000; **275**: 35669-35672
- 37 **Vos MD**, Dallol A, Eckfeld K, Allen NP, Donninger H, Hesson LB, Calvisi D, Latif F, Clark GJ. The RASSF1A tumor suppressor activates Bax via MOAP-1. *J Biol Chem* 2006; **281**: 4557-4563
- 38 **Matallanas D**, Romano D, Yee K, Meissl K, Kucerova L, Piazolla D, Baccarini M, Vass JK, Kolch W, O'Neill E. RASSF1A elicits apoptosis through an MST2 pathway directing proapoptotic transcription by the p73 tumor suppressor protein. *Mol Cell* 2007; **27**: 962-975
- 39 **Dallol A**, Agathangelou A, Tommasi S, Pfeifer GP, Maher ER, Latif F. Involvement of the RASSF1A tumor suppressor gene in controlling cell migration. *Cancer Res* 2005; **65**: 7653-7659
- 40 **To KF**, Leung WK, Lee TL, Yu J, Tong JH, Chan MW, Ng EK, Chung SC, Sung JJ. Promoter hypermethylation of tumor-related genes in gastric intestinal metaplasia of patients with and without gastric cancer. *Int J Cancer* 2002; **102**: 623-628
- 41 **Ye M**, Xia B, Guo Q, Zhou F, Zhang X. Association of diminished expression of RASSF1A with promoter methylation in primary gastric cancer from patients of central China. *BMC Cancer* 2007; **7**: 120
- 42 **Derks S**, Postma C, Moerkerk PT, van den Bosch SM, Carvalho B, Hermesen MA, Giaretti W, Herman JG, Weijnenberg MP, de Bruine AP, Meijer GA, van Engeland M. Promoter methylation precedes chromosomal alterations in colorectal cancer development. *Cell Oncol* 2006; **28**: 247-257
- 43 **Fiegl H**, Millinger S, Mueller-Holzner E, Marth C, Ensinger C, Berger A, Klocker H, Goebel G, Widschwendter M. Circulating tumor-specific DNA: a marker for monitoring efficacy of adjuvant therapy in cancer patients. *Cancer Res* 2005; **65**: 1141-1145
- 44 **Koike H**, Ichikawa D, Ikoma H, Tani N, Ikoma D, Otsuji E, Okamoto K, Ueda Y, Kitamura K, Yamagishi H. Comparison of serum aberrant methylation and conventional tumor markers in gastric cancer patients. *Hepatogastroenterology* 2005; **52**: 1293-1296

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## Reoperation of biliary tract by laparoscopy: Experiences with 39 cases

Li-Bo Li, Xiu-Jun Cai, Yi-Ping Mou, Qi Wei

Li-Bo Li, Xiu-Jun Cai, Yi-Ping Mou, Qi Wei, Department of General Surgery, Sir Run Run Shaw Hospital, Institute of Microinvasive Surgery, Medical College of Zhejiang University, Hangzhou, No. 3 East Qingchun Road, Hangzhou 310016, Zhejiang Province, China

**Author contributions:** Li LB designed the research; Li LB, Cai XJ, Mou YP, Wei Q performed the research and contributed to reagents, materials and analytic work; Li LB and Cai XJ analyzed the data; Li LB wrote the paper.

**Correspondence to:** Li-Bo Li, MD, Department of General Surgery, Sir Run Run Shaw Hospital, Medical college of Zhejiang University, No. 3 East Qingchun Road, Hangzhou 310016, Zhejiang Province, China. [lilb@srrsh.com](mailto:lilb@srrsh.com)

Telephone: +86-571-86995056 Fax: +86-571-86044822

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laparoscopic surgeons, and is an alternative choice for patients with choledocholithiasis who fail in endoscopic sphincterectomy.

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### Abstract

**AIM:** To evaluate the safety and feasibility of biliary tract reoperation by laparoscopy for the patients with retained or recurrent stones who failed in endoscopic sphincterotomy.

**METHODS:** A retrospective analysis of data obtained from attempted laparoscopic reoperation for 39 patients in a single institution was performed, examining open conversion rates, operative times, complications, and hospital stay.

**RESULTS:** Out of the 39 cases, 38 (97%) completed laparoscopy, 1 required conversion to open operation because of difficulty in exposing the common bile duct. The mean operative time was 135 min. The mean post-operative hospital stay was 4 d. Procedures included laparoscopic residual gallbladder resection in 3 cases, laparoscopic common bile duct exploration and primary duct closure at choledochotomy in 13 cases, and laparoscopic common bile duct exploration and choledochotomy with T tube drainage in 22 cases. Duodenal perforation occurred in 1 case during dissection and was repaired laparoscopically. Retained stones were found in 2 cases. Postoperative asymptomatic hyperamylasemia occurred in 3 cases. There were no complications due to port placement, postoperative bleeding, bile or bowel leakage and mortality. No recurrence or formation of duct stricture was observed during a mean follow-up period of 18 mo.

**CONCLUSION:** Laparoscopic biliary tract reoperation is safe and feasible if it is performed by experienced

### INTRODUCTION

In the past, laparoscopic surgery was contraindicated for patients undergone any prior abdominal surgery. With the advances in laparoscopic instrumentation and skills, increasingly complex procedures can be performed for patients with or without prior operations<sup>[1-5]</sup>. Prior open biliary surgery in particular is associated with difficulty in placing the initial trocar and obtaining adequate exposure of the biliary tract. Two major concerns that have prevented surgeons from using a laparoscopic approach when performing a repeated biliary tract surgery include the risk of injury to organs adherent to the abdominal wall when Veress needle or trocar is inserted, and the complications associated with adhesiolysis. With the increased experience in our institution, we have attempted laparoscopic surgery for patients with retained or recurrent stones who failed in endoscopic sphincterotomy. We reviewed the data collected from our cases to study the effect of prior biliary surgery on biliary tract reoperation using laparoscopy.

### MATERIALS AND METHODS

#### Patients

Laparoscopic cholecystectomy was introduced in our institution in 1993. Based on the experiences with 16 605 laparoscopic cholecystectomies, 658 laparoscopic common bile duct explorations, and 851 laparoscopic



Table 1 Diagnosis and prior surgery of 39 patients

Diagnosis	Prior surgery			
	LC	OC	OC+ CBDE	OC+CBDE+left lateral lobectomy
Stones in residual gallbladder	1	2		
Stones in CBD		22	11	3

LC: Laparoscopic cholecystectomy; OC: Open cholecystectomy; CBDE: Common bile duct exploration; CBD: Common bile duct.

cholecystectomies for patients with prior upper or lower abdominal surgery, we attempted laparoscopic biliary tract reoperation for patients with retained or recurrent stones who failed in endoscopic sphincterotomy.

A total of 39 patients including 26 females and 13 males, with a mean age of 46.4 years (ranging 13-76 years) were underwent to laparoscopic biliary tract reoperations by two surgical teams between January 2001 and June 2007. Retained or recurrent stones were found at a prior biliary surgery for biliary stones. None of them had any other previous abdominal surgery. A prior surgery was performed at other hospitals for 36 of them. The time between prior surgery and reoperation ranged from 7 d to 28 years, with a mean time of 2 years. Right subcostal scars were present in 18 cases, while midline or right paramidline scars were present in 21 cases. The diagnosis and prior surgery history of the 39 cases are listed in Table 1.

Diagnosis of retained stones or recurrent stones was made by pre-operative ultrasonography, CT, and MRCP. Endoscopic sphincterotomy failed or was contraindicated in the 39 cases. As the study was begun at a time when our experience with endoscopic sphincterotomy was limited, endoscopic sphincterotomy was either contraindicated or failed due to stones greater than 1.5 cm in diameter in 16 cases, the presence of more than four stones in 12 cases, tortuous ducts in 4 cases, and periampullary duodenal diverticula in 7 cases, respectively. There were no contraindications for general anesthesia. The diameter of the common bile duct ranged from 1 cm to 2.2 cm in 36 cases of choledocholithiasis. Biliary stricture or neoplasms were ruled out by radiological examination and serological tumor markers.

### Operative procedure

General endotracheal anesthesia was used. The abdominal cavity was accessed near the umbilicus. If the previous scar was more than 3 cm from the umbilicus, the blind technique was used to insert the Veress needle. If the scar was less than 3 cm from the umbilicus, the open (Hasson) technique was used. Adhesions under the umbilical incision were dissected using blunt finger dissection.

After pneumoperitoneum was established, intraperitoneal adhesions were evaluated by a 30-degree angled laparoscopy. A 5 mm port was placed under direct vision into the right or left lower abdomen, 5 cm from the adhesions, allowing dissection of the prior surgical adhesions located below the scar using scissors, a harmonic scalpel. One 10 mm operative port and two 5 mm accessory ports were placed as a standard four-trocar

Table 2 Results of laparoscopic biliary tract reoperation for 39 cases

	Laparoscopic biliary tract reoperation (n = 39)
Mean operating time (min)	135 (45-185)
Conversion rate	1 (2.5%)
Postoperative hospital stay (d)	4 (1-6)
Intra-operative complication rate	2.5% (1/39)
Post-operative complication rate	5.1% (2/39)

technique of laparoscopic cholecystectomy.

To approach the hepatic-duodenal ligament, we freed the lateral parietes and then began dissection on the right side along the lateral inferior border of the liver, dissecting the adhesions on the right side of hepatic round ligament down to the hepatic-duodenal ligament. The common bile duct was identified by touching the stones, needle aspiration of bile from the duct, or by laparoscopic ultrasound.

After identification of the common bile duct, choledochotomy was performed. Stones in the common bile duct were retrieved by spontaneous evacuation at the incision of the duct, instrumental exploration with forceps, flushing of the common bile duct with saline, or Fogarty balloon catheter. Next, a fifth port (10 mm) was placed at the right subcostal margin, just above the gallbladder, through which a 5.0 mm fiberoptic choledochoscope (Olympus) was inserted to check the biliary duct and remove the stones.

As long as choledochoscopy certified a patent common bile duct and absence of stones, the incision was closed using absorbable 4/0 sutures with a running suture and intracorporeal knotting, otherwise a T-tube was placed for drainage, and intraoperative cholangiography was performed through the T tube. A No. 10 Jackson-Pratt drain tube was placed in the subhepatic space for all patients.

## RESULTS

Of the 39 cases, 38 were underwent to laparoscopic operation and 1 was converted to an open operation because of difficulty in exposing the common bile duct. The mean operative time was 135 min (range, 45-185 min) and the mean postoperative hospital stay was 4 d (ranging 1-6 d, Table 2). Procedures included laparoscopic residual gallbladder resection in 3 cases, laparoscopic common bile duct exploration and primary duct closure at choledochotomy in 13 cases and laparoscopic common bile duct exploration and choledochotomy with T tube drainage in 22 cases. The mean number of removed stones was 3 (ranging 1-15) and the mean diameter of removed stones was 1 cm (ranging 1-2.6 cm). The mean time of T tube drainage was 38 d (ranging 28-47 d).

There were no complications due to port placement. In one patient with a history of open cholecystectomy and common bile duct exploration, the duodenum perforation occurred during dissection was repaired laparoscopically. There were no mortality, postoperative bleeding, bile

or bowel leakage in any of the 38 cases. Asymptomatic hyperamylasemia present in 3 cases postoperatively was treated with conservative therapy. Retained stones found in 2 cases were removed by choledochoscopy through the sinus tract of the T tube. No recurrent stones or duct stricture formation was found during a mean follow-up period of 18 mo.

## DISCUSSION

Most patients with common bile duct stones are cured by minimally invasive endoscopic sphincterotomy<sup>[6-10]</sup>. In the absence of a remaining T-tube from a prior operation, endoscopic sphincterotomy is considered the procedure of choice for patients with retained or recurrent stones, and should be attempted before pursuing biliary tract reoperation. However, endoscopic sphincterotomy cannot be performed, and is itself associated with a significant morbidity<sup>[11-15]</sup>. Contraindications for endoscopic sphincterotomy, as mentioned above, include size of stones, number of stones, presence of tortuous ducts or presence of periampullary duodenal diverticula, *etc* and vary depending on institutional and individual techniques and experiences. With the advances in laparoscopic skills and instrumentation, laparoscopic common bile duct exploration<sup>[16-20]</sup> and other laparoscopic procedures have become an increasingly popular option for patients undergone any prior abdominal surgery<sup>[21-25]</sup>, making laparoscopic reoperation of the biliary tract a reasonable choice for patients with a history of prior biliary surgery who have failed in endoscopic sphincterotomy. The results of our study indicate that laparoscopic surgery was not only minimally invasive, but also safe and feasible in cases of biliary tract reoperation, suggesting that it is the best method for patients who have failed in endoscopic sphincterotomy.

A primary concern when considering laparoscopic reoperation is the formation of adhesions after abdominal surgery, particularly after open biliary surgery. Adhesions from prior surgery are associated with difficulty in establishing pneumoperitoneum, placing the initial trocar, and obtaining adequate exposure of the biliary tract. To avoid the potential risk of injury to organs adherent to either the abdominal wall or the previous operative field, certain techniques and principles should be followed during Veress needle and trocar insertion as well as adhesiolysis.

Safe establishment of pneumoperitoneum and placement of an initial trocar are the prerequisite to any laparoscopic biliary tract reoperation and related with half of the complications of laparoscopic surgery<sup>[26-29]</sup>. In our study, blind Veress needle and initial trocar insertion more than 3 cm from the previous scar were safe for patients with previous biliary surgery. The open Hasson procedure performed in a previously unoperated field can avoid potential underlying adhesions or injury. In our study, no complications were related to the entrance into the peritoneum, indicating that previous biliary surgery is not a contraindication for minimally invasive procedures.

After access has been achieved, sufficient adhesiolysis

should be performed to allow the insertion of a second port to aid in visualization, retraction and dissection, and to allow for additional ports as needed. The laparoscope can be moved to different port sites without the need to perform total adhesiolysis of all visible adhesions. Only the adhesions interfering with adequate access to the operative field or the performance of the procedure need to be lysed. Adhesions close to the abdominal wall should be dissected to avoid injury to the intestine. By using a harmonic scalpel to dissect adhesions, the operative time can be reduced, thus decreasing blood loss<sup>[30]</sup>.

Once the gallbladder has been removed or the common bile duct has been explored, dense adhesions are usually found during reoperation in the healed fossa and near the common duct. In many instances, the upper edge of the duodenum is tented sharply cephalad into the gallbladder fossa. At times, because it is difficult to recognize the anatomy or identify the common bile duct, one should approach to the hepatic hilum by freeing the lateral parietes, and then begin dissection on the right side along the lateral inferior border of the liver. This gives a better mobility of structures so the hepatic flexure of the colon and the lateral edge of the second part of the duodenum can be identified before beginning dissection in the area of dense adhesions. The adhesions on the right side of the hepatic round ligament should be dissected from Glisson's capsule down to the hepatic-duodenal ligament. When adhesions are dissected from Glisson's capsule, attempts at blunt dissection with heavy retraction can easily avulse the capsule and expose the bleeding liver parenchyma. Consequently, careful sharp dissection is a more expedient technique. To prevent thermal injury of the gastrointestinal tract, electrical cautery should be avoided. After exposure of the hepatic-duodenal ligament, the common bile duct can be identified by touching the stones and needle aspiration of bile or by laparoscopic ultrasound.

In summary, laparoscopic biliary tract reoperation has a reasonable operating time, low conversion rate, low intra-operative and postoperative complication rate, and short postoperative hospital stay. Given these results, a laparoscopic approach to biliary tract reoperation appears to be a minimally invasive, safe, feasible, and effective procedure when done by expert laparoscopic surgeons, and is a first choice of treatment for patients who have failed in endoscopic sphincterotomy.

## COMMENTS

### Background

In the past, a history of prior biliary tract surgery was considered a contraindication for performing a repeat biliary operation. In the absence of a remaining T-tube from a prior operation, endoscopic sphincterotomy is considered the procedure of choice for patients with retained or recurrent stones, and should be attempted before pursuing biliary tract reoperation. However, endoscopic sphincterotomy cannot be performed on everyone, and is itself associated with a significant morbidity. With the advances in laparoscopic skills and instrumentation, increasingly complex procedures have been performed in patients with or without prior operations.

### Research frontiers

It has previously been reported that laparoscopic common bile duct (CBD)

exploration is a common method for the management of choledocholithiasis, and laparoscopic procedures are safe for patients undergone prior abdominal surgery. Few studies are available on the safety and feasibility of reoperation of biliary tract by laparoscopy for the patients with retained or recurrent stones who have failed in whom endoscopic sphincterotomy.

### Innovations and breakthroughs

This study showed laparoscopic biliary tract reoperation appears to be a minimally invasive, safe, feasible, and effective method when done by expert laparoscopic surgeons.

### Applications

Laparoscopic biliary tract reoperation is an alternative method for patients with choledocholithiasis who have failed in endoscopic sphincterectomy.

### Peer review

The authors describe, in this paper, their experience in laparoscopic biliary tract reoperation, which is of a certain clinical value.

## REFERENCES

- Cai XJ, Yu H, Liang X, Wang YF, Zheng XY, Huang DY, Peng SY. Laparoscopic hepatectomy by curettage and aspiration. Experiences of 62 cases. *Surg Endosc* 2006; **20**: 1531-1535
- Karayiannakis AJ, Polychronidis A, Perente S, Botaitis S, Simopoulos C. Laparoscopic cholecystectomy in patients with previous upper or lower abdominal surgery. *Surg Endosc* 2004; **18**: 97-101
- Palanivelu C, Jani K, Senthilnathan P, Parthasarathi R, Rajapandian S, Madhankumar MV. Laparoscopic pancreaticoduodenectomy: technique and outcomes. *J Am Coll Surg* 2007; **205**: 222-230
- Hur H, Jeon HM, Kim W. Laparoscopic pancreas- and spleen-preserving D2 lymph node dissection in advanced (cT2) upper-third gastric cancer. *J Surg Oncol* 2008; **97**: 169-172
- Donati M, Memming M, Donati A, Calò PG, Nicolosi A. [Indications and limits of laparoscopic treatment for diverticular disease of the colon: personal experience] *Chir Ital* 2008; **60**: 63-73
- Escourrou J, Cordova JA, Lazorthes F, Frexinos J, Ribet A. Early and late complications after endoscopic sphincterotomy for biliary lithiasis with and without the gall bladder 'in situ'. *Gut* 1984; **25**: 598-602
- Leese T, Neoptolemos JP, Carr-Locke DL. Successes, failures, early complications and their management following endoscopic sphincterotomy: results in 394 consecutive patients from a single centre. *Br J Surg* 1985; **72**: 215-219
- Heo JH, Kang DH, Jung HJ, Kwon DS, An JK, Kim BS, Suh KD, Lee SY, Lee JH, Kim GH, Kim TO, Heo J, Song GA, Cho M. Endoscopic sphincterotomy plus large-balloon dilation versus endoscopic sphincterotomy for removal of bile-duct stones. *Gastrointest Endosc* 2007; **66**: 720-726; quiz 768, 771
- Teoh AY, Poon MC, Leong HT. Role of prophylactic endoscopic sphincterotomy in patients with acute biliary pancreatitis due to transient common bile duct obstruction. *J Gastroenterol Hepatol* 2007; **22**: 1415-1418
- Wojtun S, Gil J, Gietka W, Gil M. Endoscopic sphincterotomy for choledocholithiasis: a prospective single-center study on the short-term and long-term treatment results in 483 patients. *Endoscopy* 1997; **29**: 258-265
- Tranter SE, Thompson MH. Comparison of endoscopic sphincterotomy and laparoscopic exploration of the common bile duct. *Br J Surg* 2002; **89**: 1495-1504
- Kim HJ, Choi HS, Park JH, Park DI, Cho YK, Sohn CI, Jeon WK, Kim BI, Choi SH. Factors influencing the technical difficulty of endoscopic clearance of bile duct stones. *Gastrointest Endosc* 2007; **66**: 1154-1160
- Szyca R, Tomaszewski S, Jasiński A, Leksowski K. [Late complication of endoscopic sphincterotomy] *Pol Merkuriusz Lekarski* 2007; **22**: 414-415
- Cheon YK, Lehman GA. Identification of risk factors for stone recurrence after endoscopic treatment of bile duct stones. *Eur J Gastroenterol Hepatol* 2006; **18**: 461-464
- Lai KH, Peng NJ, Lo GH, Cheng JS, Huang RL, Lin CK, Huang JS, Chiang HT, Ger LP. Prediction of recurrent choledocholithiasis by quantitative cholescintigraphy in patients after endoscopic sphincterotomy. *Gut* 1997; **41**: 399-403
- Decker G, Borie F, Millat B, Berthou JC, Deleuze A, Drouard F, Guillon F, Rodier JG, Fingerhut A. One hundred laparoscopic choledochotomies with primary closure of the common bile duct. *Surg Endosc* 2003; **17**: 12-18
- Petelin JB. Laparoscopic common bile duct exploration. *Surg Endosc* 2003; **17**: 1705-1715
- Paganini AM, Feliciotti F, Guerrieri M, Tamburini A, Campagnacci R, Lezoche E. Laparoscopic cholecystectomy and common bile duct exploration are safe for older patients. *Surg Endosc* 2002; **16**: 1302-1308
- Topal B, Aerts R, Penninckx F. Laparoscopic common bile duct stone clearance with flexible choledochoscopy. *Surg Endosc* 2007; **21**: 2317-2321
- Gholipour C, Shalchi RA, Abassi M. Efficacy and safety of early laparoscopic common bile duct exploration as primary procedure in acute cholangitis caused by common bile duct stones. *J Laparoendosc Adv Surg Tech A* 2007; **17**: 634-638
- Chen B, Hu SY, Wang L, Wang KX, Zhang GY, Zhang HF. Reoperation of biliary tract by laparoscopy: a consecutive series of 26 cases. *Acta Chir Belg* 2007; **107**: 292-296
- Dexter SP, Miller GV, Davides D, Martin IG, Sue Ling HM, Sagar PM, Larvin M, McMahon MJ. Relaparoscopy for the detection and treatment of complications of laparoscopic cholecystectomy. *Am J Surg* 2000; **179**: 316-319
- Kwon AH, Inui H, Imamura A, Kaibori M, Kamiyama Y. Laparoscopic cholecystectomy and choledocholithotomy in patients with a previous gastrectomy. *J Am Coll Surg* 2001; **193**: 614-619
- Ballesta Lopez C, Ruggiero R, Poves I, Bettonica C, Procaccini E, Corsale I, Mandato M, De Luca L. Laparoscopic procedures in patients who have previously undergone laparotomic operations. *Minerva Chir* 2003; **58**: 53-56
- Leister I, Becker H. [Relaparoscopy as an alternative to laparotomy for laparoscopic complications] *Chirurg* 2006; **77**: 986-997
- Chandler JG, Corson SL, Way LW. Three spectra of laparoscopic entry access injuries. *J Am Coll Surg* 2001; **192**: 478-490; discussion 490-491
- Johnston K, Rosen D, Cario G, Chou D, Carlton M, Cooper M, Reid G. Major complications arising from 1265 operative laparoscopic cases: a prospective review from a single center. *J Minim Invasive Gynecol* 2007; **14**: 339-344
- Altun H, Banli O, Kavlakoglu B, Kavlakoglu B, Kelesoglu C, Erez N. Comparison between direct trocar and Veress needle insertion in laparoscopic cholecystectomy. *J Laparoendosc Adv Surg Tech A* 2007; **17**: 709-712
- Marakis GN, Pavlidis TE, Ballas K, Aimonioutou E, Psarras K, Karvounaris D, Rafailidis S, Demertzidis H, Sakantamis AK. Major complications during laparoscopic cholecystectomy. *Int Surg* 2007; **92**: 142-146
- Langer C, Markus P, Liersch T, Füzesi L, Becker H. UltraCision or high-frequency knife in transanal endoscopic microsurgery (TEM)? Advantages of a new procedure. *Surg Endosc* 2001; **15**: 513-517

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# Is infliximab safe to use while breastfeeding?

Joel Z Stengel, Hays L Arnold

Joel Z Stengel, Hays L Arnold, Brooke Army Medical Center, 3851 Roger Brooke Drive, Fort Sam Houston, TX 78234, United States

Author contributions: Stengel JZ and Arnold HL contributed equally to this work.

Correspondence to: Joel Z Stengel, MD, Gastroenterology Service, Brooke Army Medical Center, 3851 Roger Brooke Drive, Fort Sam Houston, TX 78234

United States. [joel.stengel@us.army.mil](mailto:joel.stengel@us.army.mil)

Telephone: +1-210-916-5244 Fax: +1-210-916-3195

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## Abstract

Inflammatory bowel disease (IBD) often affects women around the age of conception and pregnancy. Most drugs used to treat IBD are safe in pregnancy, but physicians must consider the clinical implications of certain treatment regimens in young, fertile females. We report an informative case of a pregnant patient with IBD who underwent treatment with infliximab during her pregnancy and while nursing her infant. Serum and breast milk infliximab levels were monitored throughout this time period. This case report suggests that targeted monoclonal antibodies and other biologic agents can be used with caution in pregnant and breastfeeding patients.

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**Key words:** Inflammatory bowel disease; Ulcerative colitis; Crohn's disease; Pregnancy; Breast-feeding; Monoclonal antibodies

**Peer reviewer:** Akira Andoh, MD, Department of Internal Medicine, Shiga University of Medical Science, Seta Tulinowa, Otsu 520-2192, Japan

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## INTRODUCTION

In recent years, targeted monoclonal antibodies and other biologic agents have been at the forefront of the numerous therapeutic options available to treat many immune-mediated disorders. A large number of young and fertile patients are afflicted with disorders like inflammatory

bowel disease (IBD), rheumatologic diseases, asthma, and multiple sclerosis. These circumstances force patients and physicians to consider the safety of biologic agents during the peripartum time period.

## CASE REPORT

A 22-year-old female (G<sub>1</sub>P<sub>1</sub>) was referred to the Gastroenterology Clinic for treatment of fistulizing ileocolonic Crohn's disease (CD). The patient was initially treated with high dose corticosteroids, 6-mercaptopurine, metronidazole, and mesalamine with only mild improvement in her symptoms. The patient was eventually treated with infliximab and had a positive clinical response allowing her to be weaned off corticosteroids. Unfortunately, her 6-mercaptopurine was discontinued because of high thiopurine methyltransferase (TMPT) activity resulting in excessive production of the hepatotoxic 6-methylmercaptopurine metabolite. The patient's CD continued to respond modestly to 3.6 mg/d mesalamine (1200 mg tid) and 5 mg/kg infliximab (500 mg) IV infusions every 8 wk. The patient responded well to the medications but continued to have progressive symptoms requiring a stepwise increase in the maintenance dose of infliximab to 10 mg/kg (1000 mg) IV infusions every 4 wk.

Three years after her diagnosis with CD, the patient was discovered to be pregnant with her second child. The patient was successfully treated with mesalamine and infliximab when she was discovered to be 12 wk pregnant. The patient was informed that her disease could potentially worsen, nutritional deficiencies could develop, and that her medications could be potentially harmful to the fetus. The patient understood the risks and decided to proceed with the pregnancy after multiple discussions regarding the side effects and potential teratogenicity of her medications. She continued to take daily mesalamine and received a total of six doses of infliximab during her pregnancy with the last infusion occurring approximately 2 wk before delivery.

A healthy male infant weighing 7 pounds 6 ounces was born at thirty-nine weeks' gestation by an uncomplicated caesarian birth. The patient desired to breastfeed the infant while continuing to receive her mesalamine and infliximab. Again, the potential dangers of her medications were discussed with particular emphasis on their impact on breastfeeding. After taking the discussion under advisement, the patient decided to attempt to begin breastfeeding and to continue treatment with infliximab.

In an effort to determine if the infliximab was actually excreted into the breast milk, the patient's breast milk was collected and sent to the laboratory for analysis



Table 1 IBD medications during pregnancy

Low risk	Limited data	Not recommended	Contraindicated
Oral mesalamine	Olsalazine	Tetracycline	Methotrexate
Topical mesalamine	Azathioprine	Sulfonamides	
Sulfasalazine	6-Mercaptopurine		
Ampicillin	Metronidazole		
Cephalosporins	Ciprofloxacin		
Corticosteroids	Infliximab		
Cyclosporine	Adalimumab		
Loperamide			

(Prometheus Laboratories, San Diego, CA) with an enzyme-linked immunosorbent assay. A spike and recovery study was performed to investigate whether any non-specific binding by breast milk components was interfering with the assay. A sample of breast milk was spiked with 40 ng/mL solution of infliximab, a concentration comparable to the mother's serum concentration. A dilutional analysis (1:2, 1:4, and 1:8) was also performed and the infliximab was detected by the laboratory in all the spiked breast milk samples, but was not identified in her regular breast milk. The patient then received her regularly scheduled infliximab infusion (10 mg/kg) and her breast milk was collected daily for 30 d. No infliximab was identified in any of the breast milk samples, even with dilutional analysis. At 27 mo, no developmental abnormalities were noted in the child.

## DISCUSSION

New medications and aggressive treatment approaches to medical management have put more women with IBD in the position of being healthy enough to consider pregnancy. In women with IBD, the key to a healthy pregnancy is adequate control of disease activity throughout pregnancy<sup>[1]</sup>. Biologic agents are increasingly becoming a mainstay in the treatment regimens of both CD and ulcerative colitis (UC). Unfortunately, little information is available about the short-term and the long-term consequences of treatment with target monoclonal antibodies on the maturing fetus<sup>[2,3]</sup>. The safety of IBD medications during pregnancy and nursing are summarized in Tables 1 and 2.

Infliximab (Remicade; Centocor Inc, Malvern, PA) is a chimeric monoclonal antibody to tumor necrosis factor- $\alpha$  (TNF- $\alpha$ )<sup>[4]</sup>. It is indicated for inducing and maintaining clinical remission in moderately to severely active CD and UC patients that have had an inadequate response to conventional therapy and maintenance of remission<sup>[5]</sup>. Infliximab is increasingly used to treat pregnant women and data on its safety during pregnancy are scarce. Infliximab is listed as a pregnancy category B medication and the product label states that "It is not known whether infliximab can cause fetal harm when administered to a pregnant woman<sup>[4]</sup>". Most clinicians believe that the chimeric structure of the infliximab molecule containing a human IgG1 constant region, limits placental transfer during the first trimester<sup>[6]</sup>. However, the safety of infliximab beyond the first trimester

Table 2 IBD medications during nursing

Low risk	Limited data	Not recommended	Contraindicated
Oral mesalamine	Olsalazine	Tetracycline	Methotrexate
Topical mesalamine	Infliximab	Sulfonamides	Cyclosporine
Sulfasalazine	Adalimumab	Azathioprine	
Corticosteroids		6-Mercaptopurine	
		Loperamide	
		Metronidazole	
		Ciprofloxacin	

is unknown because IgG subclasses are readily passed into the fetus during the second and third trimesters<sup>[7]</sup>. Until recently, the medical literature contained no evidence that engineered therapeutic antibodies could cross the placenta when administered to expectant mothers. A recent case report documents clinically significant fetal exposure to infliximab *via* placental transfer and a prolonged half-life of the medication in newborns<sup>[2]</sup>. The presumed mechanism of fetal exposure to infliximab is transplacental maternal IgG antibody transfer beginning in the second trimester and peaking at term. No fetal abnormalities were apparent in this case, but the long-term implications of infliximab exposure during early childhood development are unknown. These findings suggest that pregnant patients should avoid therapeutic antibody treatments after thirty weeks' gestation and if necessary, the expectant mother can be bridged with steroids to control the disease activity until delivery<sup>[2,8]</sup>.

Limited clinical data are available on the safety of infliximab in pregnancy, because no controlled study is available in pregnant women. The manufacturer's safety database contains information on the outcomes of 131 pregnant women who received infliximab for rheumatoid arthritis or IBD<sup>[9]</sup>. An analysis performed on this safety database suggests no significant difference in pregnancy outcomes in women with infliximab exposure<sup>[7]</sup>. A published retrospective review of 10 pregnancies in CD patients in which infliximab was continued throughout the course of the pregnancy reported favorable fetal and maternal outcomes<sup>[7]</sup>. The limited clinical results available suggest that the benefits of infliximab in attaining response and maintaining remission in pregnant IBD patients might outweigh the risks of drug exposure to the fetus<sup>[10]</sup>.

The primary concern of the case we report is the safety of infliximab while breastfeeding, because many drugs and immunoglobulins are excreted in human milk. The infliximab product label states that "It is not known whether infliximab is excreted in human milk or absorbed systemically after ingestion<sup>[4]</sup>". A commercially available infliximab assay was used to measure drug levels in breast milk taken daily from our patient over a 30 d time period. No infliximab was detected in our patient's breast milk. Other published reports only tested breast-feeding mothers for one or two days but the results were consistent with our data<sup>[2]</sup>. We believe the daily testing performed on our patient's breast milk before and immediately after receiving an infliximab infusion clearly demonstrates that infliximab is not excreted in breast milk in any clinically significant amount.

Several case reports have recently emerged describing

the off-label usage of other biologics during pregnancy. A pregnant woman with treatment-refractory CD who failed treatment with infliximab was successfully treated with adalimumab (Humira; Abbott Laboratories, Chicago, IL), a recombinant human IgG1 monoclonal anti-TNF antibody<sup>[11,12]</sup>. The pregnancy was uncomplicated and at 6 mo, the infant showed normal growth and development<sup>[13]</sup>. Another case reported the use of etanercept (Enbrel; Amgen, Thousand Oaks, CA), a soluble TNF receptor fusion protein that binds to and inactivates TNF, in an uneventful pregnancy of a patient with refractory rheumatoid arthritis<sup>[14]</sup>. Etanercept has been shown to be excreted in breast milk, but it is not known whether the drug can be absorbed orally because it is such a large protein<sup>[15]</sup>.

In conclusion, therapeutic monoclonal antibodies and other biologic agents are used to a greater extent to treat immune-mediated disorders in pregnant patients. The limited clinical data currently available show no significant difference in pregnancy outcomes of patients exposed to infliximab during pregnancy compared to a healthy population. Physicians should be aware that the fetus may be exposed to therapeutic monoclonal antibodies when administered to pregnant patients and the long term implications on the child's developing immune system are unknown at this time. While physicians must remain cautious about maternofetal exposure to medications like therapeutic monoclonal antibodies, additions to the literature from reports like this one will hopefully assuage some of the fears faced by gastroenterologists, obstetricians, and patients, alike.

## REFERENCES

- 1 **Jospe ES**, Peppercorn MA. Inflammatory bowel disease and pregnancy: a review. *Dig Dis* 1999; **17**: 201-207
- 2 **Vasiliauskas EA**, Church JA, Silverman N, Barry M, Targan SR, Dubinsky MC. Case report: evidence for transplacental transfer of maternally administered infliximab to the newborn. *Clin Gastroenterol Hepatol* 2006; **4**: 1255-1258
- 3 **Srinivasan R**. Infliximab treatment and pregnancy outcome in active Crohn's disease. *Am J Gastroenterol* 2001; **96**: 2274-2275
- 4 **Remicade product information**. In: Physicians desk reference. 58th ed. Montvale, NJ: Medical Economics Company, Inc, 2004: 1145-1148
- 5 **Reddy JG**, Loftus EV Jr. Safety of infliximab and other biologic agents in the inflammatory bowel diseases. *Gastroenterol Clin North Am* 2006; **35**: 837-855
- 6 **Simister NE**. Placental transport of immunoglobulin G. *Vaccine* 2003; **21**: 3365-3369
- 7 **Mahadevan U**, Kane S, Sandborn WJ, Cohen RD, Hanson K, Terdiman JP, Binion DG. Intentional infliximab use during pregnancy for induction or maintenance of remission in Crohn's disease. *Aliment Pharmacol Ther* 2005; **21**: 733-738
- 8 **Friedman S**, Regueiro MD. Pregnancy and nursing in inflammatory bowel disease. *Gastroenterol Clin North Am* 2002; **31**: 265-73, xii
- 9 **Katz JA**, Antoni C, Keenan GF, Smith DE, Jacobs SJ, Lichtenstein GR. Outcome of pregnancy in women receiving infliximab for the treatment of Crohn's disease and rheumatoid arthritis. *Am J Gastroenterol* 2004; **99**: 2385-2392
- 10 **Tursi A**. Effect of intentional infliximab use throughout pregnancy in inducing and maintaining remission in Crohn's disease. *Dig Liver Dis* 2006; **38**: 439-440
- 11 **Humira (adalimumab) [prescribing information]**. North Chicago, IL: Abbott Laboratories, 2005
- 12 **Sanchez Munoz D**, Hoyas Pablos E, Ramirez Martin Del Campo M, Nunez Hospital D, Guerrero Jimenez P. [Term pregnancy in a patient with Crohn's disease under treatment with adalimumab] *Gastroenterol Hepatol* 2005; **28**: 435
- 13 **Vesga L**, Terdiman JP, Mahadevan U. Adalimumab use in pregnancy. *Gut* 2005; **54**: 890
- 14 **Sills ES**, Perloe M, Tucker MJ, Kaplan CR, Palermo GD. Successful ovulation induction, conception, and normal delivery after chronic therapy with etanercept: a recombinant fusion anti-cytokine treatment for rheumatoid arthritis. *Am J Reprod Immunol* 2001; **46**: 366-368
- 15 **Ostensen M**, Eigenmann GO. Etanercept in breast milk. *J Rheumatol* 2004; **31**: 1017-1018

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## CASE REPORT

# Abscesses of the spleen: Report of three cases

Constantin Fotiadis, Giagkos Lavranos, Pavlos Patapis, Gabriel Karatzas

Constantin Fotiadis, Giagkos Lavranos, Pavlos Patapis, Gabriel Karatzas, 3rd Department of Surgery, Attikon University Hospital, Chaidari 12462, Greece

**Author contributions:** Fotiadis C and Lavranos G contributed equally to this work and wrote the paper; Patapis P and Karatzas G analyzed the data.

**Correspondence to:** Giagkos Lavranos, Medical Doctor, 3rd Department of Surgery, Attikon University Hospital, 34 Falireos Street, 18547 Neo Faliro, Piraeus 18538, Greece. [glavran@med.uoa.gr](mailto:glavran@med.uoa.gr)

Telephone: +30-210-7462350 Fax: +30-210-7462105

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## Abstract

Abscess of the spleen is a rare discovery, with about 600 cases in the international literature so far. Although it may have various causes, it is most usually associated with trauma and infections of the spleen. The latter are more common in the presence of a different primary site of infection, especially endocarditis or in cases of ischemic infarcts that are secondarily infected. Moreover, immunosuppression is a major risk factor. Clinical examination usually reveals a combination of fever, left-upper-quadrant abdominal pain and vomiting. Laboratory findings are not constant. Imaging is a necessary tool for establishing the diagnosis, with a choice between ultrasound and computed tomography. Treatment includes conservative measures, and surgical intervention. In children and in cases of solitary abscesses with a thick wall, percutaneous catheter drainage may be attempted. Otherwise, splenectomy is the preferred approach in most centers. Here, we present three cases of splenic abscess. In all three, splenectomy was performed, followed by rapid clinical improvement. These cases emphasize that current understanding of spleen abscess etiology is still limited, and a study for additional risk factors may be necessary.

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**Key words:** Spleen; Abscess; Splenectomy; Infections; Trauma

**Peer reviewer:** Ronan A Cahill, Dr, Department of General Surgery, Waterford Regional Hospital, Waterford, Cork, Ireland

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## INTRODUCTION

Abscess of the spleen is a rather rare clinical entity. About 600 cases have been described so far in the international literature<sup>[1]</sup>. Most of these refer to patients with recognized risk factors. These include the synchronous presence of conditions that compromise the immune system, such as endocarditis, diabetes mellitus, congenital or acquired immunodeficiency and the administration of immunosuppressive medication (e.g. post-transplantation or as part of the treatment of connective tissue disorders)<sup>[2-5]</sup>. Trauma is an additional predisposing factor for splenic abscesses<sup>[6]</sup>. Instances of splenic abscesses are relatively increased among intravenous drug addicts. On the other hand, splenic abscesses are most uncommon in the general population. From an epidemiological point of view, they are more frequently detected in middle-aged and older individuals, with no obvious preference for either sex<sup>[1-3]</sup>.

The clinical manifestations of splenic abscesses usually include abdominal pain, exclusively located or, at least, more intensely described in the upper-left-quadrant area. Fever, nausea, vomiting and anorexia may be also present in various combinations<sup>[7-9]</sup>. Laboratory findings are consistent with the acute phase of infection, but their exact nature is determined by the pathogen isolated from the abscess<sup>[10,11]</sup>. The most common pathogens detected include *Staphylococcus* and *Streptococcus*<sup>[2,12]</sup>. Imaging by common abdominal X-ray or ultrasound may be suggestive, but the lesion is usually revealed *via* computed tomography (CT). Due to the seriousness of the potential implications, including a threat to life itself, the most usual treatment currently applied is splenectomy, which is followed by rapid clinical improvement<sup>[13-15]</sup>.

## CASE REPORTS

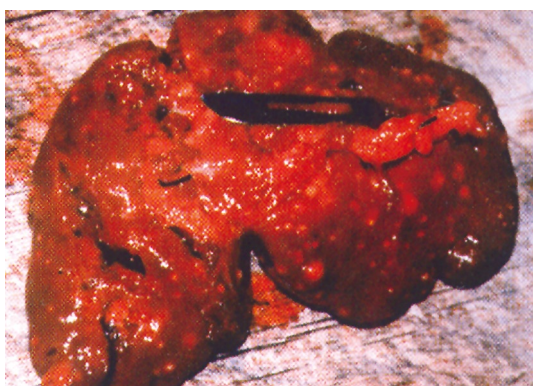
### Case 1

A 45-year-old man presented to our hospital's outpatient clinic with persistent pain in the upper-left-quadrant area of the abdomen. He was working as a clerk, having previously spent 10 years as a member of embassy personnel in Africa. The referred pain had initiated about 1 mo previously, with periods of temporary improvement and relapse. The pain was not altered after food intake or sleep. The patient recognized no other major symptoms, such as vomiting, nausea or fever. Moreover, the patient was not treated for any other disease at the time (including recent infection or operation), nor had he ever been admitted to the hospital in the past. Clinical examination reproduced localized sensitivity in the area of the spleen, with no other significant findings. Laboratory testing (standard hematological and





**Figure 1** Abdominal CT scan of a 45-year-old man. The spleen contained a large single abscess of 8 cm  $\times$  4 cm.

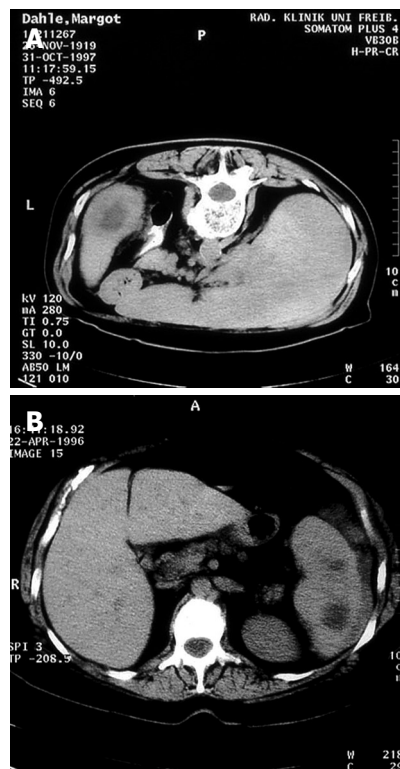


**Figure 2** Macroscopic image of the dissected spleen. Notice the extent of the pathological tissue.

biochemical controls) revealed a mild increase in the number of leukocytes, which was otherwise within the normal range. Blood and urine cultures failed to reveal any pathogens. Imaging included chest and abdominal X-ray, followed by a CT scan of the upper abdomen. The latter detected a large abscess of the spleen, of an average size of 8 cm  $\times$  4 cm (Figure 1). Aspiration of the abscess was performed under CT guidance and the material obtained was cultured, which led to the development of several colonies identified as *Streptococcus* spp. No other pathogen of any kind was detected in the cultures. Owing to the abscess being symptomatic and of considerable size, the decision to perform splenectomy was made. The operation was completed successfully (Figure 2). Follow-up 1 year later has revealed a completely asymptomatic postoperative period.

## Case 2

A 50-year-old woman visited the outpatient clinic of our hospital with referred acute abdominal pain, which was not related to dietary habits and/or sleep. She also reported mild fever for about 1 wk (up to 38.5°C). Prior to surgery, she had been evaluated by a physician at the internal medicine clinic, who reported no clinical findings other than localized pain in the left-upper-quadrant area of the abdomen. Our clinical examination verified this finding. The patient had no history of recent infection or surgery, nor had she received medication of any kind. Moreover, she did not suffer from diabetes, human immunodeficiency virus (HIV) infection, or any other condition that would justify a degree



**Figure 3** A: CT scan of a 50-year-old woman. The spleen contained a single abscess of 3 cm  $\times$  3 cm; B: CT scan of a 40-year-old male sailor, which featured multiple splenic abscesses.

of immunosuppression/immunocompromise. Laboratory testing was performed, which showed signs of on-going bacterial infection (leukocytosis, increased C-reactive protein and fibrinogen, and a mild decrease in blood albumin). Blood and urine cultures failed to reveal microbial infection. Imaging was also performed, including thoracic and abdominal X-ray, followed by CT of the upper and lower abdomen. The latter detected a single, small abscess of the spleen of 3 cm  $\times$  3 cm (Figure 3A). Subsequently, percutaneous aspiration of the lesion was performed and the material was sent for culture. The microbiological report referred to the presence of *Staphylococci*, a rather common finding in splenic abscesses. Due to the symptomatic nature of the lesion, the patient was advised to undergo surgical treatment, which she accepted. She was operated a week later and total splenectomy was performed under general anesthesia. A follow-up session took place 1 year after the operation, which revealed complete resolution of all symptoms. The patient has remained asymptomatic ever since.

## Case 3

A 40-year-old male sailor visited our hospital as part of a yearly check-up, as instructed by his employers. During a brief review of his medical history, he mentioned a persistent chronic pain in the upper-left quadrant of the abdomen. The pain was not directly related to food intake or sleep, although it was occasionally combined with mild episodes of nausea and anorexia. Moreover, the patient referred to moderate fever (up to 38.8°C) that had occurred 3-4 times in the past 3 mo, each time lasting for about 3 d, and retreated after the use of non-steroidal anti-inflammatory drugs, administered every 6 h. The patient also had a long history of infections of the respiratory





**Figure 4** Macroscopic postoperative image of the dissected spleen. Abscess areas are easily recognized.

and gastrointestinal tracts, although he reported that all of these had been treated successfully, leaving no remnant disease. Clinical examination revealed nothing significant, other than sensitivity in the abdomen, which was more intense in the upper-left quadrant. Laboratory findings were consistent with acute-phase reaction (leukocytosis, and increased acute-phase proteins). Imaging included thoracic and abdominal X-ray, abdominal ultrasound, and finally, CT. The latter revealed multiple abscesses in the spleen (Figure 3B). No abscesses were detected via imaging in any other organ examined, including the liver. Cultures from blood and urine samples were negative, but the sample obtained from the abscesses themselves developed numerous colonies of bacteria identified as *Streptococcus* spp. Evaluation of the clinical condition, laboratory and imaging data, as well as professional danger (minimal access to immediate medical referral in case of an emergency), led to the decision to perform splenectomy (Figure 4). The surgery was completed without any complications and the patient has been found healthy and free from all referred symptoms to this date.

## DISCUSSION

Abscesses of the spleen are rather rare, especially in developed countries in which the frequency of parasitic infections is low<sup>[2,13-17]</sup>. From this point of view, the random discovery of three such cases in a single Greek hospital in the course of two years may at first appear to signify an increased incidence. However, this may be misleading, since our hospital is a tertiary medical referral center for a densely populated area of about 4 million citizens. Moreover, it is closely located to the central port of the country, Pireaus, which makes access for foreign visitors, immigrants and Greeks returning from high-prevalence countries particularly easy<sup>[18]</sup>. Indeed, in case 3, the subject was a sailor and in case 1, there was a history of residence in Africa.

The age of the individuals in our small series is 40-50 years, which is consistent with the peak age group for initial diagnosis of splenic abscesses described in the literature<sup>[3,4,9,19,20]</sup>. However, it should be noted that in all three cases, medical care was only infrequently demanded prior to consultation by our department and therefore,

it might be suggested that the disease evolved over a considerable time prior to our diagnosis. The personal history of long-term presence abroad strengthens this assumption.

The clinical manifestations, and laboratory and imaging findings in all three individuals were similar, although some variety was definitely observed (e.g. presence, height and duration of fever, abdominal pain characteristics and blood leukocyte count). In fact, all the symptoms described by the patients are included in the most frequent clinical findings lists provided by other reported studies, which proves that the current general understanding of the disease's pathophysiology is reasonably accurate<sup>[1,10,21]</sup>.

Perhaps the most interesting parameter in our three cases of splenic abscess, however, is the lack of any obvious risk factors in any of the individuals<sup>[22,23]</sup>. Indeed, a detailed medical history and clinical examination were performed initially and post-diagnosis, in an attempt to reveal any of the factors known to be associated with the development of abscesses in the spleen and other organs. However, no such findings occurred, with the only exception of potential occupational risk in cases 1 and 3. This discovery, along with the detection of common pathogens in the abscess itself (*Streptococcus* and *Staphylococcus* spp., respectively), may imply that further factors, apart from those already described, must contribute to the etiology of the disease<sup>[6,10,24]</sup>. Their exact nature and involvement in immunity modification and regulation of the reaction to infectious agents remains to be determined in future<sup>[20]</sup>.

As far as treatment is concerned, our department proceeded to classic total splenectomy in all three cases discussed. This led to rapid and complete relief from all disease-associated symptoms, without any major complications. This policy is still considered the standard of care for splenic abscesses<sup>[1,6]</sup>. However, more recent studies have also referred to alternative options, including laparoscopic splenectomy and spleen-preserving protocols, such as percutaneous imaging-guided drainage<sup>[1,15,25,26]</sup>. These methods are minimally invasive and are expected to result in smaller operative risk and overall treatment period, although of course this may differ according to the exact cause of the abscess<sup>[27-31]</sup>. Although initial results from the application of these novel methods are most promising and the potential advantages most welcome, current experience is still rather limited to justify their place in splenic abscess treatment. Therefore, the current policy is to limit their use in centers with adequately trained surgeons and only for a selected subgroup of patients.

## REFERENCES

- 1 Carbonell AM, Kercher KW, Matthews BD, Joels CS, Sing RF, Heniford BT. Laparoscopic splenectomy for splenic abscess. *Surg Laparosc Endosc Percutan Tech* 2004; **14**: 289-291
- 2 Chang KC, Chuah SK, Changchien CS, Tsai TL, Lu SN, Chiu YC, Chen YS, Wang CC, Lin JW, Lee CM, Hu TH. Clinical characteristics and prognostic factors of splenic abscess: a review of 67 cases in a single medical center of Taiwan. *World J Gastroenterol* 2006; **12**: 460-464
- 3 Chiang IS, Lin TJ, Chiang IC, Tsai MS. Splenic abscesses: review of 29 cases. *Kaohsiung J Med Sci* 2003; **19**: 510-515

- 4 **Chulay JD**, Lankerani MR. Splenic abscess. Report of 10 cases and review of the literature. *Am J Med* 1976; **61**: 513-522
- 5 **Kim HS**, Cho MS, Hwang SH, Ma SK, Kim SW, Kim NH, Choi KC. Splenic abscess associated with endocarditis in a patient on hemodialysis: a case report. *J Korean Med Sci* 2005; **20**: 313-315
- 6 **Ulhaci N**, Meteoglu I, Kacar F, Ozbas S. Abscess of the spleen. *Pathol Oncol Res* 2004; **10**: 234-236
- 7 **Gadacz T**, Way LW, Dunphy JE. Changing clinical spectrum of splenic abscess. *Am J Surg* 1974; **128**: 182-187
- 8 **Green BT**. Splenic abscess: report of six cases and review of the literature. *Am Surg* 2001; **67**: 80-85
- 9 **Nelken N**, Ignatius J, Skinner M, Christensen N. Changing clinical spectrum of splenic abscess. A multicenter study and review of the literature. *Am J Surg* 1987; **154**: 27-34
- 10 **Cavuoti D**, Fogli M, Quinton R, Gander RM, Southern PM. Splenic abscess with *Vibrio cholerae* masking pancreatic cancer. *Diagn Microbiol Infect Dis* 2002; **43**: 311-313
- 11 **Farnsworth TA**. Enterococcus avium splenic abscess: a rare bird. *Lancet Infect Dis* 2002; **2**: 765
- 12 **Zacharoulis D**, Katsogridakis E, Hatzitheofilou C. A case of splenic abscess after radiofrequency ablation. *World J Gastroenterol* 2006; **12**: 4256-4258
- 13 **Smyrniotis V**, Kehagias D, Voros D, Fotopoulos A, Lambrou A, Kostopanagiotou G, Kostopanagiotou E, Papadimitriou J. Splenic abscess. An old disease with new interest. *Dig Surg* 2000; **17**: 354-357
- 14 **Westh H**, Reines E, Skibsted L. Splenic abscesses: a review of 20 cases. *Scand J Infect Dis* 1990; **22**: 569-573
- 15 **Zerem E**, Bergsland J. Ultrasound guided percutaneous treatment for splenic abscesses: The significance in treatment of critically ill patients. *World J Gastroenterol* 2006; **12**: 7341-7345
- 16 **Ghidirim G**, Rojnoveanu G, Misin I, Gagauz I, Gurghis R. [Splenic abscess--etiologic, clinical and diagnostic features]. *Chirurgia (Bucur)* 2007; **102**: 309-314
- 17 **Krzysztof L**, Krysiak R, Basiak M, Kalina M, Mykala-Ciesla J, Kolodziej-Jaskula A, Okopien B. [Diagnostic difficulties in diagnosis of splenic abscesses]. *Wiad Lek* 2007; **60**: 83-86
- 18 **Tappe D**, Muller A, Langen HJ, Frosch M, Stich A. Isolation of *Salmonella enterica* serotype newport from a partly ruptured splenic abscess in a traveler returning from Zanzibar. *J Clin Microbiol* 2007; **45**: 3115-3117
- 19 **Andre MF**, Piette JC, Kemeny JL, Ninet J, Jegou P, Delevaux I, Wechsler B, Weiller PJ, Frances C, Bletry O, Wismans PJ, Rousset H, Colombel JF, Aumaitre O. Aseptic abscesses: a study of 30 patients with or without inflammatory bowel disease and review of the literature. *Medicine (Baltimore)* 2007; **86**: 145-161
- 20 **Rudiger T**, Hartmann M, Muller-Hermelink HK, Marx A. [Inflammatory reactions of the spleen]. *Pathologe* 2008; **29**: 121-128
- 21 **Thapa R**, Mukherjee K, Chakrabartty S. Splenic abscess as a complication of enteric fever. *Indian Pediatr* 2007; **44**: 438-440
- 22 **Al-Tawfiq JA**. Bacteroides (Parabacteroides) distasonis splenic abscess in a sickle cell patient. *Intern Med* 2008; **47**: 69-72
- 23 **Pisanu A**, Ravarino A, Nieddu R, Uccheddu A. Synchronous isolated splenic metastasis from colon carcinoma and concomitant splenic abscess: a case report and review of the literature. *World J Gastroenterol* 2007; **13**: 5516-5520
- 24 **Matsubayashi T**, Matsubayashi R, Saito I, Tobayama S, Machida H. Splenic abscess in an infant caused by *Streptococcus intermedius*. *J Infect Chemother* 2007; **13**: 423-425
- 25 **Martinez DG**, Sanchez AW, Garcia AP. Splenic abscess after laparoscopic nissen fundoplication: a consequence of short gastric vessel division. *Surg Laparosc Endosc Percutan Tech* 2008; **18**: 82-85
- 26 **Kogo H**, Yoshida H, Mamada Y, Taniai N, Bando K, Mizuguchi Y, Ishikawa Y, Yokomuro S, Akimaru K, Tajiri T. Successful percutaneous ultrasound-guided drainage for treatment of a splenic abscess. *J Nippon Med Sch* 2007; **74**: 257-260
- 27 **Hasan M**, Sarwar JM, Bhuiyan JH, Islam SM. Tubercular splenic abscess. *Mymensingh Med J* 2008; **17**: 67-69
- 28 **Agarwal N**, Dewan P. Isolated tubercular splenic abscess in an immunocompetent child. *Trop Gastroenterol* 2007; **28**: 83-84
- 29 **Sharma SK**, Smith-Rohrberg D, Tahir M, Mohan A, Seith A. Radiological manifestations of splenic tuberculosis: a 23-patient case series from India. *Indian J Med Res* 2007; **125**: 669-678
- 30 **Rechner J**, Nowak L, Hess F, Mebold A, De Lorenzi D. [A rare splenic involvement by *Echinococcus multilocularis* - case report]. *Zentralbl Chir* 2007; **132**: 158-160
- 31 **Jabr FI**, Skeik N. Splenic abscess caused by actinomycosis. *Intern Med* 2007; **46**: 1943-1944

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## CASE REPORT

# Hepatic cyst misdiagnosed as a gastric submucosal tumor: A case report

Joong-Min Park, Jin Kim, Ho-Il Kim, Chong-Suk Kim

Joong-Min Park, Jin Kim, Ho-Il Kim, Chong-Suk Kim, Department of Surgery, Korea University College of Medicine, Korea University Anam Hospital, 126-1 Anam-dong 5ga, Sungbuk-gu, Seoul 136-705, South Korea

**Author contributions:** Park JM, Kim J, Kim HI and Kim CS contributed equally to this work.

**Correspondence to:** Chong-Suk Kim, Department of Surgery, Korea University Anam Hospital, 126-1 Anam-dong 5ga, Sungbuk-gu, Seoul 136-705, South Korea. [chongsuk@korea.ac.kr](mailto:chongsuk@korea.ac.kr)  
Telephone: +82-2-9205866 Fax: +82-2-9281631

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## Abstract

We describe here a case of 51-year-old woman with a symptomatic hepatic cyst that was misdiagnosed as a gastric submucosal tumor (SMT) with endoscopic ultrasound (EUS) and CT scan. The patient presented with an epigastric pain for two months. On endoscopy, a submucosal tumor was found on the cardia of the stomach. Based on EUS and abdominal CT scan, the lesion was diagnosed as a gastric duplication cyst or a gastrointestinal stromal tumor (GIST). The operative plan was laparoscopic wedge resection for the GIST of the gastric cardia. A cystic mass arising from the left lateral segment of the liver was found at the laparoscopic examination. There was no abnormal finding at the gastric cardia. She was treated by laparoscopic hepatic wedge resection including the hepatic cyst using an endoscopic linear stapler.

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**Key words:** Hepatic cyst; Submucosal tumor; Stomach

**Peer reviewer:** Filip Braet, Associate Professor, Australian Key Centre for Microscopy and Microanalysis, Madsen Building (F09), The University of Sydney, Sydney NSW 2006, Australia

Park JM, Kim J, Kim HI, Kim CS. Hepatic cyst misdiagnosed as a gastric submucosal tumor: A case report. *World J Gastroenterol* 2008; 14(19): 3092-3094 Available from: URL: <http://www.wjg-net.com/1007-9327/14/3092.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3092>

## INTRODUCTION

Although submucosal tumor (SMT) is benign and

asymptomatic, it should be evaluated by follow-up examinations. Certain gastric SMTs that are considered to be gastrointestinal stromal tumor (GIST) or symptomatic SMT require operative intervention because it is very difficult to confirm its malignant potential with endoscopic biopsy<sup>[1]</sup>.

SMT is usually asymptomatic and most often discovered accidentally at surgery and autopsy or at performing diagnostic procedures. Unspecific symptoms, such as abdominal pain, obstruction, hemorrhage and intussusception, may occur. Two advanced tools have been generally accepted for the diagnosis and treatment of gastric SMTs. Endoscopic ultrasound (EUS) is one useful accurate diagnostic method, and the other is a laparoscopic procedure that allows minimally invasive treatment for SMTs.

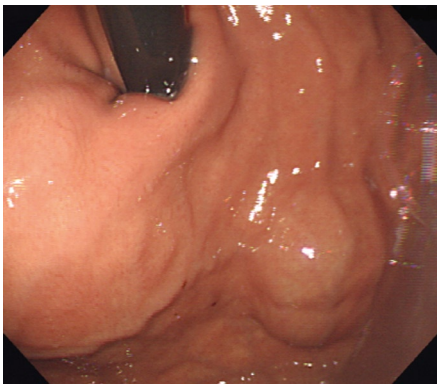
Extragastric compression may mimic the symptoms and endoscopic findings of gastric SMTs. EUS and CT scan can accurately differentiate extragastric compression from true SMTs. However, cases may arise that cannot be differentiated even after various methods are used. We report here a case of hepatic cyst which was misdiagnosed as a gastric submucosal tumor in a patient undergone various diagnostic modalities, including endoscopy, EUS and abdominal CT scan.

## CASE REPORT

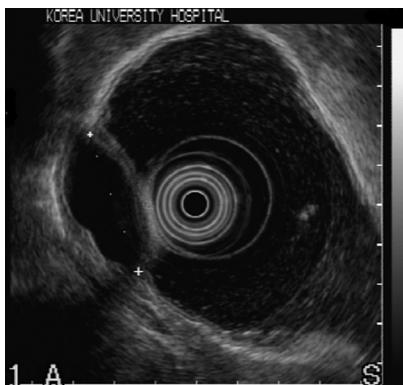
A 51-year-old woman presented with epigastric pain for two months. Initial examination showed that she had tenderness in the epigastrium. The patient was taking no medications. Her past medical history and familial history were unremarkable. Routine laboratory data on admission did not show any abnormal findings.

Gastrointestinal endoscopy revealed a submucosal tumor in the cardia of the stomach (Figure 1). On EUS, the lesion was a hypoechoic mass (3.6 cm in diameter) suggestive of a gastric duplication cyst or a GIST (Figure 2). Abdominal CT scan showed a cystic lesion at the submucosal layer of the gastric cardia, and the impression of the radiologist was a gastric duplication cyst or a GIST with necrosis (Figure 3). The operative plan was laparoscopic wedge resection for the GIST of the gastric cardia. Laparoscopic exploration was performed for the patient under general anesthesia. On the laparoscopic examination, a cystic mass arising from the left lateral segment of the liver was found (Figure 4A). There was no abnormal finding at the gastric cardia. After the

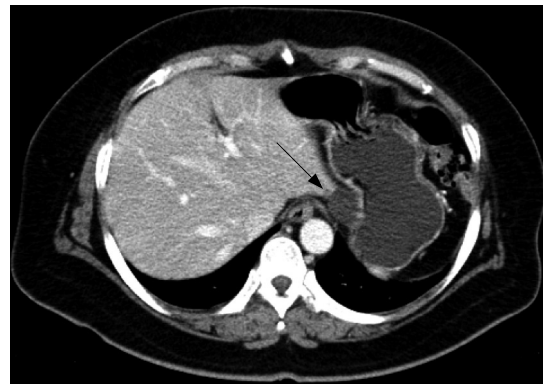




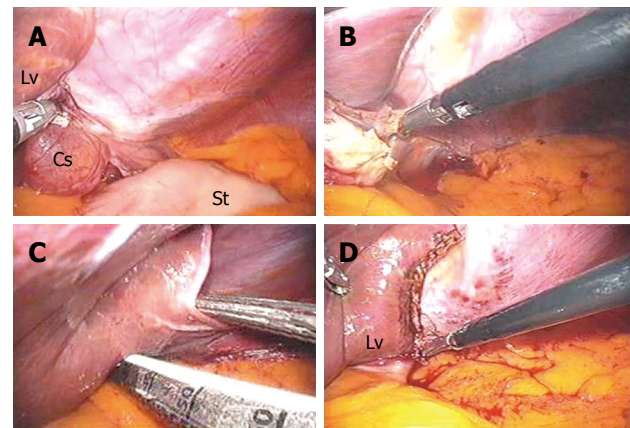
**Figure 1** Endoscopic photograph demonstrating a protruding mass on the cardia of stomach.



**Figure 2** EUS showing a hypoechoic mass (3.6 cm in diameter) which was suspicious of a gastric duplication cyst or a GIST.



**Figure 3** Abdominal CT scan showing a low density lesion in the submucosal layer of the gastric cardia (arrow).



**Figure 4** Operative procedures for the hepatic cyst in the left lateral segment of liver (A), after dissection of the triangular ligament (B), liver wedge resection using an endoscopic linear stapler (C, D). Lv: Liver, Cs: Hepatic cyst, St: Stomach.

triangular ligament was divided with electrocautery (Figure 4B), wedge resection of the left lateral segment of the liver, including the hepatic cyst, was performed using an endoscopic linear stapler (Figure 4C and D). There was no specific complication during the procedure. An oral diet was permitted on the 1st postoperative day. She was discharged from the hospital on the 3rd postoperative day. The mass was diagnosed as a simple hepatic cyst.

## DISCUSSION

Hepatic cysts are usually asymptomatic and not associated with defective hepatic function. They are incidentally found at laparotomy or laparoscopy, and even at routine ultrasound or CT scan. However, they may become symptomatic if they grow. The symptoms depend on the size and location of the cyst. The patients may have a vague upper abdominal pain, a right upper quadrant abdominal mass, postprandial fullness, dyspnea and vomiting<sup>[2]</sup>. In our case, the hepatic cyst was located at the edge of the left lateral segment of the liver and caused epigastric pain by compressing the gastric cardia.

A left hepatic cyst may rarely mimic a SMT arising from the gastric cardia or fundus<sup>[3]</sup>. Various conditions can mimic gastric SMT due to extragastric compression. The most common source of extraluminal compression in the stomach is from the spleen and splenic vessels<sup>[4,5]</sup>. Other sources of extraluminal compression include normal abdominal organ structures such as liver and gallbladder,

and pathologic lesions such as tumors, abscesses, pancreatic pseudocysts and enlarged lymph nodes.

Whether the lesion is due to intramural or extrinsic compression can be distinguished by changing the patient's position to see if the location and appearance of the mass change. Also, a change in appearance of the mass with either air insufflation or deflation is helpful in determining if the lesion is due to extrinsic compression, yet it can be difficult to differentiate. It was reported that the sensitivity and specificity of endoscopy are 87% and 29%, respectively for distinguishing intramural lesion from extramural compression<sup>[6]</sup>. On the other hand, EUS is 100% accurate for differentiating extragastric compression from submucosal tumor and for identifying the compressing organ<sup>[7]</sup>.

In our case, however, the hepatic cyst was misdiagnosed as a GIST although various diagnostic methods such as EUS and CT scan were used.

In the best of our knowledge, this is the first report of a patient with asymptomatic left hepatic cyst that was misdiagnosed as a GIST and treated by laparoscopic resection of the hepatic cyst.

Surgical treatment for hepatic cyst is indicated when the cyst causes complaints and the diameter is at least 5 cm



or rapid growth is observed. Possible surgical treatments of the cyst include unroofing, extirpation or resection of the cyst. Conservative treatment (aspiration, sclerotherapy and percutaneous drainage) is not often recommended because of frequent relapse of the disease<sup>[2,8]</sup>.

With the advances in minimally invasive surgery, laparoscopic unroofing is generally recommended for the treatment of hepatic cyst<sup>[8]</sup>. In our case, since the cyst was located at the left edge of the liver and relatively small, laparoscopic wedge resection of the hepatic cyst was easily performed by using an endoscopic linear stapler.

In conclusion, a left hepatic cyst may mimic a SMT arising from the gastric cardia and cause nonspecific abdominal symptoms. For such a case, laparoscopic procedure is a useful option for making the accurate diagnosis, and laparoscopic resection of the hepatic cyst is a minimally invasive treatment.

## REFERENCES

- 1 **Ponsaing LG**, Hansen MB. Therapeutic procedures for submucosal tumors in the gastrointestinal tract. *World J Gastroenterol* 2007; **13**: 3316-3322
- 2 **Caetano-Junior EM**, Linhares MM, Matos D, Schraibman V, Matone J, Saad SS. Laparoscopic management of hepatic cysts. *Surg Laparosc Endosc Percutan Tech* 2006; **16**: 68-72
- 3 **Park SS**, Ryu WS, Kwak JM, Lee SI, Kim WB, Mok YJ, Choi JW, Park JJ, Bak YT. Gastric fundus impression caused by a hepatic cyst mimicking gastric submucosal tumor. *South Med J* 2006; **99**: 902-903
- 4 **Hwang JH**, Kimmey MB. The incidental upper gastrointestinal subepithelial mass. *Gastroenterology* 2004; **126**: 301-307
- 5 **Rosch T**, Lorenz R, von Wichert A, Classen M. Gastric fundus impression caused by splenic vessels: detection by endoscopic ultrasound. *Endoscopy* 1991; **23**: 85-87
- 6 **Rosch T**, Kapfer B, Will U, Baronius W, Strobel M, Lorenz R, Ulm K. Accuracy of endoscopic ultrasonography in upper gastrointestinal submucosal lesions: a prospective multicenter study. *Scand J Gastroenterol* 2002; **37**: 856-862
- 7 **Motoo Y**, Okai T, Ohta H, Satomura Y, Watanabe H, Yamakawa O, Yamaguchi Y, Mouri I, Sawabu N. Endoscopic ultrasonography in the diagnosis of extraluminal compressions mimicking gastric submucosal tumors. *Endoscopy* 1994; **26**: 239-242
- 8 **Szabo LS**, Takacs I, Arkosy P, Sapy P, Szentkereszty Z. Laparoscopic treatment of nonparasitic hepatic cysts. *Surg Endosc* 2006; **20**: 595-597

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# Aortoduodenal fistula and aortic aneurysm secondary to biliary stent-induced retroperitoneal perforation

Tae Hoon Lee, Do Hyun Park, Ji-Young Park, Suck-Ho Lee, Il-Kwun Chung, Hong Soo Kim, Sang-Heum Park, Sun-Joo Kim

Tae Hoon Lee, Ji-Young Park, Suck-Ho Lee, Il-Kwun Chung, Hong Soo Kim, Sang-Heum Park, Sun-Joo Kim, Division of Gastroenterology, Department of Internal Medicine, Soonchunhyang University College of Medicine, Cheonan Hospital, 23-20 Bongmyung-dong, Cheonan, Choongnam 330-721, South Korea

Do Hyun Park, Division of Gastroenterology, Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, 388-1 Pungnap-2dong, Songpaju, Seoul 138-736, South Korea

**Author contributions:** Lee TH and Park DH contributed equally to this work; Park JY, Lee SH, Chung IK and Kim HS provided the clinical advice; Park DH, Park SH and Kim SJ designed the case report; Lee TH and Park DH wrote the paper.

**Correspondence to:** Do Hyun Park, Division of Gastroenterology, Department of Internal Medicine, University of Ulsan College of Medicine, Asan Medical Center, 388-1 Pungnap-2dong, Songpaju, Seoul 138-736, South Korea. [dhpark@amc.seoul.kr](mailto:dhpark@amc.seoul.kr)

Telephone: +82-2-30103180 Fax: +82-2-4855782

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## Abstract

Duodenal perforations caused by biliary prostheses are not uncommon, and they are potentially life threatening and require immediate treatment. We describe an unusual case of aortic aneurysm and rupture which occurred after retroperitoneal aortoduodenal fistula formation as a rare complication caused by biliary metallic stent-related duodenal perforation. To our knowledge, this is the first report describing a lethal complication of a bleeding, aortoduodenal fistula and caused by biliary metallic stent-induced perforation.

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**Key words:** Stents; Retroperitoneal perforation; Aortic aneurysm; Fistula

**Peer reviewer:** Giovanni D De Palma, Professor, Department of Surgery and Advanced Technologies, University of Naples Federico II, School of Medicine, Naples 80131, Italy

Lee TH, Park DH, Park JY, Lee SH, Chung IK, Kim HS, Park SH, Kim SJ. Aortoduodenal fistula and aortic aneurysm secondary to biliary stent-induced retroperitoneal perforation. *World J Gastroenterol* 2008; 14(19): 3095-3097 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3095.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3095>

## INTRODUCTION

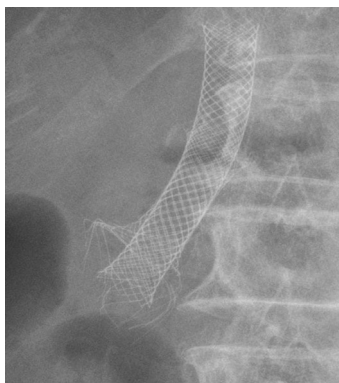
Endoscopic or percutaneous biliary stenting is the preferred method of palliative treatment for malignant biliary strictures<sup>[1-4]</sup>. As the stents are used frequently and for long periods of time, biliary stent-related duodenal perforation is not an uncommon complication, which is potentially life threatening<sup>[5-9]</sup>. Biliary metallic stent-induced retroperitoneal perforation resulting in aortoduodenal fistula has not been reported as yet. To our knowledge, this is the first report describing the lethal complication of a bleeding aortoduodenal fistula following biliary metallic stent-related duodenal perforation.

## CASE REPORT

A 69-year-old woman presented herself with symptoms of abdominal pain and melena that started to worsen 2 d or 3 d before. An uncovered biliary wall stent (Boston Scientific, Marlboro, MA), 5 cm in length, was inserted two years before when the patient was diagnosed with a locally advanced pancreatic cancer. One year later, endoscopic removal of the uncovered wall stent was attempted because of stent clogging and tumor ingrowth. However, the attempt was unsuccessful, and resulted in a partial deformity of the distal end of the stent. A covered biliary wall stent, 6 cm in length, was reinserted into the stent. The patient received gemcitabine chemotherapy for 2 mo and recently took analgesics. She also frequently received folk remedies, such as massage of the epigastrium with downward palm-pressure.

Upon presentation, clinical examination revealed mild epigastric tenderness and abdominal distension without rebound tenderness. Laboratory tests showed  $21.88 \times 10^9/L$  ( $4.0-10.8 \times 10^9/L$ ) white blood cells, 8.5 g/dL (13-18 g/dL) hemoglobin, 36 IU/L (60-160 IU/L) amylase, 10 IU/L (0-60 IU/L) lipase, and 152 IU/L (39-117 IU/L) alkaline phosphatase. Comparisons of two simple abdominal X-rays, one taken recently and the other 2 mo before, found that the stents were slightly migrated distally and the outer stent's distal tip was compressed, shooting out radially in all directions (Figure 1). Abdominal computer tomography (CT) scan demonstrated a biliary stent with lesions arising from the head of the pancreas, compressing the second part of the duodenum. Air bubble densities were traced from the pancreatic head to the lower para-aortic lesions (Figure 2).

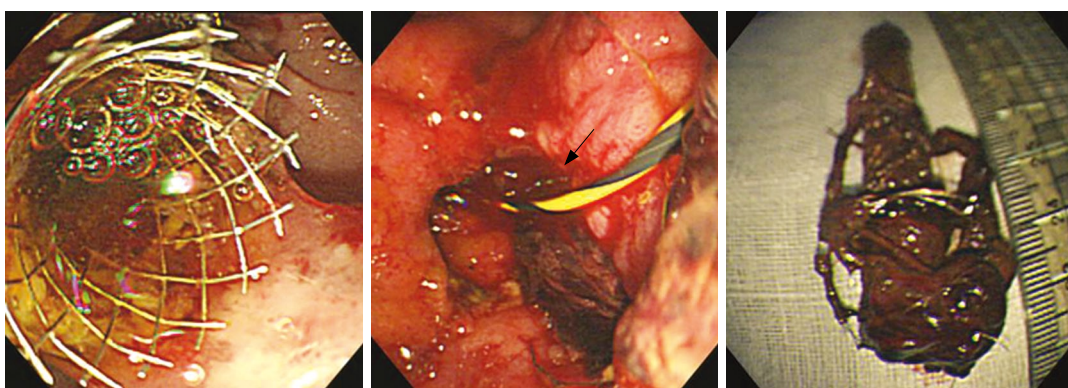
An endoscopy demonstrated that the bare metal



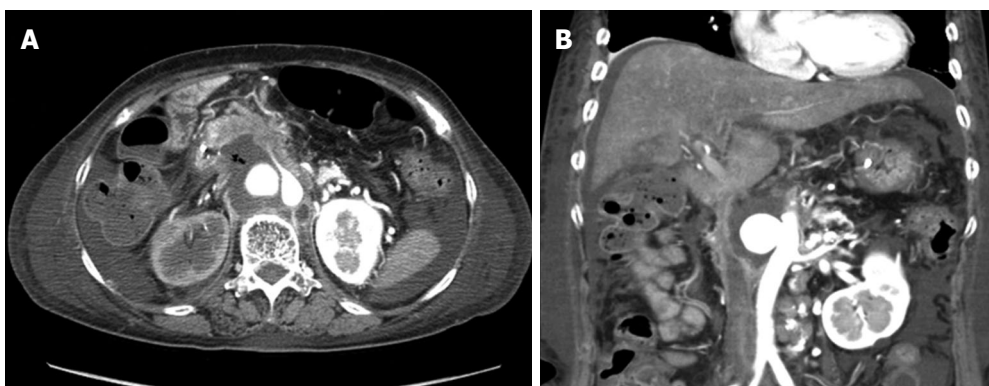
**Figure 1** Simple abdomen examination demonstrating compression of deformed distal tip of the outer biliary metallic stent shooting out radially in all directions.



**Figure 2** Abdominal CT scan showing biliary metallic stents with a lesion arising from the pancreatic head and the trajectory of air bubble densities traced from the pancreatic head to the lower paraaortic lesions.



**Figure 3** EGD. **A:** On previous admission (one year ago), placement of a stent into a stent due to clogging; **B:** On the present admission, a circular hole with bleeding (arrow) caused by stent-induced perforation following removal of stents; **C:** Retrieved biliary metallic stents showing deformed barbs of the uncovered wall stent tip on the distal portion of the covered wall stent.



**Figure 4** Abdominal CT scan showing decreased air densities in the pancreatic head to the paraaortic area (**A**) and a circular contrast collecting aneurysm of aorta (**B**).

barbs deformed in the stent were seen to have impacted and penetrated the neighboring wall of the duodenum. Following removal of the stents with a rat-tooth forceps without any additional injury, a circular hole with bleeding corresponding to the perforation was evident (Figure 3). We then planned to place a covered self-expandable metal stent into the duodenum, instead of placing a nasobiliary drainage for a short time due to desaturation and instability of the patient. After removal of the stent and treatment with parenteral nutrition and intravenous antibiotics, the patient felt well with no abdominal pain. However, 3 d later, she complained again of abdominal

pain and melena. So a follow-up abdominal CT was performed, which showed decreased air densities in the same area. However, a newly developed circular contrast collecting aortic aneurysm was found in the adjacent para-aortic lesion (Figure 4). On the following day, unexpected and massive hematemesis and hematochezia occurred, and the patient died due to bleeding.

## DISCUSSION

Early occurrence of iatrogenic duodenal perforations is generally apparent during papillotomy and stent placement.

Late presentations of duodenal perforations caused by biliary prosthesis are much rarer, but they are potentially life threatening and require immediate management<sup>[10-12]</sup>.

Although the uncovered wall stent is easily embedded in the bile duct epithelium<sup>[13,14]</sup>, the prolonged *in situ* uncovered wall stent may lose its framework, causing the stent to become weak because of its woven structures. In the present case, the stent's distal end was partially destroyed by repeated tries to remove it and it might have migrated distally during the follow-up. Under these circumstances, with repeated external abdominal pressure, the inner covered stent might have acted on the distal tip of the outer uncovered wall stent as a vehicle with a straining and compressing force. These factors are believed to have increased the intensity of trauma to the adjacent duodenal wall. Consequently, the deformed wire barbs of the outer wall stent's distal tip caused stent-induced retroperitoneal perforation and fistula.

The mainstays of treatment for early perforations without systemic upset are nasogastric suction, antibiotics, bowel rest and parenteral nutrition<sup>[15]</sup>. Our patient received conservative treatment and improved after stent removal. However, the patient suddenly bled to death due to the onset of an aortic aneurysm, the aortic wall might have been eroded as a result of retroperitoneal para-aortic irritation or inflammation through the fistula.

In summary, we can learn some lessons from this case. One is that stent-induced late perforation and related lethal complications may develop as a result of a distally migrated uncovered stent with sharp distal barbs and deformity caused by repeated stent removal trials and external abdominal pressure. The other is that early diagnosis and management are essential to prevent significant complications.

## REFERENCES

- 1 Saranga Bharathi R, Rao P, Ghosh K. Iatrogenic duodenal perforations caused by endoscopic biliary stenting and stent migration: an update. *Endoscopy* 2006; **38**: 1271-1274
- 2 Maire F, Hammel P, Ponsot P, Aubert A, O'Toole D, Hentic O, Levy P, Ruszniewski P. Long-term outcome of biliary and duodenal stents in palliative treatment of patients with unresectable adenocarcinoma of the head of pancreas. *Am J Gastroenterol* 2006; **101**: 735-742
- 3 Mutignani M, Tringali A, Costamagna G. Therapeutic biliary endoscopy. *Endoscopy* 2004; **36**: 147-159
- 4 Fogel EL, McHenry L, Sherman S, Watkins JL, Lehman GA. Therapeutic biliary endoscopy. *Endoscopy* 2005; **37**: 139-145
- 5 Humar A, Barron PT, Sekar AS, Lum A. Pancreatitis and duodenal perforation as complications of an endoscopically placed biliary stent. *Gastrointest Endosc* 1994; **40**: 365-366
- 6 Sanchez-Tembleque MD, Naranjo Rodriguez A, Ruiz Morales R, Hervas Molina AJ, Calero Ayala B, de Dios Vega JF. [Duodenal perforation due to an endoscopic biliary prosthesis] *Gastroenterol Hepatol* 2005; **28**: 225-227
- 7 Novacek G, Hormann M, Puig S, Herbst F, Puspok A, Schöfl R. Duodenal perforation secondary to placement of a biliary endoprosthesis diagnosed by multislice computed tomography. *Endoscopy* 2002; **34**: 351
- 8 Roses LL, Ramirez AG, Seco AL, Blanco ES, Alonso DI, Avila S, Lopez BU. Clip closure of a duodenal perforation secondary to a biliary stent. *Gastrointest Endosc* 2000; **51**: 487-489
- 9 Fiori E, Mazzoni G, Galati G, Lutz SE, Cesare A, Bononi M, Bolognese A, Tocchi A. Unusual breakage of a plastic biliary endoprosthesis causing an enterocutaneous fistula. *Surg Endosc* 2002; **16**: 870
- 10 Martin DF, Tweedle DE. Retroperitoneal perforation during ERCP and endoscopic sphincterotomy: causes, clinical features and management. *Endoscopy* 1990; **22**: 174-175
- 11 Enns R, Eloubeidi MA, Mergener K, Jowell PS, Branch MS, Pappas TM, Baillie J. ERCP-related perforations: risk factors and management. *Endoscopy* 2002; **34**: 293-298
- 12 Paikos D, Gatopoulou A, Moschos J, Soufleris K, Tarpagos A, Katsos I. Migrated biliary stent predisposing to fatal ERCP-related perforation of the duodenum. *J Gastrointest Liver Dis* 2006; **15**: 387-388
- 13 Park do H, Kim MH, Choi JS, Lee SS, Seo DW, Kim JH, Han J, Kim JC, Choi EK, Lee SK. Covered versus uncovered wallstent for malignant extrahepatic biliary obstruction: a cohort comparative analysis. *Clin Gastroenterol Hepatol* 2006; **4**: 790-796
- 14 Yoon WJ, Lee JK, Lee KH, Lee WJ, Ryu JK, Kim YT, Yoon YB. A comparison of covered and uncovered Wallstents for the management of distal malignant biliary obstruction. *Gastrointest Endosc* 2006; **63**: 996-1000
- 15 Putcha RV, Burdick JS. Management of iatrogenic perforation. *Gastroenterol Clin North Am* 2003; **32**: 1289-1309

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## CASE REPORT

# Tuberculous lymphadenitis as a cause of obstructive jaundice: A case report and literature review

Radoje Colovic, Nikica Grubor, Rada Jesic, Marjan Micev, Tanja Jovanovic, Natasa Colovic, Henry Dushan Atkinson

Radoje Colovic, Nikica Grubor, Rada Jesic, Marjan Micev, Natasa Colovic, Institute for Digestive Diseases, First Surgical Clinic, Clinical Center of Serbia, Belgrade 11000, Serbia

Tanja Jovanovic, Institute of Microbiology, Belgrade School of Medicine, Belgrade 11000, Serbia

Henry Dushan Atkinson, Imperial College School of Medicine, St Mary's Hospital, Praed Street, London W2 1NY, United Kingdom

**Author contributions:** Colovic R, Grubor N, Jesic R and Colovic N undertook the surgery and clinical care of the patient; Jovanovic T made PCR analysis; Micev M performed histopathological analysis; Colovic R, Atkinson HD, Grubor N and Colovic N wrote the paper.

**Correspondence to:** Nikica Grubor, MD, Institute for Digestive Diseases, First Surgical Clinic, Clinical Center of Serbia, Koste Todorovica 6, Belgrade 11000, Serbia. [ngrubor@eunet.yu](mailto:ngrubor@eunet.yu)

Telephone: +381-11-3610715 Fax: +381-11-3615569

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**Peer reviewers:** William Dickey, Altnagelvin Hospital, Londonderry, Northern Ireland BT47 6SB, United Kingdom; Serdar Karakose, Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey; Qin Su, Department of Pathology, Cancer Hospital and Cancer Institute, Chinese Academy of Medical Sciences and Peking Medical College, PO Box 2258, Beijing 100021, China

Colovic R, Grubor N, Jesic R, Micev M, Jovanovic T, Colovic N, Atkinson HD. Tuberculous lymphadenitis as a cause of obstructive jaundice: A case report and literature review. *World J Gastroenterol* 2008; 14(19): 3098-3100 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3098.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3098>

## Abstract

Obstructive jaundice secondary to tuberculosis (TB) is extremely rare. It can be caused by TB enlargement of the head of the pancreas, TB lymphadenitis, TB stricture of the biliary tree, or a TB mass of the retroperitoneum. A 29-year-old man with no previous history of TB presented with abdominal pain, obstructive jaundice, malaise and weight loss. Ultrasonography (US), computer tomography (CT) scan and endoscopic retrograde cholangiopancreatography (ERCP) were suggestive of a stenosis of the distal common bile duct (CBD) caused by a mass in the posterior head of the pancreas. Tumor markers, CEA and CA19-9 were within normal limits. At operation, an enlarged, centrally caseous lymph node of the posterior head of the pancreas was found, causing inflammatory stenosis and a fistula with the distal CBD. The lymph node was removed and the bile duct resected and anastomosed with the Roux-en Y jejunal limb. Histology and PCR based-assay confirmed tuberculous lymphadenitis. After an uneventful postoperative recovery, the patient was treated with anti-tuberculous medication and remained well 2.5 years later. Though obstructive jaundice secondary to tuberculous lymphadenitis is rare, abdominal TB should be considered as a differential diagnosis in immunocompromised patients and in TB endemic areas. Any stenosis or fistulation into the CBD should also be taken into consideration, and biliary bypass surgery be performed to both relieve jaundice and prevent further stricture.

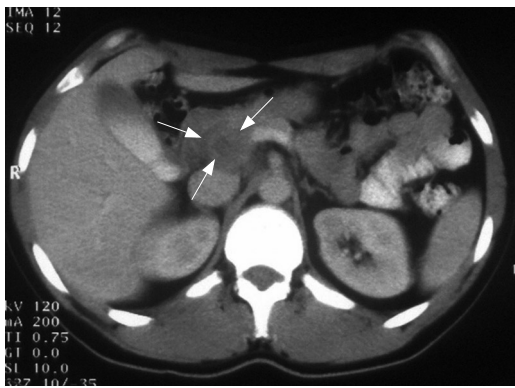
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## INTRODUCTION

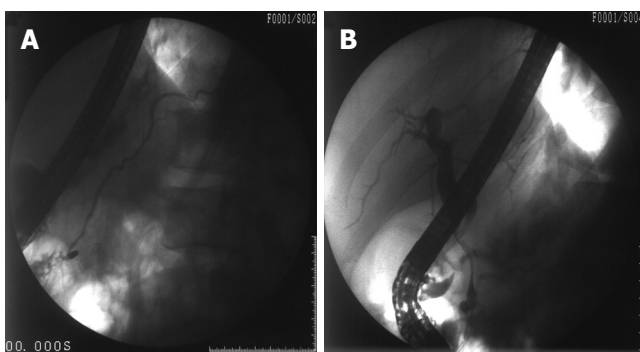
Abdominal tuberculosis (ATB) is rare and obstructive jaundice caused by tuberculosis (TB) is extremely rare. ATB can mimic more common noninfectious abdominal syndromes and is often overlooked because of its low incidence. The mechanisms by which ATB causes bile duct obstruction are varied. We describe a patient with biliary obstruction caused by enlarged tuberculous lymph nodes.

## CASE REPORT

A 29-year-old man presented to our unit with epigastric pain and tenderness on examination, and jaundice, steatorrhea, malaise and weight loss of 7 kg over the preceding 6 mo. Total bilirubin was 163  $\mu\text{mol/L}$  and direct bilirubin 88  $\mu\text{mol/L}$ ; SGOT, SGPT, gamma GT and alkaline phosphatase were moderately elevated. Other laboratory tests including the tumor markers CEA and CA19-9 were all within normal limits. HBsAg and HCV were negative. Abdominal ultrasonography (US) revealed a semi-solid hypoechogenic lesion 39 mm  $\times$  40 mm in size around the head of the pancreas, with two enlarged lymph nodes lying above this, and a common bile duct measuring 10 mm in diameter. Computer tomography (CT) scan showed a low density mass on the posterior aspect of the head of the pancreas with a contrast enhancing solid-rim (Figure 1). Pancreatography was normal, however severe narrowing of the distal common bile duct (CBD) was seen on endoscopic retrograde cholangiopancreatography (ERCP) (Figure 2A and B).



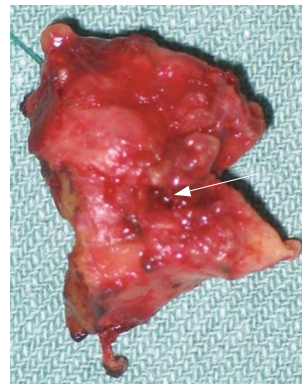
**Figure 1** Abdominal CT-scan showing a low density mass on the posterior aspect of the head of the pancreas with contrast enhancing solid rim (arrow).



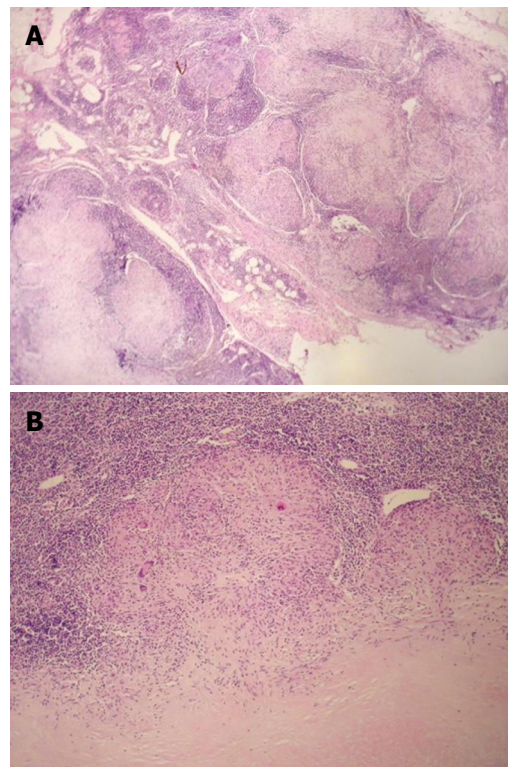
**Figure 2** A: ERCP with a normal pancreatogram; B: A smooth long severe narrowing of the distal common bile duct.

The patient underwent open surgery, and at operation the liver was found to be slightly firm, gallbladder moderately dilated, Lund's lymph node enlarged (about 1.5 cm), common bile duct moderately dilated and two lymph nodes close to the common hepatic artery also enlarged (2 cm and 3 cm). After mobilizing the duodenum and the head of the pancreas, an enlarged (4 cm) soft lymph node adherent to the distal CBD, was removed. The lymph node had a solid surface with a soft and caseous centre, and had a fistulous connection with the posterior aspect of the CBD. Frozen section histology of the lymph node revealed chronic granulomatous inflammation. The gallbladder, Lund's lymph node, the two other enlarged lymph nodes lying close to the common hepatic artery, and a specimen of liver was removed and sent for histology. The narrowed distal CBD was resected, the distal end over sewn, and the proximal end anastomosed with a Roux-en-Y jejunal limb. The resected specimen included the fistulous opening on the posterior wall of the CBD (Figure 3).

The patient had an uneventful postoperative recovery, and bilirubin levels normalised within two weeks. Histology of the liver and gallbladder was normal. The resected CBD showed epithelial ulceration and inflammation with a number of necrotizing granulomata. The lymph nodes had a chronic granulomatous appearance with large merged necrotic areas, and smaller epithelioid-type granulomata with occasional multinuclear giant cells (Figure 4A and B) suggestive of tuberculous lymphadenitis. The diagnosis was confirmed with a polymerase chain



**Figure 3** The resected part of the common bile duct with fistula on the posterior wall (arrow).



**Figure 4** A: Extensive chronic granulomatous lymphadenitis (HE, × 13); B: Focal tuberculoid granuloma formation (HE, × 64).

reaction (PCR) using automated analyzer Cobas/Roche/, with the Amplicor Mycobacterium tuberculosis assay. No previous specific risk for TB was found in the patient. He was treated with anti-tuberculous quadruple therapy and achieved gradual clinical improvement, with resolution of pain and malaise, and a weight gain of 10 kg over the next 6 mo. He remained well at 2.5 years postoperatively.

## DISCUSSION

Obstructive jaundice secondary to abdominal TB is extremely rare. Four mechanisms have been described: TB of the pancreas itself may cause pseudoneoplastic obstructive jaundice<sup>[1-10]</sup>; it may be secondary to TB lymphadenitis causing compression and inflammation of the lymph nodes and the CBD<sup>[6,11-19]</sup>, as in our case, with caseation of the lymph node causing fistulation into the CBD; biliary TB itself may lead to single or multiple



strictures, mimicking cholangiocarcinoma<sup>[20-25]</sup>; and TB can create a retroperitoneal mass leading to biliary tree obstruction<sup>[26]</sup>.

The diagnosis of abdominal TB should be considered in the context of a mass in the head of the pancreas in the immunocompromised patients and in countries with endemic TB<sup>[7]</sup>, after the exclusion of malignancy and other biliary inflammation. TB lymphadenitis can be suspected when a contrast-enhanced CT scan demonstrates low density masses surrounded by an enhancing solid rim<sup>[14]</sup>, or when ERCP demonstrates a normal pancreatogram with a smooth narrowing of the CBD<sup>[18]</sup>, as were seen in our patient. FDG-PET scanning has not been shown to be useful in distinguishing TB from pancreatic malignancy, as both conditions have an increased uptake of the FDG metabolite<sup>[18]</sup>. US or CT-guided percutaneous fine needle aspiration (FNA) of the enlarged lymph nodes may be useful<sup>[7]</sup>, but is often not definitive<sup>[21]</sup>. Cytology of CBD aspirate, however, obtained by ERCP, may be confirmatory in the presence of the acid-fast bacillus (*Mycobacterium tuberculosis*); alternatively PCR of the aspirate may be diagnostic<sup>[19]</sup>. However, in the case of a periportal lymphadenopathy causing obstructive jaundice, as in our patient, these FNA tests are only positive if a fistula exists between the TB lymph node and the CBD, allowing bacilli to pass into the CBD<sup>[19]</sup>. Other potential diagnostic methods include obtaining tissue specimens by laparoscopy<sup>[17]</sup> or endoscopic ultrasound with FNA<sup>[27]</sup>. Though in practice, the diagnosis is often established at operation<sup>[6,7,18,26]</sup> or even after surgery by histology<sup>[4]</sup> or PCR-based assay<sup>[2,4,6,8,10]</sup>, as was the case in our patient.

The great benefit of a preoperative diagnosis of TB causing the obstruction is that a more conservative path could be followed, involving removal of the obstructing lymph node alone, followed by anti-TB medications<sup>[18]</sup>. In our case more elaborate CBD resective surgery was undertaken for presumed malignancy.

However, even though TB lymphadenitis was suspected in our patient after intraoperative frozen section, resection of the involved part of the CBD was necessary as the bile duct was already strictured, and eventual closure of the fistula would probably result in additional stenosis or even complete obstruction of the CBD. Thus inexplicable stenosis of the CBD should be taken into consideration in the context of pancreatic or TB lymphadenitis associated with obstructive jaundice and be treated by biliary bypass surgery<sup>[7]</sup> in addition to anti-TB medication.

## REFERENCES

- Crowson MC, Perry M, Burden E. Tuberculosis of the pancreas: a rare cause of obstructive jaundice. *Br J Surg* 1984; **71**: 239
- Chen CH, Yang CC, Yeh YH, Yang JC, Chou DA. Pancreatic tuberculosis with obstructive jaundice--a case report. *Am J Gastroenterol* 1999; **94**: 2534-2536
- Shan YS, Sy ED, Lin PW. Surgical resection of isolated pancreatic tuberculosis presenting as obstructive jaundice. *Pancreas* 2000; **21**: 100-101
- Kouraklis G, Glinavou A, Karayiannakis A, Karatzas G. Primary tuberculosis of the pancreas mimicking a pancreatic tumor. *Int J Pancreatol* 2001; **29**: 151-153
- Singh B, Moodley J, Batitang S, Chetty R. Isolated pancreatic tuberculosis and obstructive jaundice. *S Afr Med J* 2002; **92**: 357-359
- Xia F, Poon RT, Wang SG, Bie P, Huang XQ, Dong JH. Tuberculosis of pancreas and peripancreatic lymph nodes in immunocompetent patients: experience from China. *World J Gastroenterol* 2003; **9**: 1361-1364
- El Mansari O, Tajdine MT, Mikou I, Janati MI. [Pancreatic tuberculosis. Report of two cases] *Gastroenterol Clin Biol* 2003; **27**: 548-550
- Panzuto F, D'Amato A, Laghi A, Cadau G, D'Ambra G, Aguzzi D, Iannaccone R, Montesani C, Caprilli R, Delle Fave G. Abdominal tuberculosis with pancreatic involvement: a case report. *Dig Liver Dis* 2003; **35**: 283-287
- Kumar R, Kapoor D, Singh J, Kumar N. Isolated tuberculosis of the pancreas: a report of two cases and review of the literature. *Trop Gastroenterol* 2003; **24**: 76-78
- Beaulieu S, Chouillard E, Petit-Jean B, Vitte RL, Eugene C. [Pancreatic tuberculosis: a rare cause of pseudoneoplastic obstructive jaundice] *Gastroenterol Clin Biol* 2004; **28**: 295-298
- Kohen MD, Altman KA. Jaundice due to a rare cause: tuberculous lymphadenitis. *Am J Gastroenterol* 1973; **59**: 48-53
- Murphy TF, Gray GF. Biliary tract obstruction due to tuberculous adenitis. *Am J Med* 1980; **68**: 452-454
- Stanley JH, Yantis PL, Marsh WH. Periportal tuberculous adenitis: a rare cause of obstructive jaundice. *Gastrointest Radiol* 1984; **9**: 227-229
- Mathieu D, Ladeb MF, Guigui B, Rousseau M, Vasile N. Periportal tuberculous adenitis: CT features. *Radiology* 1986; **161**: 713-715
- Alvarez SZ, Sollano JD Jr. ERCP in hepatobiliary tuberculosis. *Gastrointest Endosc* 1998; **47**: 100-104
- Queralt CB, Cruz JM, Comet V Jr, Almajano C, Val-Carres C. [Obstructive jaundice due to peripancreatic tuberculous adenitis] *Rev Esp Enferm Dig* 1992; **82**: 201-202
- Poon RT, Lo CM, Fan ST. Diagnosis and management of biliary obstruction due to periportal tuberculous adenitis. *Hepatogastroenterology* 2001; **48**: 1585-1587
- Obama K, Kanai M, Taki Y, Nakamoto Y, Takabayashi A. Tuberculous lymphadenitis as a cause of obstructive jaundice: report of a case. *Surg Today* 2003; **33**: 229-231
- Probst A, Schmidbaur W, Jechart G, Hammond A, Zentner J, Niculescu E, Messmann H. Obstructive jaundice in AIDS: diagnosis of biliary tuberculosis by ERCP. *Gastrointest Endosc* 2004; **60**: 145-148
- Fan ST, Ng IO, Choi TK, Lai EC. Tuberculosis of the bile duct: a rare cause of biliary stricture. *Am J Gastroenterol* 1989; **84**: 413-414
- Behera A, Kochhar R, Dhavan S, Aggarwal S, Singh K. Isolated common bile duct tuberculosis mimicking malignant obstruction. *Am J Gastroenterol* 1997; **92**: 2122-2123
- Yeh TS, Chen NH, Jan YY, Hwang TL, Jeng LB, Chen MF. Obstructive jaundice caused by biliary tuberculosis: spectrum of the diagnosis and management. *Gastrointest Endosc* 1999; **50**: 105-108
- Kok KY, Yapp SK. Tuberculosis of the bile duct: a rare cause of obstructive jaundice. *J Clin Gastroenterol* 1999; **29**: 161-164
- Inal M, Aksungur E, Akgul E, Demirbas O, Oguz M, Erkocak E. Biliary tuberculosis mimicking cholangiocarcinoma: treatment with metallic biliary endoprosthesis. *Am J Gastroenterol* 2000; **95**: 1069-1071
- Prasad A, Pandey KK. Tuberculous biliary strictures: uncommon cause of obstructive jaundice. *Australas Radiol* 2001; **45**: 365-368
- Jazet IM, Perk L, De Roos A, Bolk JH, Arend SM. Obstructive jaundice and hematemesis: two cases with unusual presentations of intra-abdominal tuberculosis. *Eur J Intern Med* 2004; **15**: 259-261
- Woodfield JC, Windsor JA, Godfrey CC, Orr DA, Officer NM. Diagnosis and management of isolated pancreatic tuberculosis: recent experience and literature review. *ANZ J Surg* 2004; **74**: 368-371

# Primary lymphoblastic B-cell lymphoma of the stomach: A case report

Miao-Xia He, Ming-Hua Zhu, Wei-Qiang Liu, Li-Li Wu, Xiong-Zeng Zhu

Miao-Xia He, Ming-Hua Zhu, Wei-Qiang Liu, Li-Li Wu,  
Department of Pathology, Changhai Hospital, Second Military  
Medical University, Shanghai 200433, China

Xiong-Zeng Zhu, Department of Pathology, Tumor Hospital,  
Fudan University, Shanghai 200032, China

**Author contributions:** He MX wrote the paper and organized the  
figures and patient data; The diagnosis and differential diagnosis  
were carried out by Zhu XZ, Zhu MH, Liu WQ and Wu LL; Zhu  
XZ helped organize and correct the paper; Zhu MH supervised the  
writing and organization process.

**Correspondence to:** Miao-Xia He, Department of Pathology,  
Changhai Hospital, Second Military Medical University, Shanghai  
200433, China. [hmm26@163.com](mailto:hmm26@163.com)

Telephone: +86-21-25074851 Fax: +86-21-25074604

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## Abstract

Primary stomach lymphoblastic B-cell lymphoma (B-LBL) is a rare tumor. We describe a primary stomach B-LBL in a 38 years old female who presented with nonspecific complaints of fatigue and vomiting for 2 mo. Gastrofiberscopy revealed a large gastric ulcer, which was successfully resected. Pathology showed a lymphoblastic cell lymphoma arising from the stomach, and there was no evidence of disease at any extrastomach site. Immunohistochemical staining and gene rearrangement studies supported that the stomach tumor was a clonal B-cell lymphoma. Therefore, the diagnosis of B-LBL was made based on the stomach specimen.

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**Key words:** Primary stomach lymphoma; Lymphoblastic lymphoma; B-cell

**Peer reviewer:** Ibrahim A Al Mofleh, Professor, Department of  
Medicine, College of Medicine, King Saud University, PO Box  
2925, Riyadh 11461, Saudi Arabia

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## INTRODUCTION

Precursor B-cell lymphoblastic lymphoma (B-LBL) is a

neoplasm of lymphoblasts committed to the B-cell lineage, which is an uncommon type of lymphoma and accounts for less than 10% of the total cases of lymphoblastic lymphoma. It usually affects people at a younger age. Most cases reported in a literature review are less than 18 years of age, some patients are under 35 years of age and the median age is 20 years<sup>[1]</sup>. Unlike precursor T-cell lymphoblastic lymphoma, precursor B-cell lymphoblastic lymphoma commonly involves lymph nodes or extranodal sites, mediastinal masses are infrequent. The most frequent sites of B-LBL lesions are the skin, bone, soft tissue, and lymph nodes<sup>[1-3]</sup>.

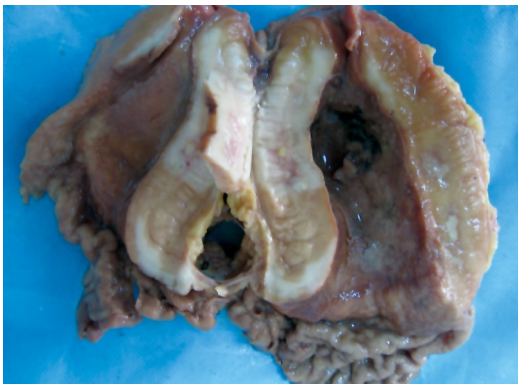
Primary stomach lymphoma is defined as an extranodal non-Hodgkin's lymphoma of any cell type, with no evidence of extrastomach dissemination. The majority of stomach cases reported in the English literature are extranodal marginal zone B cell lymphoma of mucosa-associated lymphoid tissue (MALToma), diffuse large B cell lymphoma (DLBCL), nasal type NK/T cell lymphoma, *etc*<sup>[3-5]</sup>. Primary stomach B-LBL is rare. Here we present a case of primary B-LBL involving the stomach.

## CASE REPORT

The patient was a 38 years old female who had an unremarkable past medical history. She presented with nonspecific complaints of fatigue and vomiting for 2 mo. Gastrofiberscopy revealed a large gastric ulcer. During surgery, a large neoplastic ulcer was found in the stomach and gastric wall was diffusely thickened. The tumor was successfully resected with adjacent portions of the stomach. Good macroscopic margins were obtained. No other masses or enlarged lymph nodes were present. Examination of the bone marrow showed 13% immature lymphoid cells, the leukocyte count of peripheral blood was  $12 \times 10^9/L$ , and the percentage of peripheral blood lymphocytes was 35%.

Grossly, the greater and lesser curvatures of the resected stomach measured 22 cm and 11 cm, respectively. There was a well circumscribed neoplastic ulcer measuring 10 cm × 8 cm × 2.5 cm in antro-pyloric region of the stomach (Figure 1). A small necrosis was found on surface of the neoplastic ulcer. The cut surface of the tumor was grey and firm. Tumor tissue was found in the gastric serosa. Portions of tumor tissue were fixed in formalin and embedded in paraffin and cut into sections which were stained with HE for routine histomorphology. Additional sections of paraffin-embedded tissue were used





**Figure 1** A large neoplastic ulcer in the stomach involving the whole gastric wall.

for immunohistochemical staining and gene rearrangement analysis.

Microscopically, the tumor cells were uniform, medium-sized immature lymphoid cells, their nuclei contained evenly dispersed nuclear chromatin with a high nuclear/cytoplasmic ratio. The nuclei were round or oval or convoluted in shape. A large number of mitotic figures were appreciated. The tumor cells were diffusely distributed in the gastric glands, and found in all layers of the gastric wall. There were tumor emboli within the gastric wall lymphatic vessels. Lymphoepithelial lesions were not found (Figure 2).

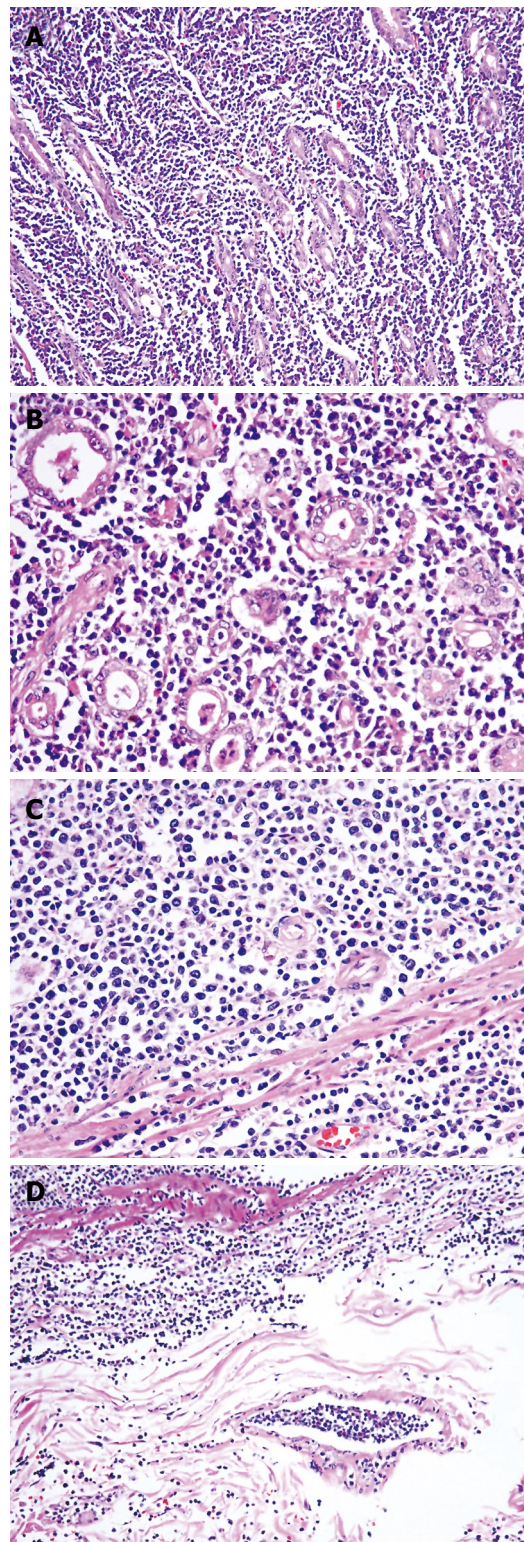
Immunohistochemistry analysis revealed immature lymphoid cells positive for cytoplasmic CD10 and CD79a, nuclear TdT and CD99 antigens, and negative for CD20 antigen. About 50%-70% of the tumor cells were reactive for Ki-67 (Figure 3). Gene rearrangement analysis showed monoclonal immunoglobulin high chain gene rearrangement (Figure 4).

The morphology, immunophenotype, and gene rearrangement of the neoplastic cells supported the diagnosis of stomach precursor B-LBL. There was no evidence that supported the diagnosis of precursor B lymphoblastic leukemia in the bone marrow and peripheral blood.

## DISCUSSION

Primary stomach lymphomas are in the minority, most of which are mucosa-associated lymphoid tissue B cell lymphoma, diffuse large B cell lymphoma, extranodal nasal type NK/T cell lymphoma, *etc.* Primary stomach B-LBL is rare<sup>[1-5]</sup>.

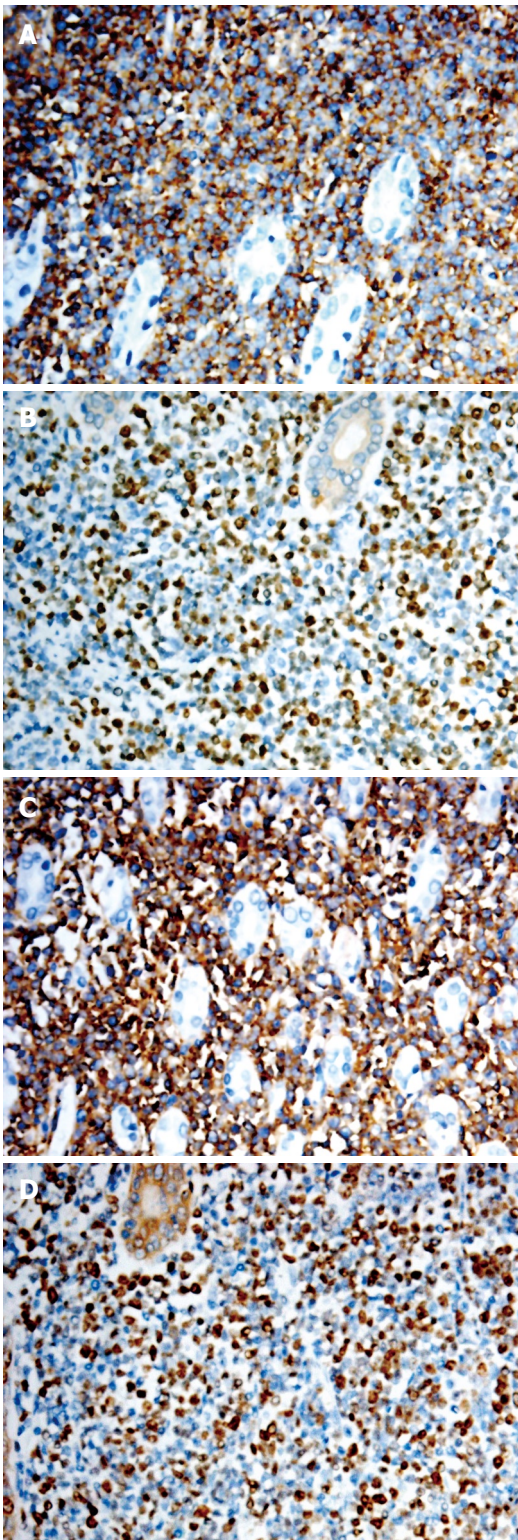
Ninety percent of lymphoblastic lymphomas are of precursor T cell lineage and only 10% are of precursor B cell lineage<sup>[1,2]</sup>. Precursor B lymphoblastic lymphoma (B-LBL)/leukemia (B-ALL) are originated from B cell lineage. ALL and LBL represent different clinical presentations of the same neoplasm and are grouped in the category of precursor B-cell lymphoblastic leukemia/lymphoma by the revised World Health Organization classification of lymphoid neoplasms<sup>[1]</sup>. Because of the biologic unity of B-ALL and B-LBL, the criteria for distinguishing B-LBL from B-ALL are also arbitrary and applied inconsistently. In some studies<sup>[2,6,7]</sup>, patients



**Figure 2** Diffuse proliferation of lymphoblastic cells between gastric glands of stomach B-LBL. A: HE, × 100; B: HE, × 200; C: HE, × 200; D: Tumor emboli within lymphatic vessels of the gastric wall (HE, × 100).

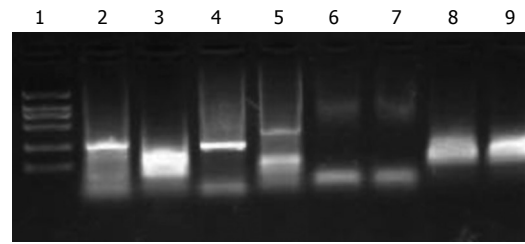
with B-LBL and acute leukemia are included, but in other studies<sup>[3,4,6]</sup>, patients with leukemic involvement are excluded. According to the new WHO classification of lymphoid neoplasms, when the process is confined to a mass lesion without any or minimal evidence of blood and marrow involvement, the diagnosis is lymphoma; when extensive marrow and blood are involved, lymphoblastic





**Figure 3** Stomach B-LBL (EnVision,  $\times 200$ ). **A:** Lymphoblasts diffusely stained with anti-CD79a; **B:** A large number of lymphoblasts positive for nuclear antigen TdT; **C:** Lymphoblasts diffusely stained with anti-CD10; **D:** A large number of lymphoblasts positive for Ki-67.

leukemia is the appropriate term; if a patient presents with a mass lesion and the number of lymphoblasts is less than 25% in the marrow, the diagnosis of lymphoma is preferred<sup>[1,3-5]</sup>. Precursor B lymphoblastic B-LBL most commonly involves the skin, bone, soft tissue, and lymph nodes, whereas stomach B-LBL is uncommon<sup>[6,8]</sup>.



**Figure 4** Polymerase chain reaction showing rearrangement of immunoglobulin heavy-chain genes. Lane 1: Marker; lane 2: FR2, sample collected from stomach B cell lymphoblastic lymphoma showing monoclonal pattern; lane 3: FR3A, sample collected from stomach B cell lymphoblastic lymphoma showing a smear; lane 4: FR2, B cell lymphoma cell line Raja used as positive control; lane 5, FR3A, B cell lymphoma cell line Raja used as positive control. Lane 6, JVI, sample collected from stomach B cell lymphoblastic lymphoma showing a negative pattern; lane 7: JVI, sample collected from stomach B cell lymphoblastic lymphoma showing a negative pattern; lane 8: JVI, T cell lymphoma cell line Jurkat used as a positive control; lane 9: JVI, T cell lymphoma cell line Jurkat used as a positive control.

Leukemic presentation with involvement of peripheral blood and bone marrow is most common. Extramedullary involvement is frequent with particular pre-direction for the central nervous system, lymph nodes, spleen, liver, and gonads. The leukocyte count may be decreased, normal or markedly elevated. The etiology of B-LBL is unknown. However, there is evidence that suggests a genetic factor in some cases<sup>[9,10]</sup>. The neoplastic cells of B-LBL are morphologically indistinguishable from those of B-ALL and typically small to medium size blast cells, with scant cytoplasm, moderately condensed to dispersed fine chromatin, inconspicuous nucleoli, and a high mitotic rate. Immunophenotypically, the neoplastic cells express terminal deoxynucleotidyl transferase (TdT) and B-cell antigens, such as CD79a, CD10, CD19, and CD22<sup>[1,2,9]</sup>.

Our review of the literature revealed few case reports of stomach B-LBL lymphoma. In this report, we describe an exceptional primary stomach precursor B-LBL with no evidence of disease at any extrastomach site. The number of lymphoblasts was less than 25% in the marrow and there was no evidence in the peripheral blood. Since immunohistochemical study showed the neoplastic lymphoid cells of precursor B-cell type, the diagnosis of B-LBL was made in this stomach case<sup>[1,9]</sup>. Precursor B-LBL, a potentially curable disease, an aggressive therapy is very important<sup>[7,11-12]</sup>. In this report, after surgical resection and final pathologic diagnosis of B-LBL, the patient was treated with chemotherapy. Now, she is followed-up at the outpatient clinic and in complete remission half a year after the initial diagnosis.

The differential diagnosis of precursor B-LBL includes myeloid lymphoma/leukemia, Burkitt's lymphoma, and precursor T-LBL, *etc.* The tumor morphology and immunophenotype help differential diagnosis. Meanwhile, the precursor B-ALL should be excluded<sup>[1,13]</sup>.

## REFERENCES

- 1 **Precursor B lymphoblastic leukemia/lymphoblastic lymphoma.** In: Jaffe ES, Harris NL, Stein H, Stein H, Vardiman JW. World Health Organization classification of tumour, Pathology and genetics of tumours of haematopoietic and

- lymphoid tissues. International agency for reseach on cancer. Lyon, France: IARC Press, 2001: 109-117
- 2 **Soslow RA**, Baergen RN, Warnke RA. B-lineage lymphoblastic lymphoma is a clinicopathologic entity distinct from other histologically similar aggressive lymphomas with blastic morphology. *Cancer* 1999; **85**: 2648-2654
  - 3 **Lin P**, Jones D, Dorfman DM, Medeiros LJ. Precursor B-cell lymphoblastic lymphoma: a predominantly extranodal tumor with low propensity for leukemic involvement. *Am J Surg Pathol* 2000; **24**: 1480-1490
  - 4 **Maitra A**, McKenna RW, Weinberg AG, Schneider NR, Kroft SH. Precursor B-cell lymphoblastic lymphoma. A study of nine cases lacking blood and bone marrow involvement and review of the literature. *Am J Clin Pathol* 2001; **115**: 868-875
  - 5 **Burkhardt B**, Zimmermann M, Oschlies I, Niggli F, Mann G, Parwaresch R, Riehm H, Schrappe M, Reiter A. The impact of age and gender on biology, clinical features and treatment outcome of non-Hodgkin lymphoma in childhood and adolescence. *Br J Haematol* 2005; **131**: 39-49
  - 6 **Bassi D**, Lentzner BJ, Mosca RS, Alobeid B. Primary cardiac precursor B lymphoblastic lymphoma in a child: a case report and review of the literature. *Cardiovasc Pathol* 2004; **13**: 116-119
  - 7 **Zinzani PL**, Bendandi M, Visani G, Gherlinzoni F, Frezza G, Merla E, Manfroi S, Gozzetti A, Tura S. Adult lymphoblastic lymphoma: clinical features and prognostic factors in 53 patients. *Leuk Lymphoma* 1996; **23**: 577-582
  - 8 **Kim JY**, Kim YC, Lee ES. Precursor B-cell lymphoblastic lymphoma involving the skin. *J Cutan Pathol* 2006; **33**: 649-653
  - 9 **Medeiros LJ**, Carr J. Overview of the role of molecular methods in the diagnosis of malignant lymphomas. *Arch Pathol Lab Med* 1999; **123**: 1189-1207
  - 10 **Hojo H**, Sasaki Y, Nakamura N, Abe M. Absence of somatic hypermutation of immunoglobulin heavy chain variable region genes in precursor B-lymphoblastic lymphoma: a study of four cases in childhood and adolescence. *Am J Clin Pathol* 2001; **116**: 673-682
  - 11 **Le Gouill S**, Lepretre S, Briere J, Morel P, Bouabdallah R, Raffoux E, Sebban C, Lepage E, Brice P. Adult lymphoblastic lymphoma: a retrospective analysis of 92 patients under 61 years included in the LNH87/93 trials. *Leukemia* 2003; **17**: 2220-2224
  - 12 **Kantarjian HM**, O'Brien S, Smith TL, Cortes J, Giles FJ, Beran M, Pierce S, Huh Y, Andreeff M, Koller C, Ha CS, Keating MJ, Murphy S, Freireich EJ. Results of treatment with hyper-CVAD, a dose-intensive regimen, in adult acute lymphocytic leukemia. *J Clin Oncol* 2000; **18**: 547-561
  - 13 **Pui CH**, Robison LL, Look AT. Acute lymphoblastic leukaemia. *Lancet* 2008; **371**: 1030-1043

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## Perivascular epithelioid cell tumour of the liver

Unne Stenram

Unne Stenram, Department of Pathology, Lund University, Lund SE-22185, Sweden

Author contribution: Stenram U contributed all to this paper.

Correspondence to: Unne Stenram, Department of Pathology, University Hospital, Lund SE-22185,

Sweden. [unne.stenram@med.lu.se](mailto:unne.stenram@med.lu.se)

Telephone: +46-46-173407 Fax: +46-46-143307

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### Abstract

Perivascular epithelioid cell tumour is not uncommon in the liver but seldom malignant.

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**Key words:** Perivascular epithelioid cell tumour; Liver

**Peer reviewer:** David Adams, Professor, Liver Research Laboratories, Institute for Biomedical Research, Queen Elizabeth Hospital, University of Birmingham, Birmingham B15 2TT, United Kingdom

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### TO THE EDITOR

I read with great interest the work by Fang, Zhou, Jin and Hu, dealing with perivascular epithelioid cell tumour (angiomyolipoma) of the liver<sup>[1]</sup>. It is right that, in mesenchymal tissues, the tumour is common in the uterus. The most frequent localization generally is, however, in the kidney<sup>[2,3]</sup>. In the liver, 110 cases had been described by 1999<sup>[3]</sup>.

It is true that the malignant cases are much fewer.

Our interest in this entity was arisen, when we scrutinized a hepatic tumour, described from our department as an oncocytic adenoma<sup>[4]</sup>. However, the tumour later turned out to be positive for the melanocytic marker HMB-45 and is in fact a perivascular epithelioid cell tumour. The journal refused to publish the revised diagnosis!

### REFERENCES

- 1 Fang SH, Zhou LN, Jin M, Hu JB. Perivascular epithelioid cell tumor of the liver: a report of two cases and review of the literature. *World J Gastroenterol* 2007; **13**: 5537-5539
- 2 Hornick JL, Fletcher CD. PEComa: what do we know so far? *Histopathology* 2006; **48**: 75-82
- 3 Tryggvason G, Blondal S, Goldin R, Albrechtsen J, Björnsson J, Jonasson J. Epithelioid angiomyolipoma of the liver: case report and review of the literature. *APMIS* 2004; **112**: 612-616
- 4 el Hag IA, Ekberg H, Tranberg KG, Lundstedt C, Johansson S, Sassner P, Hagerstrand I. Oncocytic liver tumours and arterial dilatation. Case report. *Eur J Surg* 1994; **160**: 55-59

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## LETTERS TO THE EDITOR

# Role of silis in esophageal cancer

Ali Jabbari, Sima Besharat, Shahryar Semnani

Ali Jabbari, Sima Besharat, Shahryar Semnani, Golestan University of Medical Sciences, Golestan Research Center of Gastroenterology and Hepatology, Gorgan City 49177-44563, Golestan Province, Iran

**Author contributions:** Jabbari A and Besharat S contributed equally to this work, designed and performed the research and wrote the paper; Semnani S designed the first idea of the work.

**Correspondence to:** Sima Besharat, MD, Researcher, Golestan University of Medical Sciences, Golestan Research Center of Gastroenterology and Hepatology, 21st Edalat, Vali\_e\_asr Ave, Gorgan City 49177-44563, Golestan Province, Iran. [s\\_besharat\\_gp@yahoo.com](mailto:s_besharat_gp@yahoo.com)

Telephone: +98-171-2240835 Fax: +98-171-2269210

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## Abstract

Association of silica with diseases like cancers has been determined previously. This study was designed to determine the quantity of silis in flour produced in Golestan Province, and its relation to esophageal cancer (EC). We took flour samples from all flour millings in Golestan Province. Base-melting method in nickel crucible was used at 550°C. The extract was reduced with acids. Different silis concentrations in various regions were compared.  $P < 0.05$  was considered statistically significant. The median silis concentration was 0.0030 g, the mean silis concentration was  $0.008760 \pm 0.004265$  g in each 100 g flour. The difference of mean silis concentrations in various regions was not significant. No high level of silica was found in the flour of Golestan Province. We could not find any significant difference in various areas between silica contaminations. Studies on the consumed bread and rice in various regions of Golestan Province can be helpful.

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**Key words:** Silis; Esophageal cancer; Flour; Milling; Iran

**Peer reviewer:** Satoshi Osawa, MD, First Department of Medicine, Hamamatsu University School of Medicine, 1-20-1 Handayama, Hamamatsu 431-3192, Japan

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## LETTER TO THE EDITOR

Silica ( $\text{SiO}_2$ ) is an oxide of silicon. Its existence in food

products is a presentation of contamination. Some studies revealed that silica exposure may play a role in diseases like cancer<sup>[1]</sup>, although its definite effect has not been confirmed in some cancers like esophageal cancer. The International Agency for Research on Cancer (IARC) has classified crystalline silica as a known human carcinogen in lung cancer<sup>[2]</sup>. The excess risk of esophageal cancer (EC) mortality among caisson workers with silicosis explains best by the very heavy exposure to free silica dust in their working environment<sup>[3-8]</sup> and their silicosis as an underlining disease.

A case-control study of the relationships among silica exposure, gastric cancer, and EC in Japan, suggested that gastric cancer and EC are related to silica exposure and silicosis in that area, although they did not reach a statistically significant level<sup>[9]</sup>.

O'Neill *et al*<sup>[9]</sup> reported that the contamination with fibrous silica contaminant is high in the diet of north-east of Iran. Low quality of wheat and its contamination with weed and sand are considered important<sup>[10]</sup>. The northeastern part of Iran in Golestan Province (Turkmen Sahra) is known to have the highest incidence of EC in the country and to be one of the highest areas in the world<sup>[11]</sup>. Golestan Province is located on the hot spots that are along a presumptive belt starting from northern China, extending along the southern parts of the former Soviet Union and ending in the Caspian littoral in northern Iran.

In 1982, O'Neill *et al*<sup>[9]</sup> reported an association of silica fibers in the millet bran and esophageal tumors in another study. In 1986, Newman<sup>[1]</sup> found that certain plants contain structures consisting of biogenic silica, which has been supposed as a causative agent in the high cancer areas of Southern Africa, Northeast Iran and North of China. It is hypothesized that these plant mineral fibers are involved in the etiology of EC in Iran and in other high incidence areas. *Phalaris minor* is a known common weed in the Mediterranean area, but it is not considered a region with a high incidence and prevalence of EC.

Some findings suggest that silica particles might be involved in the etiology of EC. In fact, different results are available about the significant relationship between silis exposure and EC, some are in agreement and suppose that silis plays a role in the etiology of EC, and others are in disagreement.

In our study, silis but not its compound or its biologic derivatives was considered a carcinogen. Flour samples from all flour millings in Golestan Province were taken. Base-melting method in nickel crucible was used at 550°C and the extract was reduced with acids. The complex was evaluated with a spectrophotometer (820 nanometer wavelength). Five control samples of wheat seeds and pedicles were examined, too. The different silis median concentrations in

wheat seeds, pedicles and flour were statistically significant. However, the total silis in the flour was in the normal range because a great deal of silis was omitted from the flour during the preparing process. The modern and new purification technologies may be effective in producing these results, so the previous contaminants can be supposed less important.

The mean silis concentration was 0.012, 0.01 and 0.003 in the central, western and eastern parts of Gorgan City, respectively. The differences were not statistically significant. The Golestan province was divided into 3 areas according to the incidence of EC and we matched the data with the location of flour millings on the map.

Our findings suggest that there are no significant differences in flours of various areas, revealing a less important role of silis in EC. However, from a medical point of view it is important. There is a great variation in the incidence of EC between countries and regions. The distribution of EC in Golestan Province is not concordant with the amount of silis reported in this study. Silis concentration is higher in the west part but EC is higher in the east.

Despite the high incidence of EC in Northeast Iran, no significant differences were seen between silis in wheat flour of this area and standard measures. It seems that silis could not play a major role in the etiology of EC or is considered a predisposing factor when we eat it. Perhaps, oral or inhalation absorption of silis has an effect on its carcinogenicity. This hypothesis becomes acceptable when we pay more attention to the main component of earth crust. Silis, an abundant mineral in rock, sand, and soil, is in contact with our skin, but it is not supposed as a carcinogen or a predisposing factor.

Previous studies have reported a considerable concentration of silica in the flour produced in this area and suggested a relationship between EC and silis. In this study, no high level of silica was found in the flour of Golestan Province. Thus, on the one hand, we could not confirm the hypothesis of high contamination of the flour in this area, which is considered a high risk of EC in Golestan Province. On the other hand, we could not rule out the probable role of this element in the etiology of EC. Studies on the consumed bread and rice in various regions of the province can be helpful.

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## REFERENCES

- 1 **Newman R.** Association of biogenic silica with disease. *Nutr Cancer* 1986; **8**: 217-821
- 2 **Yassin A, Yebesi F, Tingle R.** Occupational exposure to crystalline silica dust in the United States, 1988-2003. *Environ Health Perspect* 2005; **113**: 255-260
- 3 **Yu IT, Tse LA, Wong TW, Leung CC, Tam CM, Chan AC.** Further evidence for a link between silica dust and esophageal cancer. *Int J Cancer* 2005; **114**: 479-483
- 4 **Tsuda T, Mino Y, Babazono A, Shigemitsu J, Otsu T, Yamamoto E.** A case-control study of the relationships among silica exposure, gastric cancer, and esophageal cancer. *Am J Ind Med* 2001; **39**: 52-57
- 5 **Siemiatycki J, Germ M, Dewar R, Lakhani R, Begin D, Richardson L.** Silica and cancer associations from a multicenter occupational case-referent study. *IARC Sci Publ*, 1990; **97**: 129-142
- 6 **Pan G, Takahashi K, Feng Y, Liu L, Liu T, Zhang S, Liu N, Okubo T, Goldsmith DF.** Nested case-control study of esophageal cancer in relation to occupational exposure to silica and other dusts. *Am J Ind Med* 1999; **35**: 272-280
- 7 **Soutar CA, Robertson A, Miller BG, Searl A, Bignon J.** Epidemiological evidence on the carcinogenicity of silica: factors in scientific judgement. *Ann Occup Hyg* 2000; **44**: 3-14
- 8 **Johnson WM, Busnardo MS.** Silicosis following employment in the manufacture of silica flour and industrial sand. *J Occup Med* 1993; **35**: 716-719
- 9 **O'Neill C, Pan Q, Clarke G, Liu F, Hodges G, Ge M, Jordan P, Chang U, Newman R, Toulson E.** Silica fragments from millet bran in mucosa surrounding oesophageal tumours in patients in northern China. *Lancet* 1982; **1**: 1202-1206
- 10 **O'Neill CH, Hodges GM, Riddle PN, Jordan PW, Newman RH, Flood RJ, Toulson EC.** A fine fibrous silica contaminant of flour in the high oesophageal cancer area of north-east Iran. *Int J Cancer* 1980; **26**: 617-628
- 11 **Semnani SH, Besharat S, Abdolahi N, Kalavi KH, Fazeli SA, Davarian A, Danesh A, Malekzadeh R.** Esophageal cancer in northeastern Iran. *Indian J Gastroenterol* 2005; **24**: 224

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## SCIENTOMETRICS

# Variations of author origins in *World Journal of Gastroenterology* during 2001-2007

Hua Yang, Yue-Yang Zhao

Hua Yang, Yue-Yang Zhao, Library of Shengjing Hospital, China Medical University, Shenyang 110004, Liaoning Province, China  
Author contributions: Yang H designed and performed the research; Zhao YY edited the manuscript.  
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Correspondence to: Hua Yang, Library of Shengjing Hospital, China Medical University, No. 36 Sanhao Street, Heping District, Shenyang 110004, Liaoning Province, China. [yangh@cmu2h.org](mailto:yangh@cmu2h.org)  
Telephone: +86-24-83956534 Fax: +86-24-83955092  
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## Abstract

**AIM:** To discuss the variations and distributions of authors who published their papers in *World Journal of Gastroenterology* (*WJG*) during 2001-2007 and evaluate the development of *WJG* and gastroenterology core journals in recent years by comparing the contributions of the authors.

**METHODS:** *WJG* articles published in 2001-2007 were searched from *MEDLINE* database (by ISI Web of Knowledge). The variations (cooperation degree, cooperation rate) and distributions of the first authors were analyzed with bibliometric methods. SCIE was used to collect articles published in *Am J Gastroenterol*, *Gastroenterology*, *Scand J Gastroenterol* and *WJG* in 2007, and comparison of the data was made. Comparison indicators included the article number of annual journals, cooperation degree of authors, cooperation rate, mean number of articles published in each *WJG* issue, number of countries of the first *WJG* authors, geographical distribution and article contribution ratio of all *WJG* authors and domestic authors.

**RESULTS:** Of the 5851 articles covered in *MEDLINE*, 173, 236, 633, 826, 1496, 1382 and 1105 articles were cited from 2001 to 2007. The cooperation degree was 5.11, 5.56, 5.75, 5.76, 6.31, 5.90 and 5.64 respectively. The cooperation rates was 94.80%, 99.15%, 98.89%, 98.55%, 99.13%, 96.67% and 95.66%, respectively. The mean number of articles published in each *WJG* issue from 2001 to 2007 was 28, 39, 52, 34, 31, 28 and 23, respectively. The number of countries of the first *WJG* authors was 8, 8, 27, 32, 49, 61 and 56, respectively. The first authors of *WJG* came from 3 continents in 2001 and covered 6 continents in 2006-2007. The number of articles written by Asian authors was 136 (79.07%), 227 (96.19%), 575 (90.98%), 713 (87.81%), 1111 (75.32%),

712 (53.98%) and 555 (53.21%), respectively in 2001-2007. The number of articles written by European & American authors increased from 36 (20.93%) and 8 (3.39%) in 2001-2002 to 563 (42.68%) and 452 (43.34%) in 2006-2007. The number of countries except for China contributing papers was increased. The number of articles written by first authors of Japan rose from 0 (0%) in 2001-2002 to 287 (12.15%) in 2006-2007. The number of articles written by American authors increased from 6 (1.47%) in 2001-2002 to 158 (6.69%) in 2006-2007. The number of articles written by Chinese authors was 136 (79.07%), 227 (96.19%), 548 (86.71%), 669 (82.39%), 884 (59.93%), 380 (28.81%) and 320 (30.68%), respectively, in 2001 to 2007. The number of articles published in *Am J Gastroenterol*, *Gastroenterology*, *Scand J Gastroenterol* and *WJG* was 565, 586, 238 and 1118, respectively in 2007. The cooperation degree was 4.77, 6.14, 5.95 and 5.64, respectively, in 2007. The cooperation rate was 95.40%, 84.18%, 96.63% and 95.66%, respectively, in 2007. The number of countries of authors contributing papers was 44, 35, 42 and 62, respectively, in 2007.

**CONCLUSION:** The geographical distribution of *WJG* authors is wide for the past 2 years. *WJG* has made a step onto international publishing, and drawn even more attentions from gastroenterology researchers. Its authors are distributed over 74 countries in 6 global continents, and the journal has become the main intermediary for international gastroenterology researchers to demonstrate their research accomplishments.

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**Key words:** Bibliometrics; *World Journal of Gastroenterology*; Science citation index

**Peer reviewer:** Sheng-Li Ren, PhD, Department of Publication, National Natural Science Foundation of China, Beijing 100085, China

Yang H, Zhao YY. Variations of author origins in *World Journal of Gastroenterology* during 2001-2007. *World J Gastroenterol* 2008; 14(19): 3108-3111 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3108.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3108>

## INTRODUCTION

*World Journal of Gastroenterology* (*WJG*) was first published

in 1995. This English journal is edited and published by The WJG Press and can be retrieved with the following citation tools: Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, Index Medicus, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, Nature Clinical Practice Gastroenterology and Hepatology, CAB Abstracts and Global Health, *etc.* In recent years, Ma *et al*<sup>[1]</sup> has analyzed the articles covered in SCIE during 1998-2004, claiming that the self-citation rate is decreased. However, the citation rate by others is increased and the journal citation status is improved. The variations of *WJG* authors' data in 2001 to 2007 were comparatively analyzed. The cooperation degree, cooperation rate, number of countries and author publishing ratios of domestic journal issues in *American Journal of Gastroenterology*, *Gastroenterology*, *Scandinavian Journal of Gastroenterology*, were also comparatively analyzed using the SCIE database.

A bibliometric analysis of the variations in distributions of authors was made to show the improvements and shortcomings of *WJG* and speed up its development.

## MATERIALS AND METHODS

*WJG* articles were searched from MEDLINE (by ISI Web of Knowledge)<sup>[2]</sup> in 2001-2007. Variations (cooperation degree, cooperation rate) and distributions of the first authors were analyzed with bibliometric methods. Articles published in *Am J Gastroenterol*, *Gastroenterology*, *Scand J Gastroenterol* and *WJG* covered in SCIE<sup>[3]</sup> in 2007 were analyzed using Web of Science (meeting summaries were not covered). The authors, titles, addresses and other relevant data of the four journals in 2007 were processed through the SCIE's 'Refine Results' function, and countries of authors, *WJG* authors, research institutions and their distribution were closely consistent with the current status and authors of articles published in *WJG* experienced difficulties.

## RESULTS

### *WJG* articles retrieval status with MEDLINE citation in 2001-2007

*WJG* was published bimonthly in 2001-2002, monthly in 2003, semimonthly in 2004, and weekly from 2006. In 2001-2007, 173, 236, 633, 826, 1496, 1382 and 1105 articles published in *WJG* were covered in MEDLINE. The number of articles published in each issue of *WJG* was 28, 39, 52, 34, 31, 28 and 23, respectively, in 2001-2007.

### Co-author articles published in *WJG*

A total of 5851 articles published in *WJG* during 2001-2007 were cited. The number of authors of these papers was 34415 and the cooperation degree was 5.11, 5.56, 5.75, 5.76, 6.31, 5.90 and 5.64, respectively, with a mean cooperation degree of 5.88. The number of articles written by a single author was 137, accounting for 2.34% of all articles. The number of co-author articles published in 2001-2007 was 5714 and the cooperation rate was

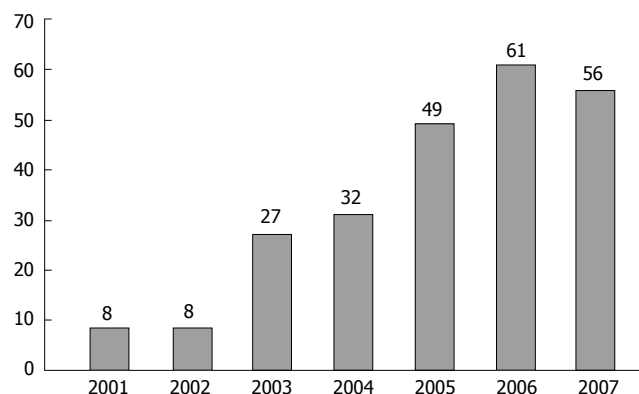


Figure 1 Distribution of first authors and countries in 2001-2007.

94.80%, 99.15%, 98.89%, 98.55%, 99.13%, 96.67% and 95.66% respectively, with a mean cooperation rate of 97.66%. The cooperation degree was slightly increased from 2001 to 2005 (Table 1).

### Distribution of first authors in *WJG*

Only addresses of the first authors were marked in MEDLINE, and 5851 articles published in *WJG* were retrieved in 2001-2007, in which only 5689 articles had available addresses. The number of countries with their articles covered in MEDLINE was 8, 8, 27, 32, 49, 61 and 56, respectively (Figure 1). The geographical distribution of *WJG* authors was increasingly broadened, especially in 2006 and 2007 during which the number of countries increased multiple folds.

The geographical distribution of the authors with addresses in 5689 articles was categorized into 6 continents (Table 2). During 2001-2005, the majority authors were from Asia, accounting for 136 (79.07%), 227 (96.19%), 575 (90.98%), 713 (87.81%) and 1111 (75.32%), respectively. During 2006-2007, the number of authors from Asia was 712 (53.98%), 555 (53.21%) respectively, showing that the number of Asian authors is declining. During 2006-2007, the geographical distribution of *WJG* authors covered all the 6 continents and the number of European and North America authors increased from 36 (20.93%) and 8 (3.39%) in 2001-2002 to 563 (42.68%) and 452 (43.34%) in 2006-2007 respectively.

### Geographical distribution of the first authors

In order to reflect the geographical distributions of authors, a comparison of the distribution of *WJG* authors was performed. The number of articles contributed to *WJG* by the top 15 countries (Table 3) was 5167 (90.82%), the number of articles contributed to *WJG* by Chinese authors was 136 (79.07%), 227 (96.19%), 548 (86.71%), 669 (82.39%), 884 (59.93%), 380 (28.81%) and 320 (30.68%), respectively, in 2001-2007. The number of articles contributed by Japanese authors increased from 0 (0%) in 2001-2002 to 287 (12.15%) in 2006-2007, the number of articles contributed by the American authors was also increased from 6 (1.47%) in 2001-2002 to 158 (6.69%) in 2006-2007. All countries, except for China showed an increased number of contributed articles.



Table 1 Co-author articles published in *WJG* during 2001-2007

Yr	Distribution of co-author articles											Total (articles)	Authors	Cooperation degree	Cooperation rate (%)
	1	2	3	4	5	6	7	8	9	10	> 11				
2001	9	18	20	33	26	20	20	14	5	2	6	173	884	5.11	94.80
2002	2	20	23	44	35	39	30	20	10	2	11	236	1313	5.56	99.15
2003	7	30	65	88	131	110	79	55	25	22	21	633	3637	5.75	98.89
2004	12	47	82	112	146	154	105	71	43	25	29	826	4755	5.76	98.55
2005	13	59	113	188	243	273	202	154	86	56	109	1496	9434	6.31	99.13
2006	46	111	149	169	210	191	150	119	74	57	106	1382	8160	5.90	96.67
2007	48	107	129	148	158	139	120	83	48	59	66	1105	6232	5.64	95.66
Total	137	392	581	782	949	926	706	516	291	223	348	5851	34415	5.88	97.66

Table 2 Geographical distribution of the authors in *WJG*

Yr	Distribution of the authors in 6 continents					
	Africa	Asia	Europe	North America	Oceania	South America
2001	0	136	32	4	0	0
2002	0	227	6	2	1	0
2003	1	575	45	6	2	3
2004	3	713	85	6	0	5
2005	7	1111	314	28	4	11
2006	11	712	453	110	10	23
2007	9	555	359	93	13	14

Among the top 15 countries, 7 are in Asia, 7 in Europe, and 1 in North America.

#### Data comparisons of gastroenterology-related journals

The articles of *Am J Gastroenterol*, *Gastroenterology* and *Scand J Gastroenterol* were selected to compare with those of *WJG*. *Am J Gastroenterol* is an official publication of the American College of Gastroenterology, and its IF was 5.608 in 2006, ranking 5<sup>th</sup> in Journal Citation Report (JCR). *Gastroenterology* is the official journal of the American Gastroenterology Association (AGA) and its IF was 12.457 in 2006, ranking 1<sup>st</sup> in JCR. *Scand J Gastroenterol* published by Taylor & Francis Group is the membership journal of the Gastroenterologic Societies of Denmark, Finland, Iceland, Norway and Sweden, and its IF was 1.869 in 2006. These four journals are most typical of all journals related to the field of gastroenterology. The number of articles published in *Am J Gastroenterol*, *Gastroenterology*, *Scand J Gastroenterol* and *WJG* covered in SCIE was 565, 586, 238 and 1118, respectively, in 2007. The cooperation degree of authors was 4.77, 6.14, 5.95 and 5.64, respectively; the cooperation rate was 95.40%, 84.18%, 96.63% and 95.66% respectively, in 2007. The geographical distribution of authors' was 44, 35, 42 and 62, respectively (Table 4). In 2007, The number of American authors contributing to *Am J Gastroenterol* and *Gastroenterology* accounted for 47.43% and 50.85% respectively, the number of Swedish authors contributing to *Scand J Gastroenterol* accounted for 18.07%, the number of Northern Europe authors contributing to *Scand J Gastroenterol* accounted for 45.38%, the number of Chinese authors contributing to *WJG* accounted for 30.4%.

## DISCUSSION

In 2001-2007, the number of articles covered in

Table 3 Distribution of the top 15 countries in *WJG* during 2001-2007

Country name	2001	2002	2003	2004	2005	2006	2007	Total
China	136	227	548	669	884	380	320	3164
Japan			6	19	133	170	117	445
Germany	10	2	4	10	68	92	66	252
Italy			9	15	56	79	56	215
United States	4	2	5	6	19	77	81	194
Turkey			17	24	31	43	62	177
South Korea			5	9	37	53	38	142
Greece			2	6	34	45	31	118
United Kingdom	18		3	1	16	28	22	88
India			5	2	7	35	30	79
Spain			1	2	13	34	21	71
Poland		1	2	6	31	17	4	61
Hungary			2	12	20	20	5	59
Iran			2	2	7	25	18	54
Thailand			1	4	14	16	13	48

*MEDLINE* was 173, 236, 633, 826, 1496, 1382 and 1105 respectively, the mean number of articles published in each issue was 28, 39, 52, 34, 31, 28 and 23 respectively. The number of articles published increased by 932 (638.73%) in 2007 compared to 2001.

In 2001-2007, the cooperation degree was 5.11, 5.56, 5.75, 5.76, 6.31, 5.90 and 5.64 respectively (mean 5.88), the cooperation rate was 94.80%, 99.15%, 98.89%, 98.55%, 99.13%, 96.67% and 95.66% respectively (mean 97.66%). The mean number of co-authors and single authors showed a tendency to increase from 2001 to 2005, while slightly decreased in 2006 to 2007.

The geographical distributions of authors in *WJG* were expanded from 4 continents in 2001 to the 6 continents in 2006-2007, the number of countries increased in multiple folds. The number of authors from Europe and North America increased while that from Asia decreased. The number of countries increased from 8, 8, 27, 32 and 49 in 2001-2005 to 61 and 56 in 2006-2007.

The number of Chinese authors accounted for 79.07%, 96.19%, 86.71%, 82.39%, 59.93%, 28.81% and 30.68% respectively, in 2001-2007, showing a maximum decrease of 67.38%. The number of Japanese, American, German and Italian authors increased greatly, showing an increasing trend of international authors contributing to *WJG*.

When compared with *Am J Gastroenterol*, *Gastroenterology*, *Scand J Gastroenterol*, the geographical distribution of

Table 4 Data comparisons of the 4 representative gastroenterology journals in 2007

Journal name	Articles published in 2007	Cooperation degree in 2007	Cooperation rate in 2007	Geographical distribution of authors	Ratio of articles contributed by domestic authors (%)
<i>Am J Gastroenterol</i>	565	4.77	95.40	44	United States 47.43
<i>Gastroenterology</i>	586	6.14	84.18	35	United States 50.85
<i>Scand J Gastroenterol</i>	238	5.95	96.63	42	Sweden 18.07
<i>WJG</i>	1118	5.64	95.66	62	China 30.4

authors in *WJG* was greatly expanded in the order of China, Asia and 6 continents. The mean number of published articles in each issue showed a prominent decrease, which may improve the quality of articles published in *WJG*. The cooperation degree and rate were reasonable, and the number of Chinese authors was slightly increased in 2007.

In conclusion, the geographical distribution of *WJG* authors is worldwide. *WJG* has made a step onto international level, thus drawing more attentions from gastroenterology researchers. The journal has become the main intermediary for international researchers in

gastroenterology to demonstrate their research accomplishments.

## REFERENCES

- 1 Ma LS, Pan BR, Li WZ, Guo SY. Improved citation status of *World Journal Gastroenterology* in 2004: Analysis of all reference citations by *WJG* and citations of *WJG* articles by other SCI journals during 1998-2004. *World J Gastroenterol* 2005; **11**: 1-6
- 2 MEDLINE. Available from: URL: <http://apps.isiknowledge.com/>
- 3 SCIE. Available from: URL: <http://www.isinet.com/cgi-bin/jrnlst/jlsubcatg.cgi?PC=D>

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### Takafumi Ando, MD, PhD

Department of Gastroenterology, Nagoya University Graduate School of Medicine, Therapeutic Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan

### Taku Aoki, MD

Division of Hepato-Biliary-Pancreatic and Transplantation Surgery, Department of Surgery, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, 113-8655, Japan

### Carla W Brady, MD, MHS

Duke University Medical Center, Division of Gastroenterology, DUMC Box 3913, Durham, NC 27705, United States

### Jordi Camps, PhD

Centre de Recerca Biomèdica, Hospital Universitari de Sant Joan, C. Sant Joan s/n, 43201 Reus, Catalunya, Spain

### Ravi S Chari, MD, Associate Professor

Division of Hepatobiliary Surgery and Liver Transplantation, Departments of Surgery and Cancer Biology, 1313 21st Avenue South Suite 801 Oxford House, Vanderbilt University Medical Center, Nashville, TN 37232-4753, United States

### Wang-Xue Chen, Dr

Institute for Biological Sciences, National Research Council Canada, 100 Sussex Drive, Room 3100, Ottawa, Ontario K1A 0R6, Canada

### John Y Chiang, MD, PhD, Professor

Department of Biochemistry and Molecular Pathology, Northeastern Ohio Univ. College of Medicine, 4209 State Route 44, P.O. Box 95, Rootstown, OH 44272, United States

### Vicente Felipo, Dr

Vicente Felipo, Laboratory of Neurobiology, Fundación C.V. Centro de Investigación Príncipe Felipe, Avda Autopista del Saler, 16, 46013 Valencia, Spain

### Zvi Fireman, MD, Associate Professor of Medicine

Head, Gastroenterology Department, Hillel Yaffe Med Ctr, POB 169, 38100, Hadera, Israel

### Jean L Frossard, Dr

Division of gastroenterology, Geneva University Hospital, Rue Micheli du Crest, 1211 Geneva 14, Switzerland

### Diego Garcia-Compean, MD, Professor

Faculty of Medicine, University Hospital, Department of Gastroenterology, Autonomous University of Nuevo Leon, Ave Madero y Gonzalitos, 64700 Monterrey, NL, México

### Subrata Ghosh, Professor

Department of Gastroenterology, Imperial College London, Hammersmith Hospital, 9 Lady Aylesford Avenue, Stanmore, Middlesex, London HA7 4FG, United Kingdom

### Salvatore Gruttadauria, MD, Assistant Professor

Abdominal Transplant Surgery, ISMETT, Via E. Tricomi, 190127 Palermo, Italy

### Kazuhiro Hanazaki, MD, Professor and Chairman

Department of Surgery, Kochi Medical School, Kochi University, Kohasu, Okohcho, Nankoku, Kochi 783-8505, Japan

### Aydin Karabacakoglu, Dr, Assistant Professor

Department of Radiology, Meram Medical Faculty, Selcuk University, Konya 42080, Turkey

### Paul Y Kwo, Professor

Gastroenterology and Hepatology Division, Indiana University School of Medicine, 975 West Walnut, IB 327, Indianapolis, Indiana 46202-5121, United States

### Shou-Dong Lee, Professor

Department of Medicine, Taipei Veterans General Hospital, 201 Shih-Pai Road, Sec. 2, Taipei 112, Taiwan, China

### Anders E Lehmann, PhD, Associate Professor

Senior Principal Scientist, Bioscience, AstraZeneca R&D Mölndal, Mölndal, Sweden

### Cynthia Levy, Dr

Division of Gastroenterology, Hepatology and Nutrition, University of Florida, MSB-Rm M 440, 1600 SW Archer Road, Gainesville, FL 32608, United States

### James D Luketich, MD, Professor and Chief

Division of Thoracic and Foregut Surgery University of Pittsburgh Medical Center Pittsburgh, PA 15213, United States

### Peter J Mannon, MD

Mucosal Immunity Section, Laboratory of Host Defense, National Institute of Allergy, Laboratory of Clinical Investigation, Building 10/CRC, Room 6-3742, 9000 Rockville Pike, Bethesda, Maryland 20892, United States

### Jose JG Marin, Professor

Head of the Departamento Physiology and Pharmacology, University of Salamanca, CIBERehd, Campus Miguel de Unamuno, ED-S09, Salamanca 37007, Spain

### Didier Merlin, PhD, Associate Professor

Department of Medicine Division of Digestive Diseases, Emory University, 615 Michael Street, Atlanta, GA 30322, United States

### Fock Kwong Ming, Professor, Senior Consultant

Department of Medicine, Changi General Hospital, 2 Simei Street 3, Singapore 529889, Singapore

### Sri P Misra, Professor

Gastroenterology, Moti Lal Nehru Medical College, Allahabad 211001, India

### Emiko Mizoguchi, MD, PhD

Department of Medicine, Gastrointestinal Unit, GRJ 702, Massachusetts General Hospital, Boston, MA 02114, United States

### Justin H Nguyen, MD

Division of Transplant Surgery, Mayo Clinic, 4205 Belfort Road, Suite 1100, Jacksonville, Florida 32256, United States

### Ramesh Roop Rai, MD, DM (Gastro.) Professor & Head

Department of Gastroenterology & Hepatology, S.M.S. Medical College & Hospital, Jaipur 302019, (Rajasthan), India

### Markus Reiser, Professor, Dr

Gastroenterology-Hepatology, Ruhr-Universität Bochum, Bürkle-de-la-Camp-Platz 1, Bochum 44789, Germany

### Ian C Roberts-Thomson, Professor

Department of Gastroenterology and Hepatology, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South 5011, Australia

### Francis Seow-Choen, Professor

Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, 3 Mt Elizabeth Medical Centre #09-10, 228510, Singapore

### Tadashi Shimoyama, MD

Hirosaki University, 5 Zaifu-cho, Hirosaki 036-8562, Japan

## Meetings

### Events Calendar 2008-2009

#### FALK SYMPOSIA 2008

January 24-25, Frankfurt, Germany  
 Falk Workshop: Perspectives in Liver Transplantation

#### International Gastroenterological Congresses 2008

February 14-16, Paris, France  
 EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
 8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
 3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

March 10-13, Birmingham, UK  
 British Society of Gastroenterology Annual Meeting  
 E-mail: [BSG@mailbox.ulcc.ac.uk](mailto:BSG@mailbox.ulcc.ac.uk)

March 14-15, HangZhou, China  
 Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
 Asian Pacific Association for the Study of the Liver  
 18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
 Shanghai - Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
 OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation - Management of Adeno- carcinomas  
 Email: [robert.giuli@oeso.org](mailto:robert.giuli@oeso.org)

April 18-22, Buenos Aires, Argentina  
 9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
 Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
 43<sup>rd</sup> Annual Meeting of the European

Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary  
 Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA  
 Digestive Disease Week 2008  
 June 4-7, Helsinki, Finland

The 39<sup>th</sup> Nordic Meeting of Gastroenterology  
[www.congex.com/ngc2008](http://www.congex.com/ngc2008)  
 June 6-8, Prague, Czech Republic  
 3<sup>rd</sup> Annual European Meeting: Perspectives in Inflammatory Bowel Diseases  
 Email: [meetings@imedex.com](mailto:meetings@imedex.com)

June 13-14, Amsterdam, Netherlands  
 Falk Symposium 165: XX International Bile Acid Meeting. B ile Acid Biology and Therapeutic Actions

June 25-28, Barcelona, Spain  
 10<sup>th</sup> World Congress on Gastrointestinal Cancer  
 Imedex and ESMO  
 Email: [meetings@imedex.com](mailto:meetings@imedex.com)

June 25-28, Lodz, Poland  
 Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatology (IAP)  
 E-mail: [office@epc-iap2008.org](mailto:office@epc-iap2008.org)  
[www.e-p-c.org](http://www.e-p-c.org)  
[www.pancreatology.org](http://www.pancreatology.org)

June 26-28, Bratislava, Slovakia  
 5<sup>th</sup> Central European Gastroenterology Meeting  
[www.ceurgem2008.cz](http://www.ceurgem2008.cz)  
 September 10-13, Budapest, Hungary

11<sup>th</sup> World Congress of the International Society for Diseases of the Esophagus  
 Email: [isde@isde.net](mailto:isde@isde.net)

September 13-16, New Delhi, India  
 Asia Pacific Digestive Week  
 E-mail: [apdw@apdw2008.net](mailto:apdw@apdw2008.net)

III FALK GASTRO-CONFERENCE  
 September 17, Mainz, Germany  
 Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany  
 Falk Symposium 166:  
 GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic  
 Prague Hepatology Meeting 2008  
[www.czech-hepatology.cz/phm2008](http://www.czech-hepatology.cz/phm2008)

September 20-21, Mainz, Germany  
 Falk Symposium 167:  
 Liver Under Constant Attack - From

Fat to Viruses  
 September 24-27, Nantes, France  
 Third Annual Meeting  
 European Society of Coloproctology  
[www.escp.eu.com](http://www.escp.eu.com)



October 8-11, Istanbul, Turkey  
 18<sup>th</sup> World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists  
 E-mail: [orkun.sahin@serenas.com.tr](mailto:orkun.sahin@serenas.com.tr)

October 18-22, Vienna, Austria  
 16<sup>th</sup> United European Gastroenterology Week  
[www.negf.org](http://www.negf.org)  
[www.acv.at](http://www.acv.at)

October 22-25, Brisbane, Australia  
 Australian Gastroenterology Week 2008  
 Email: [gesa@gesa.org.au](mailto:gesa@gesa.org.au)

October 31-November 4, Moscone West Convention Center, San Francisco, CA  
 59<sup>th</sup> AASLD Annual Meeting and Postgraduate Course  
 The Liver Meeting  
 Information: [www.aasld.org](http://www.aasld.org)

November 6-9, Lucerne, Switzerland  
 Neurogastroenterology & Motility Joint International Meeting 2008  
 Email: [ngm2008@mci-group.com](mailto:ngm2008@mci-group.com)  
[www.ngm2008.com](http://www.ngm2008.com)

November 12, Santiago de Chile, Chile  
 Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

December 7-9, Seoul, Korea  
 6<sup>th</sup> International Meeting Hepatocellular Carcinoma: Eastern and Western Experiences  
 E-mail: [sglee@amc.seoul.kr](mailto:sglee@amc.seoul.kr)

INFORMATION FOR ALL  
 FALK FOUNDATION e.V.  
 Email: [symposia@falkfoundation.de](mailto:symposia@falkfoundation.de)  
[www.falkfoundation.de](http://www.falkfoundation.de)

Advanced Courses - European Institute of Telesurgery EITS - 2008  
 Strasbourg, France  
 January 18-19, March 28-29, June 6-7, October 3-4  
 N.O.T.E.S  
 April 3-5, November 27-29  
 Laparoscopic Digestive Surgery  
 June 27-28, November 7-8  
 Laparoscopic Colorectal Surgery  
 July 3-5  
 Interventional GI Endoscopy Techniques  
 Contact address for all courses:  
[info@eits.fr](mailto:info@eits.fr)

International Gastroenterological

Congresses 2009  
 March 23-26, Glasgow, Scotland  
 Meeting of the British Society of Gastroenterology (BSG)  
 E-mail: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

May 17-20, Denver, Colorado, USA  
 Digestive Disease Week 2009

November 21-25, London, UK  
 Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.





## Instructions to authors

### GENERAL INFORMATION

*World Journal of Gastroenterology* (WJG, ISSN 1007-9327 CN 14-1219/R) is a weekly open access peer-reviewed journal supported by an editorial board consisting of 1208 experts in gastroenterology and hepatology from 60 countries. The aim of the journal is to deliver the most clinically relevant original and commentary articles to readers, and to make the full text publicly available to all clinicians, scientists, patients and biomedical students on an unrestricted platform, so that they can access and learn about the most recent key advances in the field.

In addition to the open access nature, another key characteristic of WJG is its reading guidance for each article which includes background, research frontier, related reports, breakthroughs, applications, terminology, and comments of peer reviewers for the general readers.

WJG publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidermiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

The main goal of WJG is to publish high quality commentary articles contributed by leading experts in gastroenterology and hepatology and original articles that combine the clinical practice and advanced basic research, to provide an interactive platform for clinicians and researchers in internal medicine, surgery, infectious diseases, traditional Chinese medicine, oncology, integrated Chinese and Western medicine, imaging, endoscopy, interventional therapy, pathology and other basic medical specialties, and thus eventually improving the clinical practice and healthcare for patients.

### Indexed and abstracted in

Current Contents®/Clinical Medicine, Science Citation Index Expanded (also known as SciSearch®) and Journal Citation Reports/Science Edition, Index Medicus, MEDLINE and PubMed, Chemical Abstracts, EMBASE/Excerpta Medica, Abstracts Journals, *Nature Clinical Practice Gastroenterology and Hepatology*, CAB Abstracts and Global Health. ISI JCR 2003-2000 IF: 3.318, 2.532, 1.445 and 0.993.

### Published by

The WJG Press

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Manuscripts should be typed double-spaced on A4 (297 mm × 210 mm) white paper with outer margins of 2.5 cm. Number all pages consecutively, and start each of the following sections on a new page: Title Page, Abstract, Introduction, Materials and Methods, Results, Discussion, Acknowledgements, References, Tables, Figures, and Figure Legends. Neither the editors nor the publisher are responsible for the opinions expressed by contributors. Manuscripts formally accepted for publication become the permanent property of The WJG Press, and may not be reproduced by any means, in whole or in part, without the written permission of both the authors and the publisher. We reserve the right to copy-edit and put onto our website accepted manuscripts. Authors should follow the relevant guidelines for the care and use of laboratory animals of their institution or national animal welfare committee. For the sake of transparency in regard to the performance and reporting of clinical trials, we endorse the policy of the International Committee of Medical Journal Editors to refuse to publish papers on clinical trial results if the trial was not recorded in a publicly-accessible registry at its outset. The only register now available, to our knowledge, is <http://www.clinicaltrials.gov> sponsored by the United States National Library of Medicine and we encourage all potential contributors to register with it. However, in the case that other registers become available you will be duly notified. A letter of recommendation from each author's organization should be provided with the contributed article to ensure the privacy and secrecy of research is protected.

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Full manuscript title, running title, all author(s) name(s), affiliations, institution(s) and/or department(s) where the work was carried out; author contributions; disclosure of any financial support for the research; and the name, full address, telephone and fax numbers and email address of the corresponding author should be included. Titles should be concise and informative (remove all unnecessary words), emphasize what is new, and avoid abbreviations. A short running title of less than 40 letters should be provided. List the author(s)' name(s) as follows: initial and/or first name, middle name or initial(s), and full family name.

**Author contributions:** The format of this section should be like this: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed research; Wang CL, Zou CC, Hong F and Wu XM performed research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed data; and Wang CL, Liang L and Fu JF wrote the paper.

**Peer reviewers:** All articles received are subject to peer review. Normally, three experts are invited for each article. Decision for acceptance is made only when at least two experts recommend an article for publication. Reviewers for accepted manuscripts are acknowledged in each manuscript, and reviewers of articles which were not accepted will be acknowledged at the end of each issue. To ensure the quality of the articles published in WJG, reviewers of accepted manuscripts will be announced by publishing the name, title/position and institution of the reviewer in the footnote accompanying the printed article. For example, reviewers: Professor Jing-Yuan Fang, Shanghai Institute of Digestive Disease, Shanghai, Affiliated Renji Hospital, Medical Faculty, Shanghai Jiaotong University, Shanghai, China; Professor Xin-Wei Han, Department of Radiology, The First Affiliated Hospital, Zhengzhou University, Zhengzhou, Henan Province, China; and Professor Anren Kuang, Department of Nuclear Medicine, Huaxi Hospital, Sichuan University, Chengdu, Sichuan Province, China.

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An informative, structured abstract of no more than 350 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipment, and the experimental procedures should be included. RESULTS: The observed and experimental results, including data, effects, outcome, *etc.* should be included. Authors should present *P* value where necessary, and also include any significant data. CONCLUSION: Accurate view and the value of the results should be included.

The format for structured abstracts can be found at: <http://www.wjgnet.com/wjg/help/11.doc>.

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Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

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Data that are not statistically significant should not be noted. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 should be noted (*P* > 0.05 should not be noted). If there are other series of *P* values, <sup>c</sup>*P* < 0.05 and <sup>d</sup>*P* < 0.01 are used. A third series of *P* values can be expressed as <sup>e</sup>*P* < 0.05 and <sup>f</sup>*P* < 0.01. Other notes in tables or under illustrations should be expressed as <sup>1</sup>F, <sup>2</sup>F, <sup>3</sup>F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

**Acknowledgments**

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

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The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability<sup>[1,2]</sup>". If references are cited directly in the text, they should be put together within the text, for example, "From references<sup>[19,22-24]</sup>, we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

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**Format****Journals**

*English journal article (list all authors and include the PMID where applicable)*

- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

*Chinese journal article (list all authors and include the PMID where applicable)*

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

*In press*

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

*Organization as author*

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ, Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

*No author given*

- 6 21st century heart solution may have a sting in the tail. *BMJ*

2002; 325: 184 [PMID: 12142303]

#### Volume with supplement

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment of migraine and in comparison with sumatriptan. *Headache* 2002; 42 Suppl 2: S93-99 [PMID: 12028325]

#### Issue with no volume

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (401): 230-238 [PMID: 12151900]

#### No volume or issue

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS A Careaction* 2002; 1-6 [PMID: 12154804]

### Books

#### Personal author(s)

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

#### Chapter in a book (list all authors)

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

#### Author(s) and editor(s)

- 12 **Breedlove GK**, Schorfheide AM. Adolescent pregnancy. 2nd ed. Wicczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

#### Conference proceedings

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

#### Conference paper

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

#### Electronic journal (list all authors)

Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

#### Patent (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 24.5  $\mu$ g/L; CO<sub>2</sub> volume fraction, 50 mL/L CO<sub>2</sub>, not 5% CO<sub>2</sub>; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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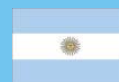
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<sup>[1]</sup>Passed away on October 20, 2007

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# Liver transplantation: Yesterday, today and tomorrow

Osman Abbasoglu

Osman Abbasoglu, Department of Surgery, Hacettepe University School of Medicine, Sıhhiye, Ankara 06100, Turkey  
Author contributions: Abbasoglu O designed research and wrote the paper.

Correspondence to: Osman Abbasoglu, Professor, Department of Surgery, Hacettepe University School of Medicine, Sıhhiye, Ankara 06100, Turkey. [osmanabbasoglu@yahoo.com](mailto:osmanabbasoglu@yahoo.com)

Telephone: +90-532-3649039 Fax: +90-312-4262421

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## Abstract

With the advances in technical skills, management of postoperative complications and improvements in immunosuppressive drugs, liver transplantation is the standard treatment for many patients with chronic liver disease. Today, shortage of donor organs seems to be the major limiting factor for the application of liver transplantation. This review focuses on five issues that are challenging to clinical practice of liver transplantation and relevant to gastroenterologists. These include living donor liver transplantation, recurrent viral hepatitis, non-heart-beating donors, hepatocellular carcinoma, and ABO incompatible liver transplantation. Living donor and non-heart beating donor transplantations were initiated as a solution to increase the donor organ pool and it is expected that there will be an increase in the number of these donors. Recurrent hepatitis C and hepatocellular carcinoma following liver transplantation are among major problems and ongoing research in these diseases may lead to better outcomes in these recipients.

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**Key words:** Liver transplantation; Hepatitis C virus; Hepatitis B virus; Hepatocellular carcinoma; ABO incompatibility; Living donor

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## INTRODUCTION

Liver transplantation is one of the most important advances in medicine. First cadaveric liver transplantation was performed by Thomas Starzl in 1963 in Denver. After this failed trial, liver transplantation was successfully performed in humans in July 1967 again by Dr. Starzl. Although rejection was a major concern, many recipients from this early era have survived for more than 20 years using immunosuppression with azathioprine, prednisone, and antilymphocyte globulin (ALG)<sup>[1]</sup>.

For clinical transplantation, the historical beginning was Medawar's recognition that rejection is an immune reaction<sup>[2]</sup> (Table 1). With the advances in immunosuppression, postoperative care and surgical technique, liver transplantation has become the golden standard in the treatment of many chronic liver diseases. Since then, the number of patients on the waiting list has increased and organ shortage appeared to be one of the major problems in clinical transplantation.

Raia of Brazil performed the first living donor liver transplantation (LDLT) in 1987 as a promising method to resolve the organ shortage, but the recipient died of a transfusion reaction despite a successful operation<sup>[3]</sup>. After this trial, LDLT has been performed by many other pioneer surgeons in other countries. In the last decade LDLT has become a widely accepted treatment modality. The most extensive experience in LDLT was initially gained in Asia. In countries such as Japan, where the availability of organs from deceased donor is limited, LDLT seems to be the only solution in the treatment of end stage liver diseases. According to the data of Japanese Liver Transplantation Society, the adult to adult LDLT is increasing per year. Despite this increase in adults, cases in children have reached a peak around 100 cases per year. The 1 and 5-year survival rates of all recipients were reported to be 81.8%, and 77%, respectively, while those of recipients of less than 18 years old was 85.6%, and 82.6% respectively. The prognosis of adult recipients was poor when compared to children<sup>[4]</sup>. It was suggested that the original disease recurrence such as hepatitis C and hepatocellular carcinoma (HCC) has influenced a significant decline in the survival of adult cases.

## MAJOR ISSUES RELATED WITH LIVER TRANSPLANTATION

### LDLT

The shortage of cadaveric liver organs has significantly

**Table 1** History of liver transplantation<sup>[1,3]</sup>

Yr	Author	Application
1943	Gibson	Defined the immunologic nature of skin allograft rejection in humans
1955	Welch	First mention of liver transplantation in the literature in a dog study
1960	Medawar	Definition of acquired transplantation tolerance
1960	Starzl	Transplantation in dogs of multiple abdominal viscera
1963	Starzl	World's first three attempts of liver transplantation in humans with maximum survival of 21 d
1968	Starzl	First long-term survival of four patients after liver transplantation
1978	Calne	Introduction of cyclosporine
1984	Bismuth	Reduced-sized adult liver transplanted into a small child
1987	Raia	First living donor liver transplantation
1988	Pichlmayr	Split one donor liver and two graft were used for two recipients
1988	Kalayoglu	Introduction of University of Wisconsin solution for preservation
1992	Starzl	Baboon to human xenotransplantation

inhibited further expansion of liver transplantation. Split liver transplantation has reduced waiting-list mortality in children, but not in adults. LDLT is currently the most effective alternative to overcome the organ shortage in adults. With the efforts of transplant surgeons in the establishment and popularization of LDLT, the number of LDLT has increased dramatically not only in Japan but also in Europe and the United States as well. Major advantages of LDLT include the good viability of the liver harvested from a healthy individual, the careful selection of the timing of the transplantation, and the potential good tissue matching. The reduced waiting period for a living donor organ may decrease the risks of decompensation or death before transplantation, thereby improving the overall chances for success. Disadvantages are the risk to healthy donors and also that, this modality has a potential psychological burden on the donor<sup>[5,6]</sup>. The surgical procedures for LDLT are technically more challenging and LDLT requires a full understanding of the hepatobiliary anatomy.

A wide range of complication rates are reported in the literature in donors after LDLT. Donor safety has a major importance in LDLT. Published reports on donor outcomes indicated a wide range of complication rates that varied between 9% and 67%<sup>[7,8]</sup>. In the Kyoto University experience 50 complications in 222 right lobe grafts have been encountered, including surgical complications is 18.5% and non-surgical complications is 3.2%<sup>[9]</sup>. On the other hand, the American Society of Transplant Surgeons reported a donor complication rate of only 10%. Thus it seems that donor morbidities have not been adequately reported and true extend of complications may be underestimated. A standardized system for reporting complications to a registry should be developed to allow meaningful data analysis. Donor mortality is also a major concern of LDLT. In the United States at least 3 deaths were confirmed. Another 3 deaths in Europe and 1 in Japan had been reported<sup>[10]</sup>.

As living donation permits transplantation to take place independent of either waiting time or the severity of liver disease, the criteria required for LDLT may be modified when compared to deceased donor liver transplantation (DDLT). Estimation of liver volume needed in individual situations is an important factor in donor selection. Aged liver, steatotic liver, and special anatomic variants have the risk of a relatively poor graft quality. Recipient factors such as metabolic load, preoperative latent infections and other organ failures have negative impact on graft survival. The minimally required quantity of graft volume has not been fully clarified, which is one of the major issues of the adult to adult LDLT. The following two methods were developed to express the graft volume: First the ratio of graft volume (GV) in the standard liver volume (SLV) of the recipient, which is calculated by the recipient's height and body weight. Second, the ratio of graft weight in the recipient's weight (GRWR: graft to recipient weight ratio). The reported safe limit of small-for-size graft is from 30% to 40% in GV/SLV, while from 0.6 to 0.8 in GRWR<sup>[11-14]</sup>.

Recipients with a small-for-size graft, suffer from graft dysfunction including hyperbilirubinemia, massive ascites, poor synthetic function that leads to serious conditions such as gastrointestinal bleeding and renal failure. When a graft size is conversely too large for a recipient such as a newborn infant, the graft necrosis occurs due to insufficient blood inflow into the graft.

As pointed out by Ghobrial and Bussuttil, future application of LDLT will be based on the accurate definition of risks imposed on donors compared with potential benefits realized by recipients<sup>[15]</sup>. As an example to this statement, the number of adult LDLT declined from approximately 400 in 2001 to 280 in 2002. Such a precipitous reduction may have occurred in response to the donor death in US in 2002 which raised increasing concerns for donor safety. While the number of LDLT in the US has declined, the number in Asia as a whole has continued to increase. LDLT accounted for less than 5% of liver transplants in the US but more than 95% of the transplants in Asia excluding mainland China. The overall number of LDLT procedures performed in Asian countries and areas with well-established programs (Japan, Korea, China Hong Kong and China Taiwan) has steadily increased over years<sup>[16]</sup>.

In summary, the overall results with good patient and graft survival, together with acceptable donor morbidity and mortality has led to the acceptance of LDLT in the transplant community. To maintain this procedure as a treatment modality in the future, satisfactory risk-benefit analysis and long-term morbidities imposed on living donors should be further investigated.

### Recurrent viral hepatitis

The most common single cause of late graft loss after liver transplantation is the recurrence of the disease for which the liver transplantation has been performed<sup>[17]</sup>. Until last decade, successful long-term outcome after liver transplantation in patients with chronic active hepatitis-B has been limited because of high rate of recurrent



of recurrent hepatitis. Long-term passive immunization with high-dose intravenous hepatitis B immunoglobulin (HBIG) led to a significant improvement in the prognosis of these patients. High-dose intravenous HBIG may prevent recurrent hepatitis B virus infection, but the cost has limited its widespread use in countries with endemic hepatitis B virus infection. Low-dose intramuscular HBIG plus nucleoside analogs such as lamivudine was shown to be equally effective and safe and in the long-term prophylaxis against recurrent hepatitis B at less than 10% the cost of the high-dose regimen<sup>[18,19]</sup>. Although lamivudine is effective in most of the patients, lamivudine resistance is becoming a major concern. With adefovir, a potent antiviral drug that became available in recent years, these patients with lamivudine-resistant tyrosine-methionine-aspartate-aspartate mutant can also be treated<sup>[20,21]</sup>. Currently, liver transplantation can be safely performed in chronic active hepatitis B patients with similar survival as for patients transplanted for other indications.

The recurrence of hepatitis C is also a great concern after transplantation. Although short-term graft and patient survival rates of chronic active hepatitis C patients are comparable to those observed in other patients undergoing liver transplantation, HCV recurrence is universal and is associated with poor graft and patient survival<sup>[22]</sup>. In contrast, survival after retransplantation for recurrent hepatitis C is poor and retransplantation for these patients is controversial<sup>[23,24]</sup>. In a previous study Abbasoglu *et al* showed that recurrent hepatitis was the most common cause of late graft loss in patients who had undergone liver transplantation for chronic active hepatitis C<sup>[17]</sup>.

Treatment of recurrent hepatitis C, whether pre-emptive or not, is an important issue. Despite recent achievements in the treatment of hepatitis C infection with pegylated interferons and ribavirin, patients with recurrent hepatitis C after liver transplantation are difficult to treat. Virological response rates in prophylactic and therapeutic approaches of hepatitis C reinfection after liver transplantation are low compared to the pre-cirrhotic hepatitis C infection. Moreover, optimal treatment duration and dosage of recurrent infection with pegylated interferon in combination with ribavirin remains to be defined<sup>[25]</sup>. Despite side effects, long-term antiviral maintenance therapy might be an effective approach for preventing progression to severe allograft fibrosis and thereby improving long-term survival in liver transplant recipients with recurrent hepatitis C<sup>[26]</sup>.

Two large studies have shown that the incidence and severity of hepatitis C recurrence do not differ between DDLT and LDLT recipients; however another study has found that the incidence of cholestatic hepatitis is significantly greater in LDLT recipients<sup>[24,27,28]</sup>. Several studies have identified a number of potential risk factors for recurrent hepatitis C infection including hepatitis C virus related factors (virus load, genotype) as well as coinfection with other viruses such as cytomegalovirus, hepatitis B virus and hepatitis D virus<sup>[29]</sup>. There are still no well-defined parameters that would predict which patients are

at risk to develop recurrent hepatitis C and those who will not. Strategies including pre- and post-transplant antiviral therapy may further improve the results.

### **Non-heart-beating donors**

The first liver transplantation from a non-heart beating donor (NHBD) was performed by Nakayama in Japan in 1964. NHBD livers are considered as a potential for expanding donor pool. The critical problem with NHBD livers is prolonged warm ischemia time. Despite calls for the use of hepatic grafts from NHBD, there are few studies examining long-term outcome. Although metabolism is slowed 1.5- to 2-fold for every 10°C drop in temperature, considerable metabolic activity still occurs during cold preservation. In NHBD organs, the effects of cold ischemia are superimposed on the injury occurred during warm ischemia. The pattern of injury sustained during warm and cold ischemia is slightly different. Cold ischemia leads to initial injury to sinusoidal endothelial cells whereas warm ischemia mainly injures the hepatocytes<sup>[30]</sup>. It seems that the additional injury resulting from warm ischemia in NHBD donation requires alternative preservation strategies to minimize the ischemic injury. Donor warm ischemic time may predispose hepatic allografts to an increased incidence of ischemic type biliary strictures. Although graft and patient survival has been reported to be similar to that of heart beating donor transplants, caution is urged with the use of these organs<sup>[31]</sup>.

Despite the increased risk of graft and patient survival, NHBD livers are being increasingly used with acceptable results. Abt *et al* analyzed data from the United Network for Organ Sharing database. In 144 NHBDs and 16856 heart beating donors (NHBDs) the 1-year (70.2% *vs* 80.4%) and 3-year (63.3% *vs* 72.1%) graft survival were inferior in the NHBD group. The primary non-function risk after transplantation was also significantly higher (11.8 % *vs* 6.4%) in the NHBD group<sup>[32]</sup>.

New strategies in organ preservation, normothermic recirculation, normothermic preservation, cytoprotection, and development of reliable markers to predict postoperative graft function may improve results in clinical transplantation with NHBD liver grafts. Based on the clinical studies and continued shortage of liver allografts, the use of NHBD organs are recommended, however, with several caveats. Careful donor (< 60 years of age) and recipient (stable, not intubated) selection, minimizing warm (< 30 min) and cold (< 8 h) ischemia, utilization of histology, and discarding organs with significant steatosis may provide acceptable results<sup>[32,33]</sup>.

### **HCC**

HCC is the most common primary liver cancer and most patients with HCC also suffer from coexisting cirrhosis. For the treatment of patients without cirrhosis, resection should be considered whenever possible. Hepatic reserve is the one of the major determinants of liver resection. When compared with resection, transplantation restores liver function and has the advantage of removing tissue with an oncogenic potential<sup>[34]</sup>. To obtain the optimal

benefit from the limited number of organs available, strict selection criteria has been developed to offer liver grafts to patients with the highest likelihood of survival after transplantation. In 1996 Mazzaferro *et al* showed that when strict criteria were applied, transplantation of patients with early HCC has resulted in excellent results with 4-year survival rate of 75%. This led to the development of Milan criteria from a retrospective analysis of 48 patients. This survival rate was achieved in patients with solitary tumor of less than 5 cm and those who have up to 3 tumor nodules each of which is smaller than 3 cm without vascular invasion or extra hepatic metastasis<sup>[35]</sup>. With the achievement of good results in HCC patients with more advanced tumors, the Milan criterion was expanded. Yao *et al* proposed UCSF criteria (solitary tumor smaller than 6.5 cm or 3 of fewer nodules with the largest lesion smaller than 4.5 cm or total tumor diameter less than 8.5 cm without vascular invasion<sup>[36]</sup>. In this study the expansion of Milan criteria did not impact on survival adversely. On the other hand, this approach reduced the availability of cadaveric grafts for patients with other liver diseases. The Barcelona Clinic Liver Cancer Group has proposed expanding the Milan criteria to single tumor of 7 cm or less, or 5 tumors of 3 cm or less, in patients who showed a partial response to any treatment lasting for more than 6 mo<sup>[37]</sup>. However organ shortage, higher drop-out rate, and less favorable results render these attempts to a controversial issue. With the expansion of listing criteria, liver transplantation could be performed in more advanced cancer patients but this lead in turn to poor survival rates. All patient selection criteria rely on radiological imaging to assess intrahepatic disease and exclude extra hepatic spread. It may be possible to improve patient selection by increasing the sensitivity of imaging studies and detection of micrometastasis<sup>[38]</sup>.

About 50% of HCC patients who are initially candidates for liver transplantation will become ineligible, if the median waiting period exceeds 1 year<sup>[39,40]</sup>. As a result of tumor progression during the waiting period, LDLT gained popularity to transplant HCC patients in a better clinical condition without a long waiting time. Although controversial, it may be claimed that LDLT can be performed in patients with HCC that exceeds the Milan criteria as 3-year survival rate of greater than 50 has been showed in other studies<sup>[41]</sup>. In two studies it was shown that LDLT is superior to DDLT for patients with HCC meeting Milan criteria, when waiting times for organs from deceased donors exceed six months<sup>[42,43]</sup>. Despite the availability of LDLT tumor progression is still a major concern and strategies like chemoembolization and radiofrequency ablation to reduce tumor growth during waiting period have shown promising results<sup>[44]</sup>. Although many studies have shown that microvascular invasion and histological grade are significant risk factors for poor prognosis, these are difficult to know clearly before transplantation. Noninvasive markers to predict the prognosis of HCC may help better patient selection in the future<sup>[45]</sup>.

### **ABO incompatible liver transplantation**

Two antigen systems (ABO and HLA) play role in trans-

plantation. In liver transplantation the ABO system is important while HLA system has a minor role. Crossing the ABO barrier in liver transplantation is usually not performed except for emergency conditions and results of ABO incompatible liver transplantation have been markedly inferior with an increased incidence of vascular and biliary complications and rejection, when compared to ABO compatible grafts. In children below the age of three years, ABO incompatible liver transplantations have been more successful<sup>[46]</sup>. In recent years, promising results with ABO incompatible liver transplantation using A<sub>2</sub> donors (subgroup of A which is less reactive and occur in approximately 20% of group A individuals) have been reported. In a Swedish study of 10 adult blood group O recipients who received A<sub>2</sub> cadaveric grafts, patient and graft survival was 10/10 and 8/10 respectively at 8.5 mo median follow up with tacrolimus based protocol and initial immunosuppression with antithymocyte globulin, interleukin-2-receptor antagonists or anti-CD20 antibody<sup>[47]</sup>. In 16 pediatric ABO-incompatible pediatric liver transplantation, Heffron *et al* reported one-year actuarial graft survival of 92% utilizing standard immunosuppression with selective post-operative plasmapheresis and without splenectomy<sup>[48]</sup>. Plasmapheresis may be useful by reducing the recipients' antibody titers before and after transplantation. ABO incompatible liver transplantation may be the only available option in LDLT, if the patients have no ABO identical or compatible donors. According to the Japanese Registry of LDLT Across ABO Blood Type Barrier, 97 ABO incompatible LDLTs were performed in Japan before 2005 and 5-year survival rate of the patients was 38% before 2001 and improved to 63% among patients who underwent transplantation after 2002<sup>[49]</sup>.

Although recent studies support the concept of ABO-incompatible liver transplantation both in adults and children, further studies are needed to draw a conclusion. More well-designed, controlled clinical trials are necessary to establish optimal pretransplantation management protocols including immunosuppressive regimens in this group of patients.

### **CONCLUSION**

Liver transplantation is the only definitive treatment modality of end stage liver diseases. Although LDLT has been widely performed with results similar to whole organ cadaveric transplantation, the benefits of the recipients *versus* the risks and long-term morbidities imposed on the donors require further studies. The overall reported donor mortality is 12 in about 6000 transplantations (0.2%)<sup>[50]</sup>. Recurrent viral hepatitis and HCC are among the major causes of late graft loss after liver transplantation. Current antiviral treatment for recurrent HCV offer limited chance of long-term success. To overcome organ shortage there is now a resurgence of interest in NHBD liver transplantation. Although ABO incompatible liver transplantation especially using A<sub>2</sub> donors is promising particularly in children, more studies are needed to draw a conclusion.

## REFERENCES

- 1 **Groth CG**, Brent LB, Calne RY, Dausset JB, Good RA, Murray JE, Shumway NE, Schwartz RS, Starzl TE, Terasaki PI, Thomas ED, van Rood JJ. Historic landmarks in clinical transplantation: conclusions from the consensus conference at the University of California, Los Angeles. *World J Surg* 2000; **24**: 834-843
- 2 **Starzl TE**. History of clinical transplantation. *World J Surg* 2000; **24**: 759-782
- 3 **Raia S**, Nery JR, Mies S. Liver transplantation from live donors. *Lancet* 1989; **2**: 497
- 4 **Japanese Liver Transplantation Society**. Liver transplantation in Japan: registry by the Japanese Liver Transplantation Society. *Jap J Transpl* 2003; **38**: 401-408
- 5 **Shneider BL**, Emre S. Pediatric liver transplantation: past, present, and future. *Liver Transpl* 2006; **12**: 511-513
- 6 **Malago M**, Burdelski M, Broelsch CE. Present and future challenges in living related liver transplantation. *Transplant Proc* 1999; **31**: 1777-1781
- 7 **Pomfret EA**, Pomposelli JJ, Lewis WD, Gordon FD, Burns DL, Lally A, Raptopoulos V, Jenkins RL. Live donor adult liver transplantation using right lobe grafts: donor evaluation and surgical outcome. *Arch Surg* 2001; **136**: 425-433
- 8 **Grewal HP**, Shokouh-Amiri MH, Vera S, Stratta R, Bagous W, Gaber AO. Surgical technique for right lobe adult living donor liver transplantation without venovenous bypass or portocaval shunting and with duct-to-duct biliary reconstruction. *Ann Surg* 2001; **233**: 502-508
- 9 **Tanaka K**. Progress and future in living donor liver transplantation. *Keio J Med* 2003; **52**: 73-79
- 10 **Sugawara Y**, Makuuchi M. Living donor liver transplantation: present status and recent advances. *Br Med Bull* 2005; **75**: 15-28
- 11 **Kawasaki S**, Makuuchi M, Matsunami H, Hashikura Y, Ikegami T, Nakazawa Y, Chisuiwa H, Terada M, Miyagawa S. Living related liver transplantation in adults. *Ann Surg* 1998; **227**: 269-274
- 12 **Lo CM**, Fan ST, Liu CL, Chan JK, Lam BK, Lau GK, Wei WI, Wong J. Minimum graft size for successful living donor liver transplantation. *Transplantation* 1999; **68**: 1112-1116
- 13 **Kiuchi T**, Kasahara M, Uryuhara K, Inomata Y, Uemoto S, Asonuma K, Egawa H, Fujita S, Hayashi M, Tanaka K. Impact of graft size mismatching on graft prognosis in liver transplantation from living donors. *Transplantation* 1999; **67**: 321-327
- 14 **Ben-Haim M**, Emre S, Fishbein TM, Sheiner PA, Bodian CA, Kim-Schluger L, Schwartz ME, Miller CM. Critical graft size in adult-to-adult living donor liver transplantation: impact of the recipient's disease. *Liver Transpl* 2001; **7**: 948-953
- 15 **Ghobrial RM**, Busuttil RW. Future of adult living donor liver transplantation. *Liver Transpl* 2003; **9**: S73-S79
- 16 **Lee SG**. Asian contribution to living donor liver transplantation. *J Gastroenterol Hepatol* 2006; **21**: 572-574
- 17 **Abbasoglu O**, Levy MF, Brkic BB, Testa G, Jeyarajah DR, Goldstein RM, Husberg BS, Gonwa TA, Klintmalm GB. Ten years of liver transplantation: an evolving understanding of late graft loss. *Transplantation* 1997; **64**: 1801-1807
- 18 **Gane EJ**, Angus PW, Strasser S, Crawford DH, Ring J, Jeffrey GP, McCaughan GW. Lamivudine plus low-dose hepatitis B immunoglobulin to prevent recurrent hepatitis B following liver transplantation. *Gastroenterology* 2007; **132**: 931-937
- 19 **Takaki A**, Yagi T, Iwasaki Y, Sadamori H, Matsukawa H, Matsuda H, Shinoura S, Umeda Y, Miyake Y, Terada R, Kobashi H, Sakaguchi K, Tanaka N, Shiratori Y. Short-term high-dose followed by long-term low-dose hepatitis B immunoglobulin and lamivudine therapy prevented recurrent hepatitis B after liver transplantation. *Transplantation* 2007; **83**: 231-233
- 20 **Gornals JB**, Casanovas T, Sabido M, Baliellis C, Casanovas A, Canas C, Serrano T, Verdura B, Chahri N, Gil-Vernet S, Figueras J. Clinical and virological effects during two years of ongoing adefovir dipivoxil in the treatment of lamivudine-resistant chronic hepatitis B infection. *Transplant Proc* 2005; **37**: 3957-3959
- 21 **Eisenbach C**, Sauer P, Mehrabi A, Stremmel W, Encke J. Prevention of hepatitis B virus recurrence after liver transplantation. *Clin Transplant* 2006; **20** Suppl 17: 111-116
- 22 **Johnson MW**, Washburn WK, Freeman RB, FitzMaurice SE, Dienstag J, Basgoz N, Jenkins RL, Cosimi AB. Hepatitis C viral infection in liver transplantation. *Arch Surg* 1996; **131**: 284-291
- 23 **Rosen HR**, O'Reilly PM, Shackleton CR, McDiarmid S, Holt C, Busuttil RW, Martin P. Graft loss following liver transplantation in patients with chronic hepatitis C. *Transplantation* 1996; **62**: 1773-1776
- 24 **Russo MW**, Galanko J, Beavers K, Fried MW, Shrestha R. Patient and graft survival in hepatitis C recipients after adult living donor liver transplantation in the United States. *Liver Transpl* 2004; **10**: 340-346
- 25 **Riediger C**, Berberat PO, Sauer P, Gotthardt D, Weiss KH, Mehrabi A, Merle U, Stremmel W, Encke J. Prophylaxis and treatment of recurrent viral hepatitis after liver transplantation. *Nephrol Dial Transplant* 2007; **22** Suppl 8: viii37-viii46
- 26 **Kornberg A**, Kupper B, Tannapfel A, Barthel E, Thrum K, Settmacher U. Antiviral maintenance treatment with interferon and ribavirin for recurrent hepatitis C after liver transplantation: pilot study. *J Gastroenterol Hepatol* 2007; **22**: 2135-2142
- 27 **Shiffman ML**, Stravitz RT, Contos MJ, Mills AS, Sterling RK, Luketic VA, Sanyal AJ, Cotterell A, Maluf D, Posner MP, Fisher RA. Histologic recurrence of chronic hepatitis C virus in patients after living donor and deceased donor liver transplantation. *Liver Transpl* 2004; **10**: 1248-1255
- 28 **Gaglio PJ**, Malireddy S, Levitt BS, Lapointe-Rudow D, Lefkowitz J, Kinkhabwala M, Russo MW, Emond JC, Brown RS Jr. Increased risk of cholestatic hepatitis C in recipients of grafts from living versus cadaveric liver donors. *Liver Transpl* 2003; **9**: 1028-1035
- 29 **Petrovic LM**. Early recurrence of hepatitis C virus infection after liver transplantation. *Liver Transpl* 2006; **12**: S32-S37
- 30 **Ikedo T**, Yanaga K, Kishikawa K, Kakizoe S, Shimada M, Sugimachi K. Ischemic injury in liver transplantation: difference in injury sites between warm and cold ischemia in rats. *Hepatology* 1992; **16**: 454-461
- 31 **Abt P**, Crawford M, Desai N, Markmann J, Olthoff K, Shaked A. Liver transplantation from controlled non-heart-beating donors: an increased incidence of biliary complications. *Transplantation* 2003; **75**: 1659-1663
- 32 **Abt PL**, Desai NM, Crawford MD, Forman LM, Markmann JW, Olthoff KM, Markmann JF. Survival following liver transplantation from non-heart-beating donors. *Ann Surg* 2004; **239**: 87-92
- 33 **Reddy S**, Zilvetti M, Brockmann J, McLaren A, Friend P. Liver transplantation from non-heart-beating donors: current status and future prospects. *Liver Transpl* 2004; **10**: 1223-1232
- 34 **Kassahun WT**, Fangmann J, Harms J, Hauss J, Bartels M. Liver resection and transplantation in the management of hepatocellular carcinoma: a review. *Exp Clin Transplant* 2006; **4**: 549-558
- 35 **Mazzaferro V**, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996; **334**: 693-699
- 36 **Yao FY**, Ferrell L, Bass NM, Bacchetti P, Ascher NL, Roberts JP. Liver transplantation for hepatocellular carcinoma: comparison of the proposed UCSF criteria with the Milan criteria and the Pittsburgh modified TNM criteria. *Liver Transpl* 2002; **8**: 765-774
- 37 **Bruix J**, Llovet JM. Prognostic prediction and treatment strategy in hepatocellular carcinoma. *Hepatology* 2002; **35**: 519-524

- 38 **Sutcliffe R**, Maguire D, Portmann B, Rela M, Heaton N. Selection of patients with hepatocellular carcinoma for liver transplantation. *Br J Surg* 2006; **93**: 11-18
- 39 **Yao FY**, Bass NM, Nikolai B, Davern TJ, Kerlan R, Wu V, Ascher NL, Roberts JP. Liver transplantation for hepatocellular carcinoma: analysis of survival according to the intention-to-treat principle and dropout from the waiting list. *Liver Transpl* 2002; **8**: 873-883
- 40 **Sarasin FP**, Giostra E, Mentha G, Hadengue A. Partial hepatectomy or orthotopic liver transplantation for the treatment of resectable hepatocellular carcinoma? A cost-effectiveness perspective. *Hepatology* 1998; **28**: 436-442
- 41 **Furukawa H**, Shimamura T, Suzuki T, Taniguchi M, Yamashita K, Kamiyama T, Matsushita M, Todo S. Living-donor liver transplantation for hepatocellular carcinoma. *J Hepatobiliary Pancreat Surg* 2006; **13**: 393-397
- 42 **Cheng SJ**, Pratt DS, Freeman RB Jr, Kaplan MM, Wong JB. Living-donor versus cadaveric liver transplantation for non-resectable small hepatocellular carcinoma and compensated cirrhosis: a decision analysis. *Transplantation* 2001; **72**: 861-868
- 43 **Sarasin FP**, Majno PE, Llovet JM, Bruix J, Mentha G, Hadengue A. Living donor liver transplantation for early hepatocellular carcinoma: A life-expectancy and cost-effectiveness perspective. *Hepatology* 2001; **33**: 1073-1079
- 44 **Bigourdan JM**, Jaeck D, Meyer N, Meyer C, Oussoultzoglou E, Bachellier P, Weber JC, Audet M, Doffoel M, Wolf P. Small hepatocellular carcinoma in Child A cirrhotic patients: hepatic resection versus transplantation. *Liver Transpl* 2003; **9**: 513-520
- 45 **Facciuto ME**, Koneru B, Rocca JP, Wolf DC, Kim-Schluger L, Visintainer P, Klein KM, Chun H, Marvin M, Rozenblit G, Rodriguez-Davalos M, Sheiner PA. Surgical Treatment of Hepatocellular Carcinoma beyond Milan Criteria. Results of Liver Resection, Salvage Transplantation, and Primary Liver Transplantation. *Ann Surg Oncol* 2008; **15**: 1383-1391
- 46 **Rydborg L**. ABO-incompatibility in solid organ transplantation. *Transfus Med* 2001; **11**: 325-342
- 47 **Skogsberg U**, Breimer ME, Friman S, Mjornstedt L, Molne J, Olausson M, Rydberg L, Svalander CT, Backman L. Successful ABO-incompatible liver transplantation using A2 donors. *Transplant Proc* 2006; **38**: 2667-2670
- 48 **Heffron T**, Welch D, Pillen T, Asolati M, Smallwood G, Hagedorn P, Nam C, Duncan A, Guy M, Martinez E, Spivey J, Douglas P, Fasola C, De Paolo J, Rodriguez J, Romero R. Successful ABO-incompatible pediatric liver transplantation utilizing standard immunosuppression with selective postoperative plasmapheresis. *Liver Transpl* 2006; **12**: 972-978
- 49 **Sugawara Y**, Makuuchi M. Adult liver transplantation using live ABO-incompatible grafts in Western countries. *Liver Transpl* 2006; **12**: 1324-1325
- 50 **Middleton PF**, Duffield M, Lynch SV, Padbury RT, House T, Stanton P, Verran D, Maddern G. Living donor liver transplantation--adult donor outcomes: a systematic review. *Liver Transpl* 2006; **12**: 24-30

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## Strategy for treatment of nonerosive reflux disease in Asia

Toru Hiyama, Masaharu Yoshihara, Shinji Tanaka, Ken Haruma, Kazuaki Chayama

Toru Hiyama, Masaharu Yoshihara, Health Service Center, Hiroshima University, Higashihiroshima 739-8521, Japan

Shinji Tanaka, Department of Endoscopy, Hiroshima University Hospital, Hiroshima 734-8551, Japan

Ken Haruma, Division of Gastroenterology, Department of Internal Medicine, Kawasaki Medical School, Kurashiki 701-0192, Japan

Kazuaki Chayama, Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima 734-8551, Japan

Author contributions: Hiyama T, Yoshihara M and Tanaka S analyzed data; Hiyama T wrote the paper; Haruma K and Chayama K supervised this review.

Correspondence to: Toru Hiyama, MD, PhD, Health Service Center, Hiroshima University, 1-7-1 Kagamiyama, Higashihiroshima 739-8521, Japan. [tohiyama@hiroshima-u.ac.jp](mailto:tohiyama@hiroshima-u.ac.jp)

Telephone: +81-82-4246191 Fax: +81-82-4227156

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Practice and Primary Care, King's College London, 5 Lambeth Walk, London SE11 6SP, United Kingdom

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### INTRODUCTION

Gastroesophageal reflux disease (GERD) is a condition that develops when reflux of stomach contents causes troublesome symptoms and/or complications<sup>[1]</sup>. GERD is more common in Western countries than in Asian countries, such as China, Korea, and Japan. Epidemiologic studies show a prevalence of GERD symptoms in Western countries ranging from 20% to 40%<sup>[2,3]</sup> and in Asian countries ranging from 5% to 17%<sup>[4]</sup>. The prevalence in Asian countries has increased gradually<sup>[4]</sup>. Esophagogastroduodenoscopy (EGD) is the gold standard for the diagnosis of erosive GERD [reflux esophagitis (RE)], and the Los Angeles (LA) classification of esophagitis is generally accepted as the best means for endoscopic assessment of GERD<sup>[5]</sup>. In Japan, the prevalence of RE (LA classification grades A, B, C, and D) is approximately 15%, and most of these cases are grade A or B<sup>[6]</sup>. The majority of GERD cases are cases of nonerosive reflux disease (NERD).

NERD was previously considered a mild/early type of RE that would progress to severe RE. However, it was reported that, regardless of therapy, only 2.7% of NERD patients develop RE after 3 years and only 3% of patients develop RE after 6 years<sup>[7]</sup>. A recent retrospective study of 2306 GERD patients found that these patients at least two separate upper endoscopic examinations during the 7-year (mean) follow-up period. Examinations revealed that 69% of the patients were unchanged, 21% were improved, and 11% became worse<sup>[8]</sup>. Another study<sup>[9]</sup> reported similar results. These studies suggest that NERD rarely progresses to RE over time. In addition, NERD is significantly more refractory to treatment than RE<sup>[3]</sup>. Therefore, it was recently suggested that the underlying mechanism of development of NERD is different from that of RE. Here we review the clinical and pathophysiologic differences between NERD and RE and propose a treatment strategy for NERD, especially for patients in Asia.

### Abstract

The paper is to review the clinical and pathophysiologic differences between of nonerosive reflux disease (NERD) and reflux esophagitis (RE), and to propose a treatment strategy for NERD, especially for patients in Asia. A Medline search was performed regarding the clinical and pathophysiologic differences between NERD and RE, and treatment of NERD and RE. The characteristics of NERD patients in Asia are as follows: (1) high proportion of female patients, (2) low frequency of hiatal hernia, (3) high frequency of *H pylori* infection, (4) severe glandular atrophy of the gastric mucosa, and (5) frequent resistance to proton pump inhibitor (PPI) therapy. In Asian NERD patients, exposure of the esophagus to acid is not increased, and esophageal motility is normal. These characteristics are similar to those of patients in Western countries. Our recommended first-choice treatment is administration of PPI in combination with a prokinetic agent. However, at present, because there is limited evidence regarding effective treatments for NERD, it is best to try several different treatment strategies to find the best treatment for each patient.

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**Key words:** Nonerosive reflux disease; Asia; Treatment

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**Table 1 Clinical characteristics of NERD and RE patients in Asia**

	NERD	RE
Male/Female	0.59-1.65	1.18-7.13
Average age (yr)	45.2-57.5	49.0-59.7
Mean body mass index (kg/m <sup>2</sup> )	22.1-23.1	21.7-24.2
Complication of hiatal hernia (%)	17.7-34.8	35.1-77.0
<i>H pylori</i> infection (%)	36.3-48.3	18.0-32.3
Glandular atrophy of the gastric mucosa (open-type) (%)	25.0-43.0	6.7-25.0
Efficacy of proton pump inhibitor (%)	29.5-64.0	55.4-90.3

## METHODS

Studies on GERD were identified by computerized and manual searches of the available literature. The Medline search (1975-2007) was performed using the following medical subject headings: reflux disease and Asia. Papers published in English were considered.

## CLINICAL AND ESOPHAGEAL MOTILITY CHARACTERISTICS OF NERD IN ASIA

Several researchers examined characteristics of NERD and RE patients in Asia<sup>[10-18]</sup>. The male/female ratios ranged from 0.59 to 1.65 in NERD patients. On the other hand, those of RE patients ranged from 1.18 to 7.13. A higher proportion of female patients was observed in NERD patients compared with RE patients. There were differences between NERD patients and RE patients in frequency of hiatal hernia, frequency of *H pylori* infection, grade of glandular atrophy of the gastric mucosa, and effect of proton pump inhibitor (PPI) therapy as well. Namely, compared with the RE patients, the characteristics of NERD patients in Asia are as follows: (1) higher proportion of female patients, (2) lower frequency of hiatal hernia, (3) higher frequency of *H pylori* infection, (4) severe glandular atrophy of the gastric mucosa, and (5) frequent resistance to PPI therapy (Table 1). In addition, Asian NERD patients are more frequently affected by functional dyspepsia, irritable bowel syndrome, and psychiatric diseases than RE patients<sup>[13]</sup>. These characteristics are similar to those of Western NERD patients. However, there are several other characteristics in Western NERD patients, such as younger age and less obese<sup>[3]</sup>. As the prevalence of *H pylori* infection in Asian populations has decreased to levels similar to those in Western populations, these additional characteristics may be observed in Asian patients in the near future.

With respect to esophageal motility, NERD patients have several characteristics that differ from those of RE patients. In NERD patients, the resting lower esophageal sphincter (LES) pressure is not decreased. In addition, exposure of the esophagus to acid is not increased, and esophageal motility is normal (Table 2)<sup>[19]</sup>. These characteristics are similar to those of patients in Western countries, although the grades of motility index abnormalities in Asian RE patients are lower than those in Western RE patients<sup>[20]</sup>.

**Table 2 Esophageal motility characteristics in NERD and RE patients in Asia**

	NERD	RE
Resting LES pressure	Mildly increased	Moderately decreased
Reflux episodes/hour	Moderately increased	Moderately increased
Primary peristalsis	Normal	Moderately decreased
Secondary peristalsis	Mildly decreased	Moderately decreased
Acid clearance	Mildly delayed	Moderately delayed

LES: Lower esophageal sphincter.

It seems that there are differences in pathophysiology between Asian RE patients and Western RE patients, because the grades of motility index abnormalities are different between them. However, there seems no significant difference in pathophysiology between Asian NERD and Western NERD patients, because clinical and esophageal motility characteristics are considerably similar between them.

## PATHOPHYSIOLOGY OF NERD

The main pathophysiology of RE is excessive exposure of the esophagus to gastric acid. Approximately 90% of patients with RE can be cured with a PPI, which is the strongest type of gastric acid suppressor<sup>[3]</sup>. In contrast, only one-third of NERD patients can be cured with a PPI. Although the cause of NERD that is responsive to PPI may be excessive exposure of the esophagus to acid, PPI-resistant NERD may be associated with the factors described below.

### Incomplete acid suppression

In some patients, even the highest approved dose of PPI cannot sufficiently suppress gastric acid secretion. In patients with insufficient gastric acid suppression, gastric juice may reflux, exposing the esophagus to acid. The time required for metabolism of PPI differs between patients possibly due to polymorphisms in the genes encoding metabolic enzymes, such as CYP2C19<sup>[21,22]</sup>. In patients with the rapid metabolic phenotype, administration of twice the approved dose of PPI and concomitant administration of PPI and H<sub>2</sub>-receptor antagonist (H<sub>2</sub>RA) may be more effective<sup>[23,24]</sup>. It has also been reported that administration of an aluminum- and magnesium-containing antacid may be effective for some NERD patients<sup>[25]</sup>.

### Esophageal hypersensitivity to acid

Some patients with severe RE do not have symptoms of acid regurgitation, even if severe esophageal acid exposure is confirmed<sup>[26]</sup>. However, many NERD patients have a normal level of esophageal acid exposure. Therefore, there appears to be significant esophageal hypersensitivity to acid exposure in PPI-resistant NERD patients, and symptoms may occur when gastric acid is refluxed<sup>[27]</sup>. Hyperosmotic foods, such as cake and chocolate, and alcoholic beverages may be the cause of this esophageal hypersensitivity<sup>[28]</sup>. Ingestion of such

foods and drinks may cause heartburn. It has been suggested that ingestion of hyperosmotic foods/drinks loosens the tight junctions between esophageal epithelial cells, and when gastric acid is refluxed, it easily intrudes between epithelial cells and stimulates the terminals of sensory nerves<sup>[26]</sup>.

### **Esophageal hypersensitivity to esophageal wall distension**

In NERD patients, heartburn symptoms are induced by distension of the esophageal wall by balloon dilatation or by pumping saline into the esophageal lumen<sup>[29]</sup>. These findings suggest the possibility that foods, air, and fluids that contain no acid may cause heartburn symptoms simply by distending of the esophageal wall.

### **Reflux of duodenal juice (bile and pancreatic juice)**

PPI suppresses gastric acid excretion but has no effect on reflux itself. Therefore, in patients with duodenogastric reflux, duodenal juice (bile and pancreatic juice) may be refluxed into the esophagus. It is possible that the refluxed duodenal juice may affect the esophageal mucosa<sup>[30]</sup>. NERD patients frequently have functional dyspepsia<sup>[4]</sup>, and significant duodenogastric reflux and delayed gastric emptying time in patients with functional dyspepsia have been reported<sup>[31,32]</sup>. These findings support the idea that reflux of duodenal juice into the esophagus causes NERD.

### **Esophageal motility abnormalities**

It has been reported that NERD patients show normal resting LES pressure and primary contraction waves but significantly reduced frequency of secondary contraction waves<sup>[19,33]</sup>. This may be due to a reduced response to distension of the esophageal wall. Secondary contraction waves are stimulated by distension of the esophageal wall and act to discharge refluxed gastric acid and air into the stomach. Heartburn symptoms may be associated with reduced motility function in the esophageal wall.

### **Sustained esophageal contraction**

Sustained contraction of the longitudinal muscles of the esophagus causes heartburn, and prolonged contraction may lead to chest pain. This phenomenon is called sustained esophageal contraction (SEC) and is identified by intraluminal ultrasonography<sup>[34,35]</sup>. SEC occurs just before the onset of heartburn symptoms. There are two types of SEC: SEC with or without subsequent acid reflux. Because patients with the latter type also have heartburn symptoms, the association of SEC with NERD is of great interest.

### **Psychological factors**

NERD patients frequently have mental disorders<sup>[13]</sup>. Psychological factors are associated with response to treatment as well as symptoms<sup>[36]</sup>. A high level of anxiety is predictive for the nonresponse to acid suppression therapy.

### **Eosinophilic esophagitis**

Eosinophilic esophagitis affects both children and adults

and is characterized by symptoms of GERD and dense esophageal eosinophilia, both of which are unresponsive to PPI<sup>[37,38]</sup>. This disease is caused by food allergies or by aeroallergens. Effective treatment include systemic/topical corticosteroids, or specific food elimination. Esophageal stricture is a potential complication, and the natural history of the disease is still unknown. Eosinophilic esophagitis may be diagnosed as PPI-resistant NERD, but should be excluded from the diagnosis of NERD.

## **TREATMENT STRATEGY FOR NERD IN ASIA**

At present, PPI-based step-down treatment is recommended for GERD patients<sup>[39,40]</sup>. In a meta-analysis, the relative risks of PPI and H<sub>2</sub>RA treatment for NERD compared with placebo were 0.69 (95% confidence interval, 0.62-0.78) and 0.84 (0.74-0.95), respectively, indicating that PPI is a more effective treatment than H<sub>2</sub>RA<sup>[41]</sup>. PPI treatment can eliminate NERD symptoms faster than H<sub>2</sub>RA treatment. In addition, PPI treatment has been reported to be more cost-effective than other treatment<sup>[42]</sup>.

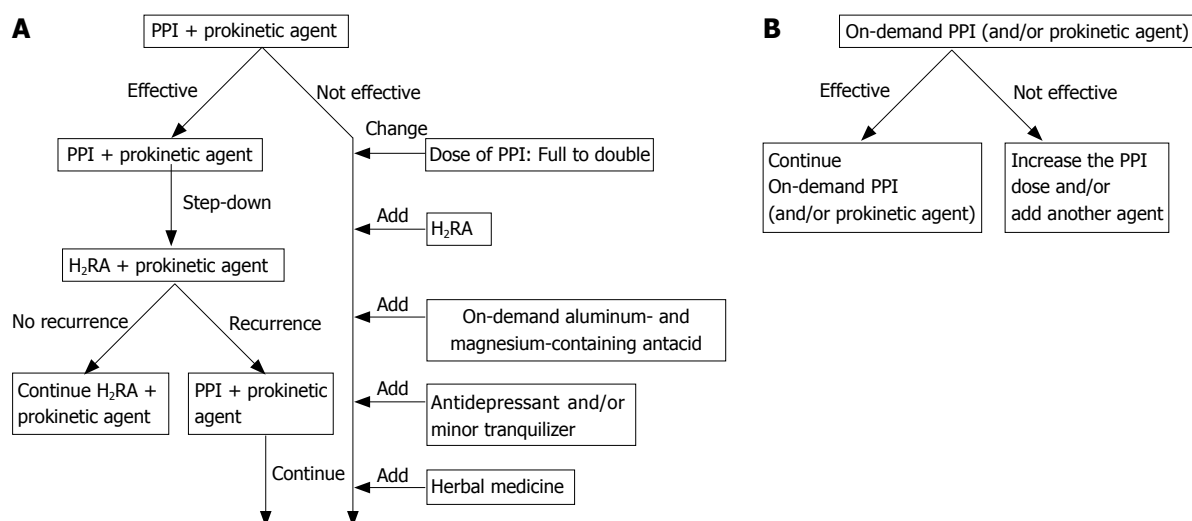
Prokinetics such as mosapride, itopride, metoclopramide, and domperidone are also effective for treatment of NERD<sup>[43-45]</sup>. Prokinetics are thought to work by reducing reflux of duodenal juice into the esophagus<sup>[31]</sup> and speeding absorption of PPI. In addition, mosapride improves esophageal motility, whereas metoclopramide and domperidone do not have this ability<sup>[46]</sup>. Mosapride shortens bolus transit time in the esophagus, reduces the duration of the longest reflux episode and reflux fraction time, and enhances the contraction strength in the lower esophagus.

Reflux of stomach contents is related to transient LES relaxation (TLESR) in NERD patients<sup>[47]</sup>. Therefore, control of TLESR is another important point for NERD treatment. 5-HT<sub>3</sub>, cholecystokinin (CCK)-A, and gamma-aminobutyric acid (GABA) receptors influence TLESR<sup>[48-50]</sup>. 5-HT<sub>3</sub> receptor antagonist, CCK-A receptor antagonist, and GABA receptor agonist reduce the frequency of TLESR. Mosapride is a selective 5-HT<sub>4</sub> receptor agonist, and the metabolite acts as a 5-HT<sub>3</sub> receptor antagonist<sup>[51,52]</sup>. Therefore, mosapride reduces the frequency of TLESR, leading to reduced gastric acid reflux in NERD patients.

Some NERD cases are refractory to PPI and/or prokinetics. In these patients, psychological factors may be associated with symptoms. In these patients, administration of an antidepressant and/or minor tranquilizer should be considered. However, evidence for the benefits of these agents in treatment of NERD is weak<sup>[53]</sup>, and further studies are needed to clarify the effects of such medications on NERD.

For NERD patients with infrequent symptoms of heartburn, on-demand therapy with PPI (and/or prokinetics) is proposed as the best treatment option<sup>[54,55]</sup>. Additional studies of the effectiveness of this treatment regimen are needed.

Here we propose a new strategy for treatment of NERD in Asia based on the basic idea of step-down



**Figure 1** Proposed treatment strategy for NERD patients in Asia. **A:** Patients with moderate or severe symptoms; **B:** Patients with infrequent symptoms.

therapy (Figure 1). The recommended first-choice treatment is administration of PPI in combination with a prokinetic agent such as mosapride. PPI can cure only one-third of NERD patients, a prokinetic agent in conjunction with the PPI can increase the efficacy. NERD is frequently associated with functional dyspepsia that can be treated with prokinetic agents. In addition, because the quality of life of NERD patients is quite low, NERD patients need quicker and more effective treatment options<sup>[56]</sup>. If this treatment is not effective, twice the recommended dose of PPI or combined treatment with PPI and an H<sub>2</sub>RA is recommended. PPI together with on-demand aluminum- and magnesium-containing antacid might be effective. If these treatments are not effective, administration of an antidepressant or minor tranquilizer should be considered. Herbal medicines such as rikkunshito may provide relief for some patients<sup>[57]</sup>, and are often administered especially in Asian countries.

For patients with infrequent symptoms, on-demand treatment with PPI and/or a prokinetic agent is recommended. However, there is not sufficient evidence for a best treatment for NERD. Further studies are needed to clarify the efficacy of treatment. Large-scale, double-blind, randomized controlled trials of PPI *vs* PPI with a prokinetic agent are also needed to clarify the benefit of the prokinetic agent.

Further trials are needed to establish the strategy for treatment of NERD. At present, because there is limited evidence regarding effective treatments for the disease, it is best to try several different treatment strategies to find the best treatment for each patient.

## REFERENCES

- Vakil N, van Zanten SV, Kahrilas P, Dent J, Jones R. The Montreal definition and classification of gastroesophageal reflux disease: a global evidence-based consensus. *Am J Gastroenterol* 2006; **101**: 1900-1920; quiz 1943
- Moayyedi P, Talley NJ. Gastro-oesophageal reflux disease. *Lancet* 2006; **367**: 2086-2100
- Fass R. Erosive esophagitis and nonerosive reflux disease (NERD): comparison of epidemiologic, physiologic, and therapeutic characteristics. *J Clin Gastroenterol* 2007; **41**: 131-137
- Wong BC, Kinoshita Y. Systematic review on epidemiology of gastroesophageal reflux disease in Asia. *Clin Gastroenterol Hepatol* 2006; **4**: 398-407
- Lundell LR, Dent J, Bennett JR, Blum AL, Armstrong D, Galmiche JP, Johnson F, Hongo M, Richter JE, Spechler SJ, Tytgat GN, Wallin L. Endoscopic assessment of oesophagitis: clinical and functional correlates and further validation of the Los Angeles classification. *Gut* 1999; **45**: 172-180
- Manabe N, Yoshihara M, Sasaki A, Tanaka S, Haruma K, Chayama K. Clinical characteristics and natural history of patients with low-grade reflux esophagitis. *J Gastroenterol Hepatol* 2002; **17**: 949-954
- Kuster E, Ros E, Toledo-Pimentel V, Pujol A, Bordas JM, Grande L, Pera C. Predictive factors of the long term outcome in gastro-oesophageal reflux disease: six year follow up of 107 patients. *Gut* 1994; **35**: 8-14
- Sontag SJ, Sonnenberg A, Schnell TG, Leya J, Metz A. The long-term natural history of gastroesophageal reflux disease. *J Clin Gastroenterol* 2006; **40**: 398-404
- Fullard M, Kang JY, Neild P, Poullis A, Maxwell JD. Systematic review: does gastro-oesophageal reflux disease progress? *Aliment Pharmacol Ther* 2006; **24**: 33-45
- Fujiwara Y, Higuchi K, Shiba M, Yamamori K, Watanabe Y, Sasaki E, Tominaga K, Watanabe T, Oshitani N, Arakawa T. Differences in clinical characteristics between patients with endoscopy-negative reflux disease and erosive esophagitis in Japan. *Am J Gastroenterol* 2005; **100**: 754-758
- Nakamura T, Shirakawa K, Masuyama H, Sugaya H, Hiraishi H, Terano A. Minimal change oesophagitis: a disease with characteristic differences to erosive oesophagitis. *Aliment Pharmacol Ther* 2005; **21** Suppl 2: 19-26
- Mishima I, Adachi K, Arima N, Amano K, Takashima T, Moritani M, Furuta K, Kinoshita Y. Prevalence of endoscopically negative and positive gastroesophageal reflux disease in the Japanese. *Scand J Gastroenterol* 2005; **40**: 1005-1009
- Wu JC, Cheung CM, Wong VW, Sung JJ. Distinct clinical characteristics between patients with nonerosive reflux disease and those with reflux esophagitis. *Clin Gastroenterol Hepatol* 2007; **5**: 690-695
- Miwa H, Sasaki M, Furuta T, Koike T, Habu Y, Ito M, Fujiwara Y, Wada T, Nagahara A, Hongo M, Chiba T, Kinoshita Y. Efficacy of rabeprazole on heartburn symptom resolution in patients with non-erosive and erosive gastro-oesophageal



- reflux disease: a multicenter study from Japan. *Aliment Pharmacol Ther* 2007; **26**: 69-77
- 15 **Kinoshita Y**, Kobayashi T, Kato M, Asahina K, Haruma K, Shimatani T, Inoue S, Kabemura T, Kurosawa S, Kuwayama H, Ashida K, Hirayama M, Kiyama S, Yamamoto M, Suzuki J, Suzuki H, Matsumoto K, Aoshima M. The pharmacodynamic effect of omeprazole 10 mg and 20 mg once daily in patients with nonerosive reflux disease in Japan. *J Gastroenterol* 2006; **41**: 554-561
  - 16 **Cheung TK**, Wong WM, Wong NY, Chan CK, Fung J, Yuen MF, Chan AO, Tong TS, Wong BC. Symptom resolution does not predict healing of erosive oesophagitis in Chinese. *Digestion* 2007; **75**: 128-134
  - 17 **Dinakaran NH**, Rajkumar JS, Potdar NP, Desai A. An open non-comparative clinical study for the evaluation of safety and efficacy of esomeprazole in patients of reflux oesophagitis in Indian population. *J Indian Med Assoc* 2002; **100**: 624-626
  - 18 **Lee YC**, Lin JT, Wang HP, Chiu HM, Wu MS. Influence of cytochrome P450 2C19 genetic polymorphism and dosage of rabeprazole on accuracy of proton-pump inhibitor testing in Chinese patients with gastroesophageal reflux disease. *J Gastroenterol Hepatol* 2007; **22**: 1286-1292
  - 19 **Wong WM**, Lai KC, Hui WM, Hu WH, Huang JQ, Wong NY, Xia HH, Chan OO, Lam SK, Wong BC. Pathophysiology of gastroesophageal reflux diseases in Chinese--role of transient lower esophageal sphincter relaxation and esophageal motor dysfunction. *Am J Gastroenterol* 2004; **99**: 2088-2093
  - 20 **Sifrim D**, Zhang X. Pathophysiology of GERD in China: the same factors at a lower scale. *Am J Gastroenterol* 2004; **99**: 2094-2097
  - 21 **Horai Y**, Kimura M, Furuie H, Matsuguma K, Irie S, Koga Y, Nagahama T, Murakami M, Matsui T, Yao T, Urae A, Ishizaki T. Pharmacodynamic effects and kinetic disposition of rabeprazole in relation to CYP2C19 genotypes. *Aliment Pharmacol Ther* 2001; **15**: 793-803
  - 22 **Sakurai Y**, Hirayama M, Hashimoto M, Tanaka T, Hasegawa S, Irie S, Ashida K, Kayano Y, Taguchi M, Hashimoto Y. Population pharmacokinetics and proton pump inhibitory effects of intravenous lansoprazole in healthy Japanese males. *Biol Pharm Bull* 2007; **30**: 2238-2243
  - 23 **Watson RG**, Tham TC, Johnston BT, McDougall NI. Double blind cross-over placebo controlled study of omeprazole in the treatment of patients with reflux symptoms and physiological levels of acid reflux--the "sensitive oesophagus". *Gut* 1997; **40**: 587-590
  - 24 **Tytgat GN**. Review article: treatment of mild and severe cases of GERD. *Aliment Pharmacol Ther* 2002; **16** Suppl 4: 73-78
  - 25 **Graham DY**, Patterson DJ. Double-blind comparison of liquid antacid and placebo in the treatment of symptomatic reflux esophagitis. *Dig Dis Sci* 1983; **28**: 559-563
  - 26 **Barlow WJ**, Orlando RC. The pathogenesis of heartburn in nonerosive reflux disease: a unifying hypothesis. *Gastroenterology* 2005; **128**: 771-778
  - 27 **Nagahara A**, Miwa H, Minoo T, Hojo M, Kawabe M, Osada T, Kurosawa A, Asaoka D, Terai T, Ohkusa T, Sato N. Increased esophageal sensitivity to acid and saline in patients with nonerosive gastro-esophageal reflux disease. *J Clin Gastroenterol* 2006; **40**: 891-895
  - 28 **Fox M**, Barr C, Nolan S, Lomer M, Anggiansah A, Wong T. The effects of dietary fat and calorie density on esophageal acid exposure and reflux symptoms. *Clin Gastroenterol Hepatol* 2007; **5**: 439-444
  - 29 **Rodriguez-Stanley S**, Robinson M, Earnest DL, Greenwood-Van Meerveld B, Miner PB Jr. Esophageal hypersensitivity may be a major cause of heartburn. *Am J Gastroenterol* 1999; **94**: 628-631
  - 30 **Tack J**. Review article: the role of bile and pepsin in the pathophysiology and treatment of gastro-oesophageal reflux disease. *Aliment Pharmacol Ther* 2006; **24** Suppl 2: 10-16
  - 31 **Kusunoki H**, Haruma K, Hata J, Tani H, Okamoto E, Sumii K, Kajiyama G. Real-time ultrasonographic assessment of antroduodenal motility after ingestion of solid and liquid meals by patients with functional dyspepsia. *J Gastroenterol Hepatol* 2000; **15**: 1022-1027
  - 32 **Aoki S**, Haruma K, Kusunoki H, Hata J, Hara M, Yoshida S, Tanaka S, Chayama K. Evaluation of gastric emptying measured with the <sup>13</sup>C-octanoic acid breath test in patients with functional dyspepsia: comparison with ultrasonography. *Scand J Gastroenterol* 2002; **37**: 662-666
  - 33 **Fass R**. Epidemiology and pathophysiology of symptomatic gastroesophageal reflux disease. *Am J Gastroenterol* 2003; **98**: S2-S7
  - 34 **Balaban DH**, Yamamoto Y, Liu J, Pehlivanov N, Wisniewski R, DeSilvey D, Mittal RK. Sustained esophageal contraction: a marker of esophageal chest pain identified by intraluminal ultrasonography. *Gastroenterology* 1999; **116**: 29-37
  - 35 **Pehlivanov N**, Liu J, Mittal RK. Sustained esophageal contraction: a motor correlate of heartburn symptom. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G743-G751
  - 36 **Wiklund I**, Carlsson R, Carlsson J, Glise H. Psychological factors as a predictor of treatment response in patients with heartburn: a pooled analysis of clinical trials. *Scand J Gastroenterol* 2006; **41**: 288-293
  - 37 **Furuta GT**, Liacouras CA, Collins MH, Gupta SK, Justinich C, Putnam PE, Bonis P, Hassall E, Straumann A, Rothenberg ME. Eosinophilic esophagitis in children and adults: a systematic review and consensus recommendations for diagnosis and treatment. *Gastroenterology* 2007; **133**: 1342-1363
  - 38 **Furuta GT**, Straumann A. Review article: the pathogenesis and management of eosinophilic oesophagitis. *Aliment Pharmacol Ther* 2006; **24**: 173-182
  - 39 **Mine S**, Iida T, Tabata T, Kishikawa H, Tanaka Y. Management of symptoms in step-down therapy of gastroesophageal reflux disease. *J Gastroenterol Hepatol* 2005; **20**: 1365-1370
  - 40 **Ofman JJ**. The economic and quality-of-life impact of symptomatic gastroesophageal reflux disease. *Am J Gastroenterol* 2003; **98**: S8-S14
  - 41 **van Pinxteren B**, Numans ME, Bonis PA, Lau J. Short-term treatment with proton pump inhibitors, H<sub>2</sub>-receptor antagonists and prokinetics for gastro-oesophageal reflux disease-like symptoms and endoscopy negative reflux disease. *Cochrane Database Syst Rev* 2006; **3**: CD002095
  - 42 **Habu Y**, Maeda K, Kusuda T, Yoshino T, Shio S, Yamazaki M, Hayakumo T, Hayashi K, Watanabe Y, Kawai K. "Proton-pump inhibitor-first" strategy versus "step-up" strategy for the acute treatment of reflux esophagitis: a cost-effectiveness analysis in Japan. *J Gastroenterol* 2005; **40**: 1029-1035
  - 43 **Miyamoto M**, Haruma K, Takeuchi K, Kuwabara M. Frequency scale for symptoms of gastroesophageal reflux disease predicts the need for addition of prokinetics to proton pump inhibitor therapy. *J Gastroenterol Hepatol* 2008; **23**: 746-751
  - 44 **Ruth M**, Hamelin B, Rohss K, Ludell L. The effect of mosapride, a novel prokinetic, on acid reflux variables in patients with gastro-oesophageal reflux disease. *Aliment Pharmacol Ther* 1998; **12**: 35-40
  - 45 **Kim YS**, Kim TH, Choi CS, Shon YW, Kim SW, Seo GS, Nah YH, Choi MG, Choi SC. Effect of itopride, a new prokinetic, in patients with mild GERD: a pilot study. *World J Gastroenterol* 2005; **11**: 4210-4214
  - 46 **Ruth M**, Finizia C, Cange L, Lundell L. The effect of mosapride on esophageal motor function and acid reflux in patients with gastro-oesophageal reflux disease. *Eur J Gastroenterol Hepatol* 2003; **15**: 1115-1121
  - 47 **Iwakiri K**, Hayashi Y, Kotoyori M, Tanaka Y, Kawakami A, Sakamoto C, Holloway RH. Transient lower esophageal sphincter relaxations (TLESRs) are the major mechanism of gastroesophageal reflux but are not the cause of reflux disease. *Dig Dis Sci* 2005; **50**: 1072-1077
  - 48 **Koutsoumbi P**, Epanomeritakis E, Tsiaoussis J, Athanasakis H, Chrysos E, Zoras O, Vassilakis JS, Xynos E. The effect of erythromycin on human esophageal motility is mediated by serotonin receptors. *Am J Gastroenterol* 2000; **95**: 3388-3392

- 49 **Adelson DW**, Million M, Kanamoto K, Palanca T, Tache Y. Coordinated gastric and sphincter motility evoked by intravenous CCK-8 as monitored by ultrasonomicrometry in rats. *Am J Physiol Gastrointest Liver Physiol* 2004; **286**: G321-G322
- 50 **McDermott CM**, Abrahams TP, Partosoedarso E, Hyland N, Ekstrand J, Monroe M, Hornby PJ. Site of action of GABA(B) receptor for vagal motor control of the lower esophageal sphincter in ferrets and rats. *Gastroenterology* 2001; **120**: 1749-1762
- 51 **Hiyama T**, Yoshihara M, Matsuo K, Kusunoki H, Kamada T, Ito M, Tanaka S, Nishi N, Chayama K, Haruma K. Meta-analysis of the effects of prokinetic agents in patients with functional dyspepsia. *J Gastroenterol Hepatol* 2007; **22**: 304-310
- 52 **Hiyama T**, Yoshihara M, Matsuo K, Kusunoki H, Kamada T, Ito M, Tanaka S, Chayama K, Haruma K. Treatment of functional dyspepsia with serotonin agonists: a meta-analysis of randomized controlled trials. *J Gastroenterol Hepatol* 2007; **22**: 1566-1570
- 53 **Tack J**, Fass R. Review article: approaches to endoscopic-negative reflux disease: part of the GERD spectrum or a unique acid-related disorder? *Aliment Pharmacol Ther* 2004; **19** Suppl 1: 28-34
- 54 **Metz DC**, Inadomi JM, Howden CW, van Zanten SJ, Bytzer P. On-demand therapy for gastroesophageal reflux disease. *Am J Gastroenterol* 2007; **102**: 642-653
- 55 **Juul-Hansen P**, Rydning A. On-demand PPI requirements in patients with endoscopy-negative GERD. *J Clin Gastroenterol* 2004; **38**: 746-749
- 56 **Prasad M**, Rentz AM, Revicki DA. The impact of treatment for gastro-oesophageal reflux disease on health-related quality of life: a literature review. *Pharmacoeconomics* 2003; **21**: 769-790
- 57 **Kawahara H**, Kubota A, Hasegawa T, Okuyama H, Ueno T, Ida S, Fukuzawa M. Effects of rikkunshito on the clinical symptoms and esophageal acid exposure in children with symptomatic gastroesophageal reflux. *Pediatr Surg Int* 2007; **23**: 1001-1005

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## Intraductal biliary and pancreatic endoscopy: An expanding scope of possibility

Joel R Judah, Peter V Draganov

Joel R Judah, Peter V Draganov, Division of Gastroenterology, Hepatology and Nutrition, University of Florida, Gainesville, Florida 32610-0214, United States

Author contributions: Judah JR wrote the first draft of the paper with guidance and support from Draganov PV.

Correspondence to: Peter Draganov, MD, Division of Gastroenterology, Hepatology and Nutrition, PO Box 100214, University of Florida, Gainesville, Florida 32610-0214, United States. [dragapv@medicine.ufl.edu](mailto:dragapv@medicine.ufl.edu)

Telephone: +1-352-3922877 Fax: +1-352-3923618

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### Abstract

Intraductal endoscopy describes the use of an endoscope to directly visualize the biliary and pancreatic ducts. For many years, technological challenges have made performing these procedures difficult. The "mother-baby" system and other various miniscopes have been developed, but routine use has been hampered due to complex setup, scope fragility and the time consuming, technically demanding nature of the procedure. Recently, the SpyGlass peroral cholangiopancreatography system has shown early success at providing diagnostic information and therapeutic options. The clinical utility of intraductal endoscopy is broad. It allows better differentiation between benign and malignant processes by allowing direct visualization and targeted sampling of tissue. Therapeutic interventions, such as electrohydraulic lithotripsy (EHL), laser lithotripsy, photodynamic therapy, and argon plasma coagulation (APC), may also be performed as part of intraductal endoscopy. Intraductal endoscopy significantly increases the diagnostic and therapeutic yield of standard endoscopic retrograde cholangiography (ERCP), and as technology progresses, it is likely that its utilization will only increase. In this review of intraductal endoscopy, we describe in detail the various endoscopic platforms and their diagnostic and clinical applications.

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**Key words:** Intraductal endoscopy; Choledochoscopy; Cholangioscopy; Pancreatography; Biliary endoscopy; Duodenoscope-assisted cholangiopancreatography;

### INTRODUCTION

Intraductal endoscopy describes the use of an endoscope to evaluate the biliary and pancreatic ducts. There are significant technological challenges encountered in creating a scope that allows direct visualization of these ducts. However, attempts have been made, and technology is developing that promises greater opportunity to provide improved diagnosis and therapy regarding lesions in the biliary and pancreatic ducts.

### HISTORY AND TYPES OF SCOPES

Cholangioscopy was considered as early as the 1950's<sup>[1]</sup>. However, technology at that time caused severe limitations. In the 1960's, intraoperative cholangioscopy was first successfully utilized<sup>[2-4]</sup>. Peroral cholangioscopy (POCS) was initially described in the mid-1970's. One of the first reports demonstrated that a fiberscope of 8.8 mm diameter could be directly inserted through the mouth, into the biliary system after an endoscopic papillotomy, without the need of using a second scope as a guide<sup>[5]</sup>. This scope did provide a biopsy channel to obtain tissue samples. Other investigators also successfully demonstrated the use of POCS to directly visualize the biliary system during this time<sup>[6-10]</sup>.

The idea of guiding a small caliber "baby" cholangioscope through the channel of a "mother" duodenoscope into the common bile duct (CBD) gained acceptance. This "mother-baby" system is also known as duodenoscope-assisted cholangiopancreatography (DACP). However, use of the early cholangioscopes was difficult since

their optical fibers were prone to break easily from pressure applied with the elevator of the duodenoscope. Regardless, Urakami demonstrated successful access to the ductal system in 25 of 30 cases in 1980 by using this technique<sup>[11]</sup>. The University of Chicago published their experience with a conventional “mother-baby” system utilizing a set of Olympus scopes (TJF-M20 and CHF-B20) (Olympus Inc, Tokyo, Japan), where the “baby” scope had a diameter of 4.5 mm, two-way deflection and included an instrument channel<sup>[12]</sup>. This system was used in patients 18 times over a 3-year study period. Initially, they demonstrated a steep learning curve, when they intubated the papilla in only 2 of 5 cases. They subsequently found it was necessary to perform a papillotomy before the “baby” scope could be passed. After this adjustment, they were successful at intubating the papilla in 13 of 13 cases. While the 1.7 mm working channel on the cholangioscope did allow for diagnostic and therapeutic intervention, the system was found to be cumbersome to use. Average time of the procedure was around 2 h. Two endoscopists were required (i.e. one for each scope). The cholangioscopes continued to be fragile and prone to breaking. Further, these cholangioscopes only had two-way deflection at the tip as opposed to the typical four-way deflection offered by other endoscopes. These limitations led this group to conclude that while this “mother and baby” system certainly offered new endoscopic potential, it would best be utilized in only select patients at highly specialized tertiary referral centers. Another study at Case Western Reserve University further validated the use of this Olympus system by successfully visualizing the biliary tree in five patients<sup>[13]</sup>. The steerable properties of the cholangioscope combined with the presence of the accessory channel allowed it to have significant advantages over past attempts at POCS.

The search for a less cumbersome technique to directly visualize the biliary tree led to a small pilot study with an attempt to perform direct visualization of the biliary tree with an ultra-slim upper endoscope<sup>[14]</sup>. This technique used endoscopic retrograde cholangiography (ERCP) to place a super-stiff 0.035-inch diameter Jagwire (Boston Scientific Corp, Natick, Mass) in the CBD. Using the wire to maintain access, the duodenoscope was removed and an ultra-slim upper endoscope (GIF-XP 160, Olympus America Inc, Center Valley, PA) with an outer diameter of 5.9 mm was back loaded over the guidewire under fluoroscopic and endoscopic control into the duodenum and then across the ampulla of Vater into the CBD. Endoscopic sphincterotomy was required in order to permit passage of the endoscope into the CBD. This procedure was successful in providing direct cholangioscopy in 3 of 3 patients. Further studies will show whether this technique may have broader application. However, this technique can be performed by only one endoscopist, and the larger working channel (2.0 mm) of the endoscope allows for larger biopsies and the potential for more therapeutic applications.

Several miniscopes have been developed which allow the ability to examine the biliary and pancreatic ducts. The extreme small size of some of these scopes,

ranging as small as 1 to 15 French in diameter, allowed for their delivery into even the smallest of ducts, and could allow access without the presence of papillotomy when the outer diameter of the scope is less than 2.5 mm<sup>[15-19]</sup>. While these very small scopes raise interesting possibilities, their use is limited by their fragility, lack of tip deflection and lack of an inner working channel. A fine-caliber flexible miniscope created by Soda<sup>[20]</sup>, allowed access to the bile duct without necessitating sphincterotomy due to its external diameter of only 2.09 mm. However, unlike many other fine-caliber miniscopes, this scope did have a central working channel of 0.72 mm.

Slightly larger miniscopes with bi-directional angulation systems and instrument channels were developed by Olympus (CHF BP 30 with 3.4 mm diameter) and Pentax (FCP-9P with 3.1 mm diameter and FCP-8P with 2.8 mm diameter)<sup>[21]</sup>. Sander and Poehl developed a new miniscope (2.3 mm in diameter) for POCS (PolyDiagnost, Reichertshausen, Germany), with a less fragile, steerable tip, which had two different degrees of stiffness. This scope has a working channel measuring 1.2 mm (3.6 Fr), through which a probe for electrohydraulic lithotripsy (EHL) and a stone extraction basket can be passed<sup>[22]</sup>. These two authors demonstrated successful pancreatoscopies with their scope in 8 of 10 cases and successful choledochoscopies in 11 of 11 cases. The presence of the instrument channel in all three of these scopes allows for therapeutic applications. Also, common to these miniscopes is their ability to be introduced through a standard therapeutic duodenoscope, hence these scopes could become part of a DACP (DACP) system. However, none of these scopes had separate air/water channels, and it is frequently necessary to continuously irrigate the bile ducts due to stone debris or sludge obscuring the view. Thus, at times, nasobiliary drainage tubes have been inserted in the bile duct along with the cholangioscope in order to allow irrigation to be effectively performed during the cholangioscopy examination.

While some of the fine-caliber miniscopes have been used to perform pancreatoscopy, one group of Japanese researchers has focused on developing a miniscope specifically designed to perform pancreatic duct visualization. Kodama and others developed a prototype peroral electronic pancreatoscope (external diameter 2.1 mm) and found its images did provide fine detail of the pancreatic duct<sup>[23]</sup>. They utilized an ultraminiature charge-coupled device with sequential color wheel method to generate images. This initial prototype scope was limited by its lack of a working channel. The group continued their development and in 2004 published their experience with another peroral electronic pancreatoscope prototype with a 2.6 mm external diameter and an inner working channel of 0.5 mm<sup>[24]</sup>. This scope was successfully inserted into the pancreatic duct without sphincterotomy in 7 of 9 cases. A duodenoscope was required to insert the scope into the pancreatic duct, and two endoscopists were required to perform the case. However, images were obtained that



provided excellent visualization of the pancreatic duct and sampling of pancreatic fluid could be performed *via* the working channel.

Recently, the SpyGlass peroral cholangio-pancreatography system (Boston Scientific Corp, Natick, Mass) has been introduced<sup>[25]</sup>. This system makes use of a reusable optical probe, a disposable access and delivery catheter (SpyScope), and disposable biopsy forceps. The outer diameter of the SpyScope is 10 French. This system offers several advantages over previous cholangioscopes. It allows for single-operator control of both the duodenoscope and the SpyScope because the SpyScope catheter is mounted on the duodenoscope by a silastic belt. The endoscopist can sequentially manipulate the controls of both the duodenoscope and the SpyScope with one hand; thus, the need for two endoscopists is eliminated. This system also uses 4-way tip deflection, which allows for improved access of tertiary ducts. Further, the irrigation channel (0.6 mm) is separate from the working channel (1.2 mm), which allows for sustained continuous irrigation regardless of whether the working channel is in use. These advances have allowed this system to be used clinically in a number of tertiary referral centers.

Clinical data regarding the SpyGlass system continues to be collected; however, an initial feasibility study is available<sup>[26]</sup>. In this study, 35 patients underwent cholangioscopy with the SpyGlass system. Procedural success defined as attaining the diagnostic or therapeutic goal of the procedure. Procedural success was documented in 91% (32 of 35 patients). Sphincterotomy was frequently required in patients, in that 8 of 10 patients with intact sphincters required sphincterotomy at the time of the SpyGlass procedure. SpyGlass directed biopsy yielded promising results in that 19 of 20 (95%) of optically guided biopsies yielded specimens with adequate tissue for histologic evaluation. EHL was successful in 5 of 5 (100%) of patients when performed *via* the SpyGlass working channel. Two patients (6%) experienced procedure-related complications, namely ascending cholangitis in one patient and cholangitis with intrahepatic abscess in the other patient. Both patients recovered without sequelae. While this initial data is promising, the prospective data currently being collected from clinical use of the SpyGlass system will provide a better analysis of its potential impact on cholangiopancreatography.

## DIAGNOSTIC APPLICATIONS

Intraductal endoscopy may be used for multiple diagnostic indications (Table 1). Direct visualization of the ducts may increase the ability to differentiate and diagnose lesions accurately in comparison with standard imaging and ERCP techniques. In 1999, Siddique reported an experience of 61 choledochoscopies performed *via* the transpapillary route for diagnostic purposes<sup>[27]</sup>. Importantly, this study showed that direct visualization provided additional unsuspected diagnostic information in 18 of the 61 (29.5%) patients,

**Table 1** Diagnostic uses of intraductal endoscopy

Optically guided biopsies of stricture
Indeterminate stricture
Dominant stricture in primary sclerosing cholangitis
Evaluate fixed filling defect noted on cholangiogram or other imaging
Differentiate benign <i>versus</i> malignant intraductal mass
Optical examination yields visual clues
Improved yield from tissue sampling under visual guidance
Precisely map intraductal tumor prior to resection
Collect significant fluid sample for cytology
Visually evaluate intraductal mucinous neoplasms
Visually evaluate choledochal cyst
Visually evaluate for post-liver transplant ductal ischemia
Visually evaluate for intraductal spread of ampullary adenoma
Evaluate with visual exam and tissue sampling for infection
Cytomegalovirus
Fungal infection

beyond that which had been achieved by previous workup. A Korean study reviewed cholangioscopic findings from 111 patients with benign or malignant bile duct tumors<sup>[28]</sup>. By evaluating mucosal changes, presence of neovascularization, and patterns of luminal narrowing, it was determined that bile duct tumors did indeed demonstrate unique optical characteristics, that could allow optical differentiation among adenocarcinoma, adenoma, hepatocellular carcinoma, mucin-hypersecreting cholangiocarcinoma, biliary cystadenocarcinoma, and squamous cell carcinoma. Thus, it was felt that cholangioscopy can provide additional information that would be useful in differentiating benign from malignant lesions and would help characterize the type of malignant lesion. Another Korean study of 63 patients<sup>[29]</sup> with indeterminate strictures reported that cholangioscopy could potentially improve the diagnosis of cholangiocarcinoma by allowing for the optical recognition of an irregularly dilated and tortuous vessel, the so-called “tumor vessel.” They found that this “tumor vessel” was noted in 25 of 41 patients with malignancy (61%), while no patients with benign stricture had this characteristic appearance. The value of direct cholangioscopy could be seen best in this study by combining the optical observation of tumor vessel with percutaneous transhepatic cholangiography-guided biopsy resulting in a diagnosis of malignancy in 39 of 41 patients (96%). This is a significantly increased rate of preoperative diagnosis when compared with percutaneous transhepatic cholangiography-guided biopsy alone (80.4% sensitivity for diagnosis in this study). In 2005, data from 97 patients showed the additive value of combining direct POCS with standard ERCP<sup>[30]</sup>. The combination of POCS and ERCP improved the sensitivity of diagnosing malignant lesions from 58% to 93%. Additionally, POCS was especially useful in evaluating 21 filling defects of uncertain etiology which had been noted on ERCP cholangiogram. POCS was able to correctly diagnose all 8 malignant lesions and all 13 benign lesions (i.e. accuracy of diagnosis was 100%). In particular, 4 fixed and immobile bile duct stones had the appearance of

tumor on ERCP, but were diagnosed correctly as benign stones at a glance with POCS.

Biliary strictures, with the exception of those clearly following surgery or trauma, are frequently concerning for malignancy. Obtaining adequate tissue from these biliary strictures, which can provide definitive diagnosis, is often challenging. Traditionally, ERCP may be of assistance in characterizing the stricture by providing tissue sampling; however, the low yield rates of ERCP-based methods for securing the pathologic diagnosis of malignancy has been demonstrated in multiple studies. The diagnostic yield is variable in the range of 35% to 70%<sup>[31-43]</sup>. Percutaneous transhepatic cholangioscopy (PTCS) and POCS have both been used to obtain visually guided biopsies. However, a risk of percutaneous cholangioscopy is the potential for tumor seeding along the tract. In 1997, Sato published results obtained from 25 bile duct carcinomas showing carcinomas and invasive carcinomas were diagnosed histologically from biopsy specimens obtained with PTCS guidance in 96% and 91% of the cases, respectively<sup>[44]</sup>. However, the sensitivity of a single biopsy for diagnosis for invasive carcinoma was only 62%, which demonstrated the need for multiple biopsies in order to obtain a higher diagnostic yield. In 2003, Somogyi reported the feasibility of using POCS with visually-guided biopsy to successfully directly biopsy an intraductal papillary mucinous tumor within the common hepatic duct<sup>[45]</sup>. Cholangioscopy additionally allowed precise mapping of the tumor in preparation for surgical resection. A 2006 report further details the usefulness of cholangioscopy in patients with indeterminate pancreaticobiliary pathology by evaluating 62 patients<sup>[46]</sup>. If a lesion was initially observed with direct POCS, biopsies were obtained under direct visualization (cholangioscopy-directed) or through the duodenoscope (cholangioscopy-assisted). Overall in this study, sensitivity to detect malignancy by utilizing POCS was 89%, and specificity was 96%, which continues to mark a significant improvement over utilization of only ERCP techniques to obtain tissue. As mentioned previously, the SpyGlass system has also been used for optically guided biopsy<sup>[26]</sup>. The sensitivity and specificity for diagnosis utilizing SpyGlass-directed biopsy was 71% and 100%, respectively, in evaluation of 20 patients' intraductal lesions. Current multi-center trials will shed more light on the use of this new system.

Attempts have been made to utilize POCS in patients with primary sclerosing cholangitis (PSC). A study from the University of Colorado examined 41 PSC patients with POCS<sup>[47]</sup>. In order to evaluate dominant strictures, POCS-directed biopsies were obtained. In cases where the cholangioscopic biopsy forceps could not pass through the operating channel due to angulation, POCS-assisted biopsies were obtained. Impressively, tissue samples were adequate for histologic evaluation in 32 of 33 patients. The median follow-up period of 17 mo, has shown that this method of evaluation was able to successfully exclude cancer in 31 of 31 patients (100%) where biopsies were negative. The predominant difficulty in this study came due to limitations of technology with

the cholangioscopes which were used (Olympus CHF BP30, Olympus CHF B160, Pentax FCP 9P), in that the stricture of interest could not be traversed in 14 cases. Another study detailing the use of POCS in PSC was published by a German group in 2006<sup>[48]</sup>. In this study, 53 PSC patients with dominant bile duct stenoses underwent transpapillary cholangioscopy and POCS-assisted tissue sampling in addition to ERCP. This study found that utilization of cholangioscopy was statistically significantly superior to ERCP for detecting malignancy in terms of its specificity (93% *vs* 51%) and accuracy (93% *vs* 55%). Thus, this group concluded that transpapillary cholangioscopy significantly increases the ability to distinguish between malignant and benign dominant bile duct stenoses in patients with PSC.

Direct pancreatoscopy can also play a diagnostic role in differentiating pancreatic duct lesions<sup>[49]</sup>. Pancreatoscopy can visualize chronic scarring and stenosis of the duct, pancreatic duct stones, and intraductal papillary-mucinous neoplasms (IPMN's) of the pancreas. In 1997, peroral pancreatoscopy was utilized to evaluate carcinoma *in situ* of the pancreas<sup>[50]</sup>. The carcinoma *in situ* in the main duct had the optical appearance of papillary mucosa, irregular mucosa, or nodular mucosa. Pancreatic juice collected during pancreatoscopy provided a better yield than traditional catheter collection, in that fluid collected during pancreatoscopy from all 11 patients with carcinoma *in situ* yielded positive cytology, while only 7 of 11 patients' cytology was positive when collected without direct pancreatoscopy. Thus, this study concluded that peroral pancreatoscopy and pancreatoscopic cytology are indeed useful for locating and diagnosing carcinoma *in situ* of the pancreas. In 1998, further evidence of the additive value of pancreatoscopy to supplement traditional diagnostic techniques was published<sup>[51]</sup>. In this report, pancreatoscopy was performed in 24 patients with intraductal mucinous neoplasms of the pancreas. Pancreatoscopy was able to detect 10 cases of intraductal mucinous neoplasms (IPMN's) that were not diagnosed with endoscopic ultrasound (EUS) or ERCP. Multiple other studies have evaluated the benefits of pancreatoscopy, especially in regard to evaluating intraductal mucinous neoplasms<sup>[52-58]</sup>. However, more recently, peroral pancreatoscopy has been combined with narrow-band imaging to emphasize certain image features often seen with IPMN's, such as mucosal structures and capillary vessels<sup>[59]</sup>. It is thought that the addition of narrow band imaging may aid in the diagnosis of the primary tumor and help in the determination of the extent of the tumor.

Other diagnostic uses of intraductal endoscopy include the evaluation of choledochal cysts<sup>[60-62]</sup>. Hemobilia of unknown etiology has been evaluated by cholangioscopy<sup>[63]</sup>. Infectious etiologies of bile duct pathology, such as cytomegalovirus (CMV) and fungal infections, have also been exposed by the use of direct cholangioscopy<sup>[27,64]</sup>. There also may be a role for evaluation of the biliary tree after liver transplant. A case report exists detailing the use of methylene blue-

**Table 2** Current therapeutic applications of intraductal endoscopy

Stone extraction
Electrohydraulic lithotripsy (EHL)
Laser lithotripsy
Argon plasma coagulation (APC)
Photodynamic therapy
Nd-YAG laser ablation
Cystic duct stent placement

aided chromoendoscopy *via* POCS to optically diagnose extensive bile duct necroses and inflammation consistent with ischemic-type biliary lesions after transplant<sup>[65]</sup>. Other diagnostic uses of POCS will become evident as better technology allows for greater use of this modality.

## THERAPEUTIC APPLICATIONS

Intraductal endoscopy is useful not only for diagnostic purposes, but it also has therapeutic applications (Table 2). Intraductal endoscopy has been frequently used to remove stones from within the ducts that cannot be removed by standard ERCP techniques in 5% to 10% of cases, due to size, location, or adherence to biliary epithelium<sup>[66]</sup>. EHL has been used in combination with POCS in multiple reports. EHL employs the use of a bipolar electrode in an aqueous medium. The probe is placed at the surface of the stone and directly observed using the cholangioscope. The probe emits spark discharges, which create a shock wave that fragments the stone<sup>[67]</sup>. Binmoeller reported, in 1993, that this technique was successful in removing stones where standard mechanical lithotripsy had failed in 64 of 65 patients<sup>[68]</sup>. Arya reported, in 2004, on experience with 94 patients who received POCS combined with EHL<sup>[69]</sup>. Of this group, 93 patients had failed previous standard stone extraction with ERCP. In this retrospective review, POCS combined with EHL was successful in performing stone fragmentation in 96% of cases, and stones were completely removed in 90% of cases. In both of these studies, there were no significant complications associated with the procedures. In elderly patients where biliary stone removal with traditional methods is unsuccessful, permanent biliary stenting has been attempted. However, Hui demonstrated in a prospective study of 36 high-risk patients with difficult CBD stones that POCS guided lithotripsy, when compared to stenting alone, allows for significantly less mortality and cholangitis<sup>[70]</sup>. Another study using EHL with POCS reported a 100% success rate for large bile duct stone removal after failure to remove the stone with a mechanical lithotripter during ERCP<sup>[71]</sup>. In 2002, data from 36 patients who had strictly intrahepatic stones underwent POCS guided lithotripsy<sup>[72]</sup>. Indeed, this form of therapy was successful in these difficult cases to achieve complete stone removal in 64% of cases. Most recently, the SpyGlass-directed EHL system allowed for success in 5 of 5 patients, although after the initial procedure two patients did require repeat SpyGlass-directed EHL and one patient required repeat ERCP in

order to achieve complete stone clearance<sup>[26]</sup>.

Standard surgical management has been difficult for patients with gallstones which erode into the common hepatic duct and form a cholecystobiliary fistula (i.e. Mirizzi types 2-4). In 25 patients (23 patients with Mirizzi type 1 syndrome and two with Mirizzi type 2 syndrome), POCS combined with EHL allowed for successful treatment of the stone in all patients with type 2 Mirizzi syndrome, while it failed in both patients with type 1 Mirizzi syndrome<sup>[73]</sup>. Thus, it was felt that POCS guided therapy may offer a safe and effective alternative to surgery in patients with type 2 Mirizzi syndrome.

There are other therapeutic interventions which have been coupled with POCS. Multiple reports describe the use of cholangioscopy along with laser lithotripsy<sup>[12,74,75]</sup>. Laser lithotripsy may be used under fluoroscopic or direct cholangioscopy guidance. Current evidence indicates that POCS-guided laser lithotripsy is especially preferred in cases of intrahepatic stones or in patients with stones situated proximal to a bile duct stenosis<sup>[76]</sup>. Photodynamic therapy, under peroral cholangioscopic guidance, has also been utilized for patients with biliary tumors. In 1998, Ortner reported on the use of photodynamic therapy under cholangioscopic guidance to treat nonresectable Bismuth type III and IV cholangiocarcinoma<sup>[77]</sup>. In this study, therapy was successful at restoring biliary drainage, improving mortality and enhancing quality of life. In 2003, Ortner reported results of a randomized trial of cholangioscopically guided photodynamic therapy with stenting *versus* stenting only for nonresectable cholangiocarcinoma<sup>[78]</sup>. The improvement of survival in the group receiving photodynamic therapy was so impressive that it was considered unethical to continue with randomization after the first 39 patients. Specifically, the photodynamic therapy group had median survival to 493 d, while the stenting only group had median survival to 98 d ( $P < 0.0001$ ). Treatment with photodynamic therapy and stenting also led to improvement of cholestasis and quality of life compared with endoscopic stenting alone. Argon plasma coagulation (APC) has also been utilized under direct optical guidance to treat an intraductal papillary mucinous neoplasm involving the extrahepatic bile ducts<sup>[79]</sup>. However, in this case, after cholangioscopic evaluation, a thin gastroscope (Olympus GIF-H180, Olympus America Inc, Center Valley, PA) was introduced across the papilla into the bile duct, since the APC probe would not fit down the working channel of the cholangioscope. Other therapeutic applications reported in concert with cholangioscopy include Nd-YAG laser ablation of tumor stent ingrowth and biliary angiodysplastic lesions<sup>[27]</sup>.

## COMPLICATIONS AND SAFETY

There are no large trials specifically addressing the safety of intraductal endoscopy. Most information regarding safety and complications comes from individual case series, often with small numbers of patients enrolled. However, intraductal endoscopy is generally believed to

be a safe procedure with relatively few complications. Complications typically include minor bleeding at the time of sphincterotomy or lithotripsy<sup>[73]</sup>. There was one report of bile duct perforation following POCS guided EHL in 1993<sup>[68]</sup>. Obviously, the incidence of cholangitis is increased in patients with incomplete biliary drainage, from causes such as a biliary stricture or residual biliary stones; however, cholangitis has not been reported as a direct cause POCS<sup>[73]</sup>. Reports in the literature generally demonstrate a low threshold to give antibiotics in POCS guided procedures, but the use of antibiotics is based on the needs of an individual clinical situation. Pancreatitis has been reported in 2 of 52 (3.8%) of pancreatoscopy cases<sup>[49]</sup>. Complication rates will be better calculated as more intraductal endoscopic procedures are performed and further prospective data is collected.

## COMPARATIVE PROCEDURES

There are two other significant methods which allow optical examination of the ductal systems and deserve brief mention due to their association with POCS. PTCS, also known as percutaneous choledochoscopy, and laparoscopic choledochoscopy have both been used extensively to for diagnostic and therapeutic purposes. While PTCS is more invasive than POCS, there are times when it allows excellent visualization, even in difficult anatomic situations where a POCS technique has failed<sup>[80]</sup>. Many of the same diagnostic and therapeutic techniques utilized with POCS are also used with PTCS, including targeted biopsy and management of stones with lithotripsy. One unique use of PTCS was documented, where a push-type sphincterotome was used *via* PTCS to create a papillary sphincterotomy and allow drainage of obstructing biliary stones in 3 patients who each had an endoscopically inaccessible papilla<sup>[81]</sup>. There are no reports of percutaneous pancreatoscopy. There have been no significant randomized studies directly comparing PTCS *versus* POCS. Generally, POCS is preferred as the initial therapy, due to its less invasive nature. However, if POCS is not available, or if POCS techniques fail, then PTCS may be used.

Laparoscopic choledochoscopy has been utilized to explore the CBD. Frequently, this technique has been utilized at the time of laparoscopic cholecystectomy, when intraoperative cholangiogram shows concern for retained CBD stones<sup>[82]</sup>. There are multiple surgical techniques which have been used to explore the CBD, but choledochoscopy *via* the cystic duct appears to be the safest and most effective approach, with success rates of 90%<sup>[83]</sup>. A benefit of this procedure is that the papilla may be left intact without sphincterotomy<sup>[84]</sup>. There is minimal experience with using laparoscopic techniques to perform pancreatoscopy; however, reports do exist<sup>[85]</sup>.

## CONCLUSION

Experience with intraductal endoscopy has shown its advantages over conventional ERCP in regards to

the diagnosis and treatment of biliary and pancreatic disease. Direct optical examination may provide significant additional information about ductal lesions. Furthermore, the ability to guide instrumentation in the ducts under direct optical guidance provides significant advantages. As technology advances, the utilization of this endoscopic modality will only increase and new uses for this technology will likely develop.

## REFERENCES

- 1 Roca J, Flichtentrei R, Parodi M. [Progress in the radiologic study of the biliary tract in surgery; cholangioscopy and cholangiography; utilization of apparatus; preliminary note.] *Dia Med* 1951; **23**: 3420
- 2 Allegaert W. [Report concerning cholangioscopy, using closed-circuit television, during surgical operations on the biliary tract.] *Acta Gastroenterol Belg* 1961; **24**: 599-606
- 3 Deister J. [Intraoperative cholangioscopy, an improvement in bile duct diagnosis.] *Langenbecks Arch Klin Chir Ver Dtsch Z Chir* 1963; **303**: 111-122
- 4 Haberland P. [Cholangioscopy] *Helv Chir Acta* 1966; **33**: 78-80
- 5 Urakami Y, Seifert E, Butke H. Peroral direct cholangioscopy (PDCS) using routine straight-view endoscope: first report. *Endoscopy* 1977; **9**: 27-30
- 6 Nakajima M, Akasaka Y, Fukumoto K, Mitsuyoshi Y, Kawai K. Peroral cholangiopancreatocopy (PCPS) under duodenoscopic guidance. *Am J Gastroenterol* 1976; **66**: 241-247
- 7 Rosch W, Koch H, Demling L. Peroral cholangioscopy. *Endoscopy* 1976; **8**: 172-175
- 8 Popiela T, Karcz D, Kulig J. Significance of intraoperative fibercholangioscopy in the diagnosis of biliary tract disorders. *Endoscopy* 1978; **10**: 275-278
- 9 Nakajima M, Fukumoto K, Mitsuyoshi Y, Kato S, Aoike A. [Peroral cholangiopancreatocopy (PCPS): its development and clinical application] *Nippon Shokakibyo Gakkai Zasshi* 1976; **73**: 1381-1388
- 10 Nakajima M, Akasaka Y, Yamaguchi K, Fujimoto S, Kawai K. Direct endoscopic visualization of the bile and pancreatic duct systems by peroral cholangiopancreatocopy (PCPS). *Gastrointest Endosc* 1978; **24**: 141-145
- 11 Urakami Y. Peroral cholangiopancreatocopy (PCPS) and peroral direct cholangioscopy (PDCS). *Endoscopy* 1980; **12**: 30-37
- 12 Bogardus ST, Hanan I, Ruchim M, Goldberg MJ. "Mother-baby" biliary endoscopy: the University of Chicago experience. *Am J Gastroenterol* 1996; **91**: 105-110
- 13 Ponsky JL, Scheeres DE, Simon I. Endoscopic retrograde cholangioscopy. An adjunct to endoscopic exploration of the common bile duct. *Am Surg* 1990; **56**: 235-237
- 14 Larghi A, Waxman I. Endoscopic direct cholangioscopy by using an ultra-slim upper endoscope: a feasibility study. *Gastrointest Endosc* 2006; **63**: 853-857
- 15 Kozarek RA. Direct cholangioscopy and pancreatoscopy at time of endoscopic retrograde cholangiopancreatography. *Am J Gastroenterol* 1988; **83**: 55-57
- 16 Foerster EC, Schneider MU, Stommer P, Runge U, Domschke W. Miniscopes in gastroenterological endoscopy--inspection of the gallbladder and the biliary and pancreatic duct systems in autopsy specimens. *Endoscopy* 1988; **20**: 316-320
- 17 Kozarek RA. Direct pancreatoscopy. *Gastrointest Endosc Clin N Am* 1995; **5**: 259-267
- 18 Bourke MJ, Haber GB. Transpapillary choledochoscopy. *Gastrointest Endosc Clin N Am* 1996; **6**: 235-252
- 19 Neuhaus H, Schumacher B. Miniscopes. *Baillieres Best Pract Res Clin Gastroenterol* 1999; **13**: 33-48
- 20 Soda K, Shitou K, Yoshida Y, Yamanaka T, Kashii A, Miyata M. Peroral cholangioscopy using new fine-caliber flexible scope for detailed examination without papillotomy. *Gastrointest Endosc* 1996; **43**: 233-238



- 21 **Technology Status Evaluation Report: Duodenoscope-Assisted Cholangiopancreatography.** *Gastrointest Endosc* 1999; **50**: 943-945
- 22 **Sander R**, Poesl H. Initial experience with a new babyscope for endoscopic retrograde cholangiopancreatography. *Gastrointest Endosc* 1996; **44**: 191-194
- 23 **Kodama T**, Sato H, Horii Y, Tatsumi Y, Uehira H, Imamura Y, Kato K, Koshitani T, Yamane Y, Kashima K. Pancreatography for the next generation: development of the peroral electronic pancreatoscope system. *Gastrointest Endosc* 1999; **49**: 366-371
- 24 **Kodama T**, Tatsumi Y, Sato H, Imamura Y, Koshitani T, Abe M, Kato K, Uehira H, Horii Y, Yamane Y, Yamagishi H. Initial experience with a new peroral electronic pancreatoscope with an accessory channel. *Gastrointest Endosc* 2004; **59**: 895-900
- 25 **Chen YK**. Preclinical characterization of the Spyglass peroral cholangiopancreatography system for direct access, visualization, and biopsy. *Gastrointest Endosc* 2007; **65**: 303-311
- 26 **Chen YK**, Pleskow DK. SpyGlass single-operator peroral cholangiopancreatography system for the diagnosis and therapy of bile-duct disorders: a clinical feasibility study (with video). *Gastrointest Endosc* 2007; **65**: 832-841
- 27 **Siddique I**, Galati J, Ankoma-Sey V, Wood RP, Ozaki C, Monsour H, Rajman I. The role of choledochoscopy in the diagnosis and management of biliary tract diseases. *Gastrointest Endosc* 1999; **50**: 67-73
- 28 **Seo DW**, Lee SK, Yoo KS, Kang GH, Kim MH, Suh DJ, Min YI. Cholangioscopic findings in bile duct tumors. *Gastrointest Endosc* 2000; **52**: 630-634
- 29 **Kim HJ**, Kim MH, Lee SK, Yoo KS, Seo DW, Min YI. Tumor vessel: a valuable cholangioscopic clue of malignant biliary stricture. *Gastrointest Endosc* 2000; **52**: 635-638
- 30 **Fukuda Y**, Tsuyuguchi T, Sakai Y, Tsuchiya S, Saisyo H. Diagnostic utility of peroral cholangioscopy for various bile-duct lesions. *Gastrointest Endosc* 2005; **62**: 374-382
- 31 **Desa LA**, Akosa AB, Lazzara S, Domizio P, Krausz T, Benjamin IS. Cytodiagnosis in the management of extrahepatic biliary stricture. *Gut* 1991; **32**: 1188-1191
- 32 **Foutch PG**, Kerr DM, Harlan JR, Kummert TD. A prospective, controlled analysis of endoscopic cytotechniques for diagnosis of malignant biliary strictures. *Am J Gastroenterol* 1991; **86**: 577-580
- 33 **Glasbrenner B**, Ardan M, Boeck W, Prelik G, Moller P, Adler G. Prospective evaluation of brush cytology of biliary strictures during endoscopic retrograde cholangiopancreatography. *Endoscopy* 1999; **31**: 712-717
- 34 **Howell DA**, Parsons WG, Jones MA, Bosco JJ, Hanson BL. Complete tissue sampling of biliary strictures at ERCP using a new device. *Gastrointest Endosc* 1996; **43**: 498-502
- 35 **Jailwala J**, Fogel EL, Sherman S, Gottlieb K, Flueckiger J, Bucksot LG, Lehman GA. Triple-tissue sampling at ERCP in malignant biliary obstruction. *Gastrointest Endosc* 2000; **51**: 383-390
- 36 **Kurzwinski TR**, Deery A, Dooley JS, Dick R, Hobbs KE, Davidson BR. A prospective study of biliary cytology in 100 patients with bile duct strictures. *Hepatology* 1993; **18**: 1399-1403
- 37 **Layfield LJ**, Wax TD, Lee JG, Cotton PB. Accuracy and morphologic aspects of pancreatic and biliary duct brushings. *Acta Cytol* 1995; **39**: 11-18
- 38 **Lee JG**, Leung JW, Baillie J, Layfield LJ, Cotton PB. Benign, dysplastic, or malignant--making sense of endoscopic bile duct brush cytology: results in 149 consecutive patients. *Am J Gastroenterol* 1995; **90**: 722-726
- 39 **Ponchon T**, Gagnon P, Berger F, Labadie M, Liaras A, Chavaillon A, Bory R. Value of endobiliary brush cytology and biopsies for the diagnosis of malignant bile duct stenosis: results of a prospective study. *Gastrointest Endosc* 1995; **42**: 565-572
- 40 **Pugliese V**, Conio M, Nicolo G, Saccomanno S, Gatteschi B. Endoscopic retrograde forceps biopsy and brush cytology of biliary strictures: a prospective study. *Gastrointest Endosc* 1995; **42**: 520-526
- 41 **Sugiyama M**, Atomi Y, Wada N, Kuroda A, Muto T. Endoscopic transpapillary bile duct biopsy without sphincterotomy for diagnosing biliary strictures: a prospective comparative study with bile and brush cytology. *Am J Gastroenterol* 1996; **91**: 465-467
- 42 **Schoefl R**, Haefner M, Wrba F, Pfeffel F, Stain C, Poetzi R, Gangl A. Forceps biopsy and brush cytology during endoscopic retrograde cholangiopancreatography for the diagnosis of biliary stenoses. *Scand J Gastroenterol* 1997; **32**: 363-368
- 43 **Stewart CJ**, Mills PR, Carter R, O'Donohue J, Fullarton G, Imrie CW, Murray WR. Brush cytology in the assessment of pancreatico-biliary strictures: a review of 406 cases. *J Clin Pathol* 2001; **54**: 449-455
- 44 **Sato M**, Inoue H, Ogawa S, Ohashi S, Maetani I, Igarashi Y, Sakai Y. Limitations of percutaneous transhepatic cholangioscopy for the diagnosis of the intramural extension of bile duct carcinoma. *Endoscopy* 1998; **30**: 281-288
- 45 **Somogyi L**, Dimashkieh H, Weber FL Jr, Buell J. Biliary intraductal papillary mucinous tumor: diagnosis and localization by endoscopic retrograde cholangioscopy. *Gastrointest Endosc* 2003; **57**: 620-622
- 46 **Shah RJ**, Langer DA, Antillon MR, Chen YK. Cholangioscopy and cholangioscopic forceps biopsy in patients with indeterminate pancreaticobiliary pathology. *Clin Gastroenterol Hepatol* 2006; **4**: 219-225
- 47 **Awadallah NS**, Chen YK, Piraka C, Antillon MR, Shah RJ. Is there a role for cholangioscopy in patients with primary sclerosing cholangitis? *Am J Gastroenterol* 2006; **101**: 284-291
- 48 **Tischendorf JJ**, Kruger M, Trautwein C, Duckstein N, Schneider A, Manns MP, Meier PN. Cholangioscopic characterization of dominant bile duct stenoses in patients with primary sclerosing cholangitis. *Endoscopy* 2006; **38**: 665-669
- 49 **Tajiri H**, Kobayashi M, Ohtsu A, Ryu M, Yoshida S. Peroral pancreatoscopy for the diagnosis of pancreatic diseases. *Pancreas* 1998; **16**: 408-412
- 50 **Uehara H**, Nakaizumi A, Tatsuta M, Iishi H, Kitamura T, Ohigashi H, Ishikawa O, Takenaka A. Diagnosis of carcinoma in situ of the pancreas by peroral pancreatoscopy and pancreatoscopic cytology. *Cancer* 1997; **79**: 454-461
- 51 **Kaneko T**, Nakao A, Nomoto S, Furukawa T, Hirooka Y, Nakashima N, Nagasaka T. Intraoperative pancreatoscopy with the ultrathin pancreatoscope for mucin-producing tumors of the pancreas. *Arch Surg* 1998; **133**: 263-267
- 52 **Kozarek RA**. Direct cholangioscopy and pancreatoscopy at time of endoscopic retrograde cholangiopancreatography. *Am J Gastroenterol* 1988; **83**: 55-57
- 53 **Fujita N**, Mochizuki F, Lee S, Kobayashi G, Kimura K, Watanabe H. Pancreatocopy for mucus producing pancreatic tumor. *Dig Endosc* 1990; **2**: 110-115
- 54 **Hara T**, Yamaguchi T, Ishihara T, Tsuyuguchi T, Kondo F, Kato K, Asano T, Saisho H. Diagnosis and patient management of intraductal papillary-mucinous tumor of the pancreas by using peroral pancreatoscopy and intraductal ultrasonography. *Gastroenterology* 2002; **122**: 34-43
- 55 **Yamao K**, Ohashi K, Nakamura T, Suzuki T, Sawaki A, Hara K, Fukutomi A, Baba T, Okubo K, Tanaka K, Moriyama I, Fukuda K, Matsumoto K, Shimizu Y. Efficacy of peroral pancreatoscopy in the diagnosis of pancreatic diseases. *Gastrointest Endosc* 2003; **57**: 205-209
- 56 **Kodama T**, Koshitani T, Sato H, Imamura Y, Kato K, Abe M, Wakabayashi N, Tatsumi Y, Horii Y, Yamane Y, Yamagishi H. Electronic pancreatoscopy for the diagnosis of pancreatic diseases. *Am J Gastroenterol* 2002; **97**: 617-622
- 57 **Mukai H**, Yasuda K, Nakajima M. Differential diagnosis of mucin-producing tumors of the pancreas by intraductal ultrasonography and peroral pancreatoscopy. *Endoscopy* 1998; **30** Suppl 1: A99-A102
- 58 **Yasuda K**, Sakata M, Ueda M, Uno K, Nakajima M. The use of pancreatoscopy in the diagnosis of intraductal papillary mucinous tumor lesions of the pancreas. *Clin Gastroenterol*

- Hepatol 2005; **3**: S53-S57
- 59 **Itoi T**, Sofuni A, Itokawa F, Kurihara T, Tsuchiya T, Ishii K, Tsuji S, Ikeuchi N, Arisaka Y, Moriyasu F. Initial experience of peroral pancreatoscopy combined with narrow-band imaging in the diagnosis of intraductal papillary mucinous neoplasms of the pancreas (with videos). *Gastrointest Endosc* 2007; **66**: 793-797
  - 60 **Kolodziejewski TR**, Safadi BY, Nakanuma Y, Milkes DE, Soetikno RM. Bile duct cysts in a patient with autosomal dominant polycystic kidney disease. *Gastrointest Endosc* 2004; **59**: 140-142
  - 61 **Scotiniotis IA**, Kochman ML. Intramural cyst of the bile duct demonstrated by cholangioscopy and intraductal US. *Gastrointest Endosc* 2001; **54**: 260-262
  - 62 **Huang SP**, Wang HP, Chen JH, Wu MS, Shun CT, Lin JT. Clinical application of EUS and peroral cholangioscopy in a choledochocoele with choledocholithiasis. *Gastrointest Endosc* 1999; **50**: 568-571
  - 63 **Kubota H**, Kageoka M, Iwasaki H, Sugimoto K, Higuchi R, Honda S, Watanabe F, Koda K, Hanai H, Kaneko E. A patient with undifferentiated carcinoma of gallbladder presenting with hemobilia. *J Gastroenterol* 2000; **35**: 63-68
  - 64 **Prasad GA**, Abraham SC, Baron TH, Topazian MD. Hemobilia caused by cytomegalovirus cholangiopathy. *Am J Gastroenterol* 2005; **100**: 2592-2595
  - 65 **Hoffman A**, Kiesslich R, Moench C, Bittinger F, Otto G, Galle PR, Neurath MF. Methylene blue-aided cholangioscopy unravels the endoscopic features of ischemic-type biliary lesions after liver transplantation. *Gastrointest Endosc* 2007; **66**: 1052-1058
  - 66 **Classen M**, Hagenmuller F, Knyrim K, Frimberger E. Giant bile duct stones--non-surgical treatment. *Endoscopy* 1988; **20**: 21-26
  - 67 **Wamsteker EJ**. Updates in biliary endoscopy. *Curr Opin Gastroenterol* 2006; **22**: 300-304
  - 68 **Binmoeller KE**, Bruckner M, Thonke F, Soehendra N. Treatment of difficult bile duct stones using mechanical, electrohydraulic and extracorporeal shock wave lithotripsy. *Endoscopy* 1993; **25**: 201-206
  - 69 **Arya N**, Nelles SE, Haber GB, Kim YI, Kortan PK. Electrohydraulic lithotripsy in 111 patients: a safe and effective therapy for difficult bile duct stones. *Am J Gastroenterol* 2004; **99**: 2330-2334
  - 70 **Hui CK**, Lai KC, Ng M, Wong WM, Yuen MF, Lam SK, Lai CL, Wong BC. Retained common bile duct stones: a comparison between biliary stenting and complete clearance of stones by electrohydraulic lithotripsy. *Aliment Pharmacol Ther* 2003; **17**: 289-296
  - 71 **Farrell JJ**, Bounds BC, Al-Shalabi S, Jacobson BC, Brugge WR, Schapiro RH, Kelsey PB. Single-operator duodenoscope-assisted cholangioscopy is an effective alternative in the management of choledocholithiasis not removed by conventional methods, including mechanical lithotripsy. *Endoscopy* 2005; **37**: 542-547
  - 72 **Okugawa T**, Tsuyuguchi T, K C S, Ando T, Ishihara T, Yamaguchi T, Yugi H, Saisho H. Peroral cholangioscopic treatment of hepatolithiasis: Long-term results. *Gastrointest Endosc* 2002; **56**: 366-371
  - 73 **Tsuyuguchi T**, Saisho H, Ishihara T, Yamaguchi T, Onuma EK. Long-term follow-up after treatment of Mirizzi syndrome by peroral cholangioscopy. *Gastrointest Endosc* 2000; **52**: 639-644
  - 74 **Jakobs R**, Pereira-Lima JC, Maier M, Kohler B, Benz C, Adamek HE, Riemann JF. [Endoscopic laser lithotripsy for difficult calculi after unsuccessful extracorporeal shock wave lithotripsy] *Arq Gastroenterol* 1996; **33**: 145-150
  - 75 **Adamek HE**, Maier M, Jakobs R, Wessbecher FR, Neuhauser T, Riemann JF. Management of retained bile duct stones: a prospective open trial comparing extracorporeal and intracorporeal lithotripsy. *Gastrointest Endosc* 1996; **44**: 40-47
  - 76 **Jakobs R**, Pereira-Lima JC, Schuch AW, Pereira-Lima LF, Eickhoff A, Riemann JF. Endoscopic laser lithotripsy for complicated bile duct stones: is cholangioscopic guidance necessary? *Arq Gastroenterol* 2007; **44**: 137-140
  - 77 **Ortner MA**, Liebetruht J, Schreiber S, Hanft M, Wruck U, Fusco V, Muller JM, Hortnagl H, Lochs H. Photodynamic therapy of nonresectable cholangiocarcinoma. *Gastroenterology* 1998; **114**: 536-542
  - 78 **Ortner ME**, Caca K, Berr F, Liebetruht J, Mansmann U, Huster D, Voderholzer W, Schachschal G, Mossner J, Lochs H. Successful photodynamic therapy for nonresectable cholangiocarcinoma: a randomized prospective study. *Gastroenterology* 2003; **125**: 1355-1363
  - 79 **Brauer BC**, Fukami N, Chen YK. Direct cholangioscopy with narrow-band imaging, chromoendoscopy, and argon plasma coagulation of intraductal papillary mucinous neoplasm of the bile duct (with videos). *Gastrointest Endosc* 2008; **67**: 574-576
  - 80 **Shim CS**, Neuhaus H, Tamada K. Direct cholangioscopy. *Endoscopy* 2003; **35**: 752-758
  - 81 **Itoi T**, Shinohara Y, Takeda K, Nakamura K, Sofuni A, Itokawa F, Moriyasu F, Tsuchida A. A novel technique for endoscopic sphincterotomy when using a percutaneous transhepatic cholangioscope in patients with an endoscopically inaccessible papilla. *Gastrointest Endosc* 2004; **59**: 708-711
  - 82 **Shuchleib S**, Chousleb A, Mondragon A, Torices E, Licon A, Cervantes J. Laparoscopic common bile duct exploration. *World J Surg* 1999; **23**: 698-701; discussion 702
  - 83 **Lyass S**, Phillips EH. Laparoscopic transcystic duct common bile duct exploration. *Surg Endosc* 2006; **20** Suppl 2: S441-S445
  - 84 **Dion YM**, Ratelle R, Morin J, Gravel D. Common bile duct exploration: the place of laparoscopic choledochotomy. *Surg Laparosc Endosc* 1994; **4**: 419-424
  - 85 **Balalykin AS**, Avaliani MV. [Laparoscopic pancreatoscopy] *Vestn Khir Im I I Grek* 1985; **135**: 132-136

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Peter V Draganov, Dr, Series Editor

## Pain management in chronic pancreatitis

Cathia Gachago, Peter V Draganov

Cathia Gachago, Peter V Draganov, University of Florida, Department of Gastroenterology, Hepatology and Nutrition, 1600 SW Archer Rd, Room 602, Gainesville, Florida 32610, United States

**Author contributions:** Gachago C and Draganov PV contributed equally to this work.

**Correspondence to:** Dr. Peter V Draganov, University of Florida, Department of Gastroenterology, Hepatology and Nutrition, 1600 SW Archer Rd, Room 602, Gainesville, Florida 32610, United States. [dragapv@medicine.ufl.edu](mailto:dragapv@medicine.ufl.edu)

Telephone: +1-352-3922878 Fax: +1-352-3923618

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### INTRODUCTION

Chronic pancreatitis remains an enigma in the field of gastroenterology. Challenges can be encountered in defining the etiology and pathogenesis, in securing the diagnosis, and finally in providing adequate therapy. Chronic pancreatitis is a common problem, but the exact prevalence is unclear. Many patients suffering from chronic abdominal pain may indeed have unrecognized chronic pancreatitis. The prevalence in the developed world is reported from 0.4% to 5%<sup>[1]</sup>.

In the western world alcohol abuse is the overwhelming etiologic factor. Of patients with chronic pancreatitis, 60% to 70% have 6 to 12 years history of heavy consumption of alcohol (150-175 g/d)<sup>[2]</sup>. Less common, but important etiologic factors to consider, are ductal obstruction (from tumors and strictures), autoimmune, hypercalcemia, hyperlipidemia, toxins, and genetic. In a small number of cases, there is no identifiable causative factor and the pancreatitis is deemed idiopathic. It should not be surprising in view of this array of etiologic factors that there exist uncertainties in both diagnosis and ultimately treatment of chronic pancreatitis. Adding to the perplexity of this clinical situation are the multiple treatment options that can be provided by primary care physicians, gastroenterologists, interventional endoscopists, and surgeons. Despite the evolution of new medications and tools in the last two decades no clear consensus has emerged on the management of chronic pancreatitis. Most reports are either anecdotal or collected experiences of a single approach.

It is the purpose of this review to discuss the different modalities that are currently being used for the treatment of pain in chronic pancreatitis and to attempt to integrate them in a patient centered comprehensive approach.

### Abstract

Abdominal pain is a major clinical problem in patients with chronic pancreatitis. The cause of pain is usually multifactorial with a complex interplay of factors contributing to a varying degree to the pain in an individual patient and, therefore, a rigid standardized approach for pain control tends to lead to suboptimal results. Pain management usually proceeds in a stepwise approach beginning with general lifestyle recommendations. Low fat diet, alcohol and smoking cessation are encouraged. Analgesics alone are needed in almost all patients. Maneuvers aimed at suppression of pancreatic secretion are routinely tried. Patients with ongoing symptoms may be candidates for more invasive options such as endoscopic therapy, and resective or drainage surgery. The role of pain modifying agents (antidepressants, gabapentin, pregabalin), celiac plexus block, antioxidants, octreotide and total pancreatectomy with islet cell auto transplantation remains to be determined.

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**Peer reviewers:** Tatjana Crnogorac-Jurcevic, MD, PhD, Cancer Research UK, Molecular Oncology Unit, Barts and The London School of Medicine and Dentistry, John Vane Science Centre, Charterhouse Square, London EC1M 6BQ, United Kingdom; Giuseppe Brisinda, MD, Department of Surgery, Catholic School of Medicine "Agostino Gemelli", Largo Agostino Gemelli, 8-00168 Rome, Italy

### PATHOPHYSIOLOGY OF PAIN IN CHRONIC PANCREATITIS

At least 85% of patients with chronic pancreatitis

develop pain at some point during the course of their disease. Painless chronic pancreatitis is rare, and more commonly late in the natural history of idiopathic chronic pancreatitis<sup>[3]</sup>. The frequency, severity and other characteristics of pain in chronic pancreatitis have a major impact on its management, the number of treatments, and the choice between medical and surgical interventions.

Several hypotheses exist as to the basis for pain in chronic pancreatitis; however, the exact mechanism is still not completely known. Possible mechanisms for pain include acute inflammation of the pancreas, increased pressure within the ductal system and parenchyma, neuritis, recurrent ischemia of the parenchyma; intra-pancreatic causes such as acute pseudocysts; and extra-pancreatic causes such as common bile duct or duodenal stenosis<sup>[4,5]</sup>. The relative contribution of each factor is unknown.

## THERAPY OF PAIN IN CHRONIC PANCREATITIS

### Medical therapy

**Nonspecific supportive therapy:** The first line in pain management is the use of medical therapy. The initial step of medical therapy usually is nonspecific supportive treatment. Supportive therapy is aimed at treating the concurrent symptoms and not the underlying factors in pain causation. Analgesic drugs are still the most commonly adopted method for pain relief. The obvious problem with this method of treatment is that patients often become dependant on heavy narcotic use. Most patients with chronic pancreatitis have their pain treated with analgesics on an episodic or continuing basis. Although the use of narcotics for the treatment of chronic pancreatitis is widespread, there are no controlled trials testing their efficacy as compared to the other modalities. Time intervals and doses of drug application must be adapted to the individual pain pattern. Although reluctance to use of narcotics is understandable, it should not be withheld if the treatment would otherwise not lead to adequate pain control<sup>[6]</sup>.

There may be significant psychiatric, psychological, or psychosomatic contributions to the pain syndrome in these patients. Many physicians and surgeons use antidepressant medications as concomitant therapy, acknowledging the difficulty in assessing the psychological contributors to patients' pain syndrome. The benefits are anecdotal and variable in any individual experience and have never been rigorously assessed. It has been suggested that the natural path of chronic pancreatitis is toward progressive glandular insufficiency and calcification, and with the eventual 'burnout' would come spontaneous remission of pain<sup>[7]</sup>. There is a school of thought against conservative therapy. Pain is endured until burnout. This theory sheds light on the uncertainty regarding the duration of clinical pain, and if burnout is indeed a certainty and not solely a proposed hypothesis<sup>[8,9]</sup>. In conclusion, a strategy of waiting

for spontaneous pain relief is not reliable and may be unreasonable advice for the patient with persistent or frequent severe pain.

**Pancreatic enzymes:** The presumed mechanism for pain relief after the administration of oral pancreatic enzymes is thought to involve the negative feedback inhibition to the pancreas. A cholecystokinin (CCK)-releasing peptide in the duodenum is normally denatured by pancreatic trypsin. In chronic pancreatitis, damage to acinar cells results in decreased secretion of pancreatic trypsin and consequently insufficient denaturing of the CCK-releasing peptide. This then leads to the potentiation and increased release of CCK, which causes pancreatic pain related to an increase in pancreatic enzyme output. When pancreatic enzymes are administered orally, there is more complete denaturing of the CCK-releasing peptide, thereby diminishing the release of CCK<sup>[10,11]</sup>. The results of studies examining the use of pancreatic enzymes that are administered orally to treat the pain of chronic pancreatitis have been variable, in part because of a high placebo response rate of over 35%, the potential for exogenously administered digestive enzymes to be inactivated by gastric acid and pancreatic proteases, and the lack of efficacy of enteric coated preparations<sup>[12-16]</sup>.

In one of the earliest double-blind randomized trials of pancreatic enzymes, Isakson *et al* showed the pain relieving effect of oral enzyme preparations in a proportion of patients with chronic pancreatitis<sup>[16]</sup>. They took 19 patients with chronic pancreatitis, and treated them for 1 wk with a granulated pancreatic enzyme preparation (Pankreon<sup>®</sup>; five times daily 7.5 mL) or placebo and vice versa. Pain was evaluated using an analog scale and by questioning. A 30% pain reduction was seen after treatment with pancreatic extract compared to placebo. Fifteen of the nineteen patients had less pain during the week of treatment with pancreatic extracts. These results could not be confirmed by Halgreen, who conducted a 4-wk double-blind cross-over study with pancreatic enzymes (Pancrease<sup>®</sup>) in 20 chronic pancreatitis patients. There was no significant pain reduction<sup>[17]</sup>. In a placebo-controlled, double-blind, crossover study, pancrelipase (Viokase), in a dose of six tablets taken four times per day for one month, significantly reduced pain in 75% of patients with mild-to-moderate disease<sup>[15]</sup>. The best response was in young women with idiopathic chronic pancreatitis, whereas patients with advanced disease, including those with steatorrhea, had no response.

Of the 6 randomized trials published to date two studies using a non-enteric coated enzyme preparation reported benefit and four studies using an enteric-coated capsule showed no effect on pain in chronic pancreatitis. The conflicting study results led to investigators questioning the mechanism of negative feedback inhibition in the proximal small bowel<sup>[18]</sup>. As noted, the presumed mechanism for pain relief with administration of oral pancreatic enzymes is thought to involve feedback inhibition of the exocrine pancreas by the



degradation of CCK-releasing peptide in the duodenum. The administered enzymes would need to release activated serine proteases into the duodenum. This is much more likely with the non-enteric coated than the enteric-coated preparations, and hence the suspicion that the former are more effective. A meta-analysis of the six randomized, double-blind, placebo-controlled trials for the treatment of chronic pancreatitis with pancreatic enzymes showed no benefit in improving pain. The pooled estimate of the percentage of patients per study who preferred enzymes relative to placebo was 52% (95% confidence interval 45%-60%). This was not statistically different from 50%. Thus, this analysis demonstrates no significant benefit of pancreatic enzyme therapy to relieve chronic pancreatitis-associated pain<sup>[19]</sup>. It should be noted that this meta-analysis combines studies using enteric-coated and studies using non enteric-coated preparations. In that way, the potential benefit of non enteric-coated enzymes may have been negated by the lack of positive effect with non-enteric-coated preparation. The role of oral pancreatic enzymes in reducing pain in chronic pancreatitis, therefore, remains unclear. Additional studies are required to establish the effectiveness of this modality of treatment and to define whether certain subsets of pain: chronic *versus* intermittent pain; patients with or without exocrine insufficiency; alcoholic *versus* idiopathic pancreatitis; minimal *versus* extensive pancreatic duct changes; are more likely to benefit from enzyme therapy than others. Non-enteric coated enzymes are certainly safe and reasonable to try before considering more invasive or risky therapies.

**Octreotide:** Cholecystokinin-receptor antagonists or somatostatin analogues, such as octreotide, have been postulated to work on the negative feedback inhibition as well as hypertension of the pancreatic duct due to outflow obstruction. Inhibition of pancreatic secretion using somatostatin might, therefore, be effective in reducing pain in chronic pancreatitis. Octreotide is a synthetic somatostatin-analogue with an increased half-life, higher potency and the possibility of subcutaneous application. Experimental data suggest that octreotide increases the contractibility of the sphincter of Oddi, while somatostatin decreases it. This has, however, not consistently been demonstrated<sup>[20]</sup>. Normally, the release of cholecystokinin from specific intestinal cells is regulated by a cholecystokinin-releasing peptide in the proximal small intestine that is luminally active and trypsin-sensitive<sup>[13]</sup>. In chronic pancreatitis, exocrine insufficiency may lead to increased cholecystokinin-mediated stimulation of the pancreas. Theoretically, this process could be interrupted by the administration of cholecystokinin-receptor antagonists, or somatostatin. In a multicenter pilot study, octreotide, in a dose of 200 µg administered subcutaneously three times per day for 4 wk, reduced pain scores by 25% or more in 65% of patients with severe chronic pancreatitis<sup>[21]</sup>. On the other hand in a randomized, prospective, double-blind, placebo-controlled study conducted in Europe

[100 mg subcutaneously (*sc*) every 8 h] administered to 10 patients for only 3 d was no more effective than placebo in relieving pain in chronic pancreatitis<sup>[22]</sup>. In a second study<sup>[23]</sup>, octreotide (100 mg *sc* every 8 h for 3 wk) administered to six patients in a nonblinded fashion provided relief of pain in some but not all patients. In a third study<sup>[24]</sup>, octreotide was administered to 84 patients for 4 wk in a randomized, prospective, double blind trial and showed a trend toward benefit at the highest dosage used (200 mg *sc* every 8 h). However, this effect did not reach statistical significance in this dose-ranging study. The longevity of the possible benefit was not established. Clearly further studies are needed before the use of octreotide can be widely adopted.

**Antioxidant therapy:** Bhardwaj *et al*<sup>[25]</sup> reported a decreased micronutrient intake (Vitamin E, riboflavin, choline, magnesium, copper, manganese and sulphur) in patients with chronic pancreatitis. This was due to diet modifications due to pain, as well as to a lower caloric intake. This points to the possibility that micronutrients deficiency may contribute to increased oxidative stress. In a comparison between patients with chronic pancreatitis and acute pancreatitis, the antioxidant profiles appeared to be different. Patients with chronic pancreatitis had significantly lower plasma concentrations of selenium, Vitamins A and E, beta-carotene, xanthine, beta-cryptoxanthine and lycopene in comparison with patients with recurrent acute pancreatitis<sup>[26]</sup>. Cullen *et al*<sup>[27]</sup> reported a decrease in antioxidant enzyme expression in pancreatic cells from normal pancreas to chronic pancreatitis to pancreatic cancer. Another observation concerning antioxidants is the altering of antioxidant status in chronic pancreatitis patients, which is worsened in patients with diabetes mellitus<sup>[28]</sup>. A 1-year clinical trial with 10 patients studied the effect of food supplementation using a complex containing l-methionine, beta-carotene, Vitamins C and E and organic selenium<sup>[29]</sup>. This resulted in a significant decrease in the intensity of pain as well as in days of hospital admission. Based on a placebo-controlled trial, followed by a retrospective cross-sectional study in 94 patients, some authors recommend antioxidant therapy consisting of supplements of methionine, Vitamin C and selenium<sup>[30]</sup>.

Based on the observations that activation of oxygen free radicals can cause metabolic changes leading to pancreatic ischemia, antioxidant treatment with allopurinol seems a valid option. A trial with 13 patients with chronic pancreatitis investigated the effect of allopurinol on pain in a cross-over double-blind, randomized treatment trial<sup>[31]</sup>. Allopurinol, which is believed to reduce oxidative stress by inhibiting xanthine oxidase and thereby preventing the formation of oxygen derived free radicals, was given to 13 patients with pain occurring at least three times each week. Allopurinol was not effective in reducing pain or improving activities of daily living compared to placebo. In contrast, others showed that addition of allopurinol or dimethyl sulfoxide to intramuscular pethidine hydrochloride significantly

enhanced the efficacy of the analgesic regime<sup>[32]</sup>. This report suggests that removing oxygen free radicals in chronic pancreatitis may result in a beneficial therapeutic effect. The results of the most recent randomized trial presented only in abstract form showed that the combination of selenium, Vitamin C,  $\beta$ -carotene, Vitamin E, and methionine was significantly better in controlling pain compared to placebo<sup>[33]</sup>. In summary, there are conflicting data about the effectiveness of antioxidant therapy. A few trials show potential benefit, but further research is needed before it can become standard of therapy.

### Endoscopic therapy

Endoscopists have shown that they can overcome pancreatic duct obstruction caused by ampullary stenosis, strictures, or stones. However, there have been no published validated guidelines for defining significant obstruction, and methodology for assessing patients before treatment and then judging the efficacy of that treatment. It should be noted though that the alternative to endoscopy, surgical sphincterotomy and sphincteroplasty, have already proven to be less efficient<sup>[34,35]</sup>. These interventions are hardly ever used now. This may also be due to the more acceptable rate of complication with endoscopic procedures, in conjunction with stent placement and stone extraction. For the present, the decision to perform endoscopy is based partially on subjective judgments that include assessment of the need for long-term narcotic therapy, marked diminution of the quality of life because of intractable pain, or major nutritional consequences of pain. When major pain episodes cannot be controlled by major, but acceptable maintenance analgesics, intervals of narcotics, or reasonable and brief periodic hospitalizations, a trial of interventional therapy can be justified. Among three recent studies involving stent therapy in 98 patients, at times associated with other interventional therapies such as lithotripsy and/or sphincterotomy<sup>[36-38]</sup>, two studies<sup>[36,38]</sup> reported amelioration of pain and one did not<sup>[37]</sup>.

The ideal treatment for patients with pancreatic-duct stones, dilated pancreatic ducts, and pain is not known. The stones can be easily removed coincidentally with the performance of a surgical-drainage procedure, such as pancreaticojejunostomy. Alternatively, however, they can be fragmented by extracorporeal shock-wave lithotripsy (ESWL) and removed endoscopically after sphincterotomy of the pancreatic duct. Stones can be cleared by this approach in roughly 80 percent of patients, and approximately 50% of these have long-term relief of their symptoms<sup>[39,40]</sup>. Dumonceau *et al* conducted a randomized trial comparing pain relief after extracorporeal shock wave lithotripsy alone *versus* in combination with endoscopic drainage of the main pancreatic duct in patients with painful calcified chronic pancreatitis. Two years after trial intervention, 10 (38%) and 13 (45%) patients of the ESWL alone and ESWL combined with endoscopy group, respectively, had presented pain relapse. In both groups, a similar and significant decrease was seen after treatment in

the number of pain episodes/year (mean decrease 3.7 episodes). There was no difference between the treatment groups and the treatment costs per patient were three times higher in the ESWL combined with endoscopy group compared with the ESWL alone group<sup>[41]</sup>. The claims for the efficacy of stone removal for pain relief should be considered in context with the observations that the presence or absence of stones does not necessarily correlate with the existence of pain. In the absence of randomized prospective trials comparing stone ablation either with placebo or with surgical decompression, it is difficult to assess the results of pancreatic stone removal.

An alternative involves the use of endoprotheses or stents placed in the pancreatic duct endoscopically. Reports indicate that 30%-76% of patients receiving such stents had symptomatic improvement over a period of 14 to 36 mo of observation<sup>[42-46]</sup>. Cremer *et al*<sup>[42]</sup>, for example, noted initial improvement of symptoms in 94% of patients who were so treated for pancreatic-duct strictures and upstream ductal dilatation. In that group of patients, 53% remained free of symptoms over a mean follow-up period of 36 mo. Similarly, Grimm *et al*<sup>[43]</sup> showed that 57% of their patients were symptomatically improved by this treatment over a mean follow-up period of 19 mo. Although these results seem encouraging, a criticism is that most of the data reported to date were from relatively short term, nonrandomized studies. The issue is further complicated by the fact that pancreatic-duct stents may not be entirely harmless; for example, they may cause further pancreatic duct changes and potentiation of chronic pancreatitis<sup>[47-49]</sup>. Endoprosthesis occlusion and migration also seem to be relatively common.

Analyzing all the endoscopic modalities taken together it is usual to find a report of 80%-90% complete stone clearance and good immediate pain relief<sup>[47]</sup>. The long term results were not as favorable in the larger series. Delhaye *et al*<sup>[39]</sup> found that of 123 patients, only 60% experienced complete or partial pain relief during 14 mo follow-up. So far there are two randomized control trials comparing endoscopic therapy with surgery<sup>[50,51]</sup>. The study from Dite *et al* randomized 72 patients with large duct chronic pancreatitis to endoscopic therapy *versus* surgical lateral pancreaticojejunostomy. In addition, 68 patients were treated with endoscopy or surgery based on patient preference. The results between the randomized and nonrandomized study groups are similar. After 5 years of follow-up only 14% of the patients treated by endoscopy were pain free compare with 36% in the surgery group. The latest randomized controlled study comparing endoscopy with surgery (lateral pancreaticojejunostomy) enrolled 36 patients. The results are strikingly similar to the previous study. Pain was absent in 16% of patients treated with endoscopy and 40% in patients treated with surgery. Based on these trials it appears that surgery provides better pain relief compared to endoscopy, but even surgery fails to provide substantial pain relief in more than half of the patients<sup>[51]</sup>.

Endoscopic treatment may have a place in the prevention of acute relapsing pancreatitis, more so than treatment of the pain of chronic pancreatitis. To avoid this potential problem, some have suggested that endoscopically placed pancreatic-duct stents should be used only for relatively short periods. This serves as a screening procedure, to identify those patients most likely to benefit from surgical drainage<sup>[42,45,52]</sup>. At present, endoscopically placed stents should be considered an unproved, but potentially useful approach to the treatment of chronic pancreatitis.

Kozarek and Traverso<sup>[53]</sup> have analyzed collected experiences and indicate that the likelihood of symptomatic improvement with combination endotherapy is reported to be 50%-85% at 15 to 25 mo. Successful pain relief has been correlated anecdotally with stone removal and subsequent decrease in diameter of the pancreatic duct. As a rule, the focus is on stones in the main duct and the morbidity of side-branch stones has not been defined. Better selection of patients for endotherapy may be helpful in order to maximize results. Due to its low degree of invasiveness, however, endotherapy can be offered as a first-line treatment, with surgery being performed in case of failure and/or recurrence.

### **Nerve blockade**

Although this modality is thought to be medical management, it may be administered *via* endoscopic or interventional radiological means. Although widely used, there have been relatively few formally reported experiences with nerve blocks for long-term therapy of chronic pancreatitis. Leung *et al*<sup>[54]</sup> studied the use of celiac block in 23 patients with chronic pancreatitis. Twelve of the 23 had complete analgesia, whereas six had partial relief. There was no effect in five patients. The mean pain-free period in the chronic pancreatitis patients was only 2 mo, and the longest 4 mo. Benefit was least in patients with previous pancreatic surgery and repeat blocks were unhelpful.

Because of possible concerns about potential irreversible nerve injury, including very rare anecdotes of paraplegia from neurolytic agents, injection of steroids for the treatment of chronic pancreatitis has been recommended, instead of the use of alcohol injected into the celiac plexus (principally used in the treatment of cancer pain)<sup>[55,56]</sup>. In one study, steroid injection provided relief of pain (lasting two mo) in only 4 of 16 patients<sup>[57]</sup>. Eleven of the 12 patients who did not obtain relief were narcotic dependent, whereas none of the four who obtained relief were narcotic dependent. This finding emphasizes the complexity of treating pain in a population of patients with chemical dependencies and other abnormal psychological and psychosomatic behavior. In another report<sup>[58]</sup>, which investigated the mode of delivering the nerve block, only 2 of 8 patients with a CT-guided celiac plexus block experienced relief of pain compared with 6 of 14 who were treated by endoscopic ultrasonography-guided celiac plexus block with 10 mL of bupivacaine. The benefit from endoscopic ultrasonography-guided celiac plexus block

seemed to persist longer than CT-guided block. More importantly, paraplegia has not been described after endoscopic ultrasonography-guided celiac plexus block, probably because of the anterior transgastric approach taken during endoscopic ultrasonography-decreasing or even eliminating the risk of nerve or spinal cord injury. The same group of investigators more recently published their prospective experience with endoscopic ultrasonography-guided celiac plexus block with steroids in 90 patients with pain resulting from chronic pancreatitis<sup>[59]</sup>. A significant improvement in pain score occurred in 55% of the patients. The benefit persisted beyond 12 wk in 26% of patients and beyond 24 wk in only 10%. Younger patients (< 45 years) and patients with previous pancreatic surgery for chronic pancreatitis did not appear to benefit from the block.

The current evidence indicates that endoscopic ultrasonography-guided celiac plexus block is safe and well tolerated, with excellent temporary results in some patients. Unfortunately, reliable predictors of success are lacking. In the absence of long-term studies with follow-up in patients with chronic pancreatitis whose pain is chronic, the role of endoscopic ultrasonography-guided celiac plexus block should be limited to treating flares of chronic pain in patients with otherwise limited therapeutic options.

### **Surgical treatment**

Duval pioneered efforts to treat the pain of chronic pancreatitis by surgical means in the 1950s with transduodenal sphincteroplasty and with caudal pancreatojejunostomy (the Duval procedure). The results of this procedure were fraught with variable and usually poor results, perhaps only helping some of those patients with true recurrent acute pancreatitis<sup>[60]</sup>. A more extensive drainage procedure, lateral pancreatojejunostomy, described by Puestow and Gillesby<sup>[61]</sup> and subsequently modified by Partington and Rochelle<sup>[62]</sup>, was applied to the subset of patients with dilated main pancreatic duct and became the first surgical treatment widely considered to be effective for pain in this disease. At that time, however, its application was hampered because there was no way to determine preoperatively if a patient with chronic pancreatitis had the dilated ducts required for this procedure because neither ERCP nor CT was available until the 1970s. Thus, at exploration an intraoperative pancreatogram was used to select who would be candidates for lateral pancreatojejunostomy. In those without dilated ducts, the remaining options were to perform a sphincteroplasty (which was largely abandoned because of its failure) or to do nothing further. In the 1960s, surgeons began performing pancreatic resections for chronic pancreatitis, initially distal pancreatectomies (with poor results) and later distal subtotal (95%) resections, which were relatively more effective for pain, but rendered most patients diabetic<sup>[63]</sup>. Proximal resections of the head of the pancreas (i.e. Whipple procedures) were not widely applied until the 1980s, when the associated operative morbidity and mortality fell substantially<sup>[64-66]</sup>.

Patients whose pain persists in spite of aggressive noninvasive treatment should undergo endoscopic retrograde pancreatography to define the caliber and morphologic characteristics of their pancreatic ducts. Depending on the population being studied, up to half of these patients may have dilated ducts, frequently with areas of stricture-the "chain of lakes" or "string of pearls" appearance; the remainder have either ducts of normal caliber (2 to 4 mm in diameter) or small ducts that may lack side branches-the "tree in winter" appearance<sup>[67,68]</sup>. Ducts larger than 8 mm in diameter can be successfully decompressed by an internal surgical-drainage procedure, such as a longitudinal pancreaticojejunostomy (the modified Puestow procedure)<sup>[60,62]</sup>, but smaller ducts are not amenable to internal surgical drainage or resection.

Like most surgical procedures currently in use, those for chronic pancreatitis gradually became part of the armamentarium without undergoing rigorous testing and were never compared against medical treatment or no treatment. The vast majority of patients are still operated on when they continue to have intractable pain despite medical treatment. There are very few controlled trials in the surgical literature on this disease. The two randomized controlled studies comparing surgery with endoscopic therapy are discussed in the endoscopic therapy section. Surgical options include decompression/drainage operations, pancreatic resections, and denervation procedures. As with endoscopic interventional therapy, objective transferable criteria for the need for surgical intervention have not been developed or agreed upon.

**Decompression/drainage operations:** At present, the ultimate role of these various invasive approaches to the treatment of patients with large-duct, symptomatic chronic pancreatitis has not been established. Given the information available at the present time, most physicians recommend longitudinal pancreaticojejunostomy for patients with pain and dilated ducts. This operation may also retard the progression of exocrine and endocrine insufficiency<sup>[69,70]</sup>. Surgical decompression of the obstructed main pancreatic duct was for a long time the gold standard<sup>[71]</sup>. Drainage procedures today are most commonly side to side pancreaticojejunostomy. This particular procedure preserves parenchymal function. Longitudinal pancreaticojejunostomy is also used based on the concept the ductal obstruction leads to distention and that this in turn gives rise to pain and should thus be favored if the duct is widened. Ebbehøj *et al*<sup>[72]</sup> were able to show a relationship between the degree of pain and intrapancreatic pressure. Pancreatic pressure was measured by a percutaneously placed needle preoperatively, postoperatively, and one year after pancreatic duct drainage. Patients whose pressure decreased after surgery and remained low were pain free, whereas those with recurrent pain had increased pressure.

Theoretically, any procedure that improves drainage, either by improving flow into the jejunum or stomach, might be expected to relieve pain. Pancreatic decom-

pression results in immediate and lasting pain relief in a high proportion (80%-90%) of patients with non-alcoholic chronic pancreatitis<sup>[73]</sup>. These procedures have been less successful with alcoholic chronic pancreatitis with pain relief averaged at 60%<sup>[74]</sup>. Although early good results have also been reported after a lateral pancreaticojejunostomy in patients with alcoholic pancreatitis, when these patients are followed for 5 years only 38%-60% of them continue to be pain free<sup>[75]</sup>. These operations are predicated upon the presence of a widely dilated main pancreatic duct (generally taken as > 6 to 7 mm) and the presumption that the dilated ducts imply an abnormally high pressure in the duct system<sup>[75]</sup> and in the pancreatic parenchyma<sup>[72,76]</sup>. The operation most commonly performed is a variant of the Puestow procedure, which is actually the Partington-Rochelle modification (lateral pancreaticojejunostomy)<sup>[62]</sup>.

Many of the studies of lateral pancreatico-duodenectomy find that short-term pain relief is achieved in about 80% of patients and that the operation can be performed with a very low morbidity and mortality (0%-5%). Although the short-term studies shine a positive light on the procedure, long-term follow-up studies show that pain not uncommonly recurs. As time goes by, pain recurs, perhaps related to progression of the pancreatic injury and fibrosis. Pain relief for greater than two years is achieved in only 60% of patients<sup>[77,78]</sup>. Strategies for salvage in patients with persistent or recurrent pain after drainage procedures include redoing or extending the pancreatojejunostomy and resection procedures<sup>[79]</sup>. Of patients undergoing pancreatic duct drainage procedures, 25%-66% require concomitant biliary or gastric drainage, because of functionally significant obstruction of the bile duct or duodenum<sup>[80,81]</sup>. Biliary or duodenal strictures have been reported to be more likely in patients with large-duct disease than in their counterparts without dilated ducts<sup>[81]</sup>.

The only reported attempt made to compare pancreatic duct drainage with no intervention in the management of pain is that of Nealon and Thompson<sup>[70]</sup>. In a series of 143 patients with chronic pancreatitis, 85% of the 87 patients who were treated by pancreatic duct decompression achieved pain relief, whereas pain abated spontaneously in only 1.3% of the 56 nonoperative patients. The study was not randomized, however, the principal criterion to determine candidacy for the operation was the presence of a dilated pancreatic duct. Thus, what the study actually reports is the outcome of pancreatojejunostomy in patients with dilated ducts *versus* the natural history of patients with chronic pancreatitis and no duct dilation. The study also found that deterioration of pancreatic function was slower in their patients with dilated ducts than in those with small ducts. Although this effect was ascribed by the investigators to the protection or relief afforded by the surgical drainage procedure, the cause and effect relationship is uncertain because of the differences in the patient population.

The consensus, albeit based on evidence from collected experiences, states that pancreatic duct decompression *via* lateral pancreatojejunostomy (a Puestow-type operation) can be accomplished with low associated morbidity and mortality and that pain relief will be achieved in the



majority of patients. For most experienced pancreatic surgeons, it is the preferred surgical treatment option in patients whose main pancreatic duct measures 6 mm or more because of its simplicity, safety, and benefits, including the advantage that remaining pancreatic tissue and function are at least not compromised further by loss from resection.

Drainage of pancreatic pseudocysts provides another form of pancreatic decompression in conjunction and even in continuity with a lateral pancreaticojejunostomy when the main duct is also dilated. Up to 39% of patients undergoing lateral pancreaticojejunostomy have evidence of pseudocysts disease at the time of surgery<sup>[82]</sup>. Pseudocysts are found in about 25% of patients with chronic pancreatitis and have a much lower rate of spontaneous resolution than those that are a consequence of an attack of acute pancreatitis<sup>[82-84]</sup>. They can be the source of pain indistinguishable from that of the underlying chronic pancreatitis. In one study, surgical drainage resulted in complete short-term pain relief in 96% of 55 patients, and 53% remained pain free after a median follow-up of 11 years<sup>[84]</sup>. Endoscopic drainage of pseudocysts into the stomach or duodenum may be an alternative, especially in patients who do not have associated duct dilation. Studies directly comparing surgical with endoscopic drainage of pseudocyst are lacking.

It should also be mentioned that there are numerous variations of the previously mentioned operations. Frey *et al*<sup>[85,86]</sup> combined a coring out of the pancreatic head with a lateral pancreaticojejunostomy. In his series, the pain relief after 5 years was complete or improved in 87% of cases. There is also one randomized series of patients comparing the Beger and Frey procedure<sup>[86-90]</sup>, with no difference in decrease of pain, but less morbidity with the Frey procedure.

**Resection procedures:** The therapeutic principle of resection is based on the assumption that pain in chronic pancreatitis is predominantly caused by inflammation. This inflammation then becomes the nidus for qualitative and quantitative changes of nerve fibers. This is especially seen in the clinical scenario of normal sized ducts and masses of the head of the pancreas. Thirty percent of patients with chronic pancreatitis develop inflammatory enlargement of the pancreatic head with subsequent obstruction of the pancreatic duct, and sometimes also of the common bile duct and duodenum. In these cases a pancreaticoduodenectomy, "Whipple procedure", has been the procedure of choice for a long time, as it provides reasonably effective pain relief. These resections, however, have both immediate postoperative morbidity and long-term morbidity. Insulin dependent diabetes mellitus has an increase in the incidence from 20% preoperatively to 60% in the years that follow<sup>[81]</sup>. Also, postgastrectomy complications detract significantly from the overall quality of life. The long-term mortality rate and quality of life after this procedure in patients with chronic pancreatitis has not always been encouraging, and in some studies disappointing<sup>[71]</sup>.

Distal pancreatectomy alone had poor results unless the disease is largely confined to the body and tail of the gland, e.g. with an occlusion of the mid-pancreatic duct or with a pseudocyst in the tail. By contrast, resection of the pancreatic head by either a conventional or pylorus-preserving pancreaticoduodenectomy will provide pain relief in up to 85% of patients, even if the disease extends into the distal pancreas. In order to deal with these undesirable consequences of the Whipple procedure, surgeons turned to the pylorus preserving pancreaticoduodenectomy (PPPD) and the "Beger procedure"<sup>[88-91]</sup>. Russel<sup>[92]</sup>, in studying the results of preservation of the duodenum in total pancreatectomy compared with those of standard pancreaticoduodenectomy, found no difference in pain relief between the results of the two operations. He noted that 13 (14%) of the 32 still had severe pain after duodenum preserving total pancreatectomy, and that six required major analgesics. The purported benefits of better postoperative nutritional status and glucose control in the duodenum-preserving procedure were addressed in two randomized trials<sup>[93,94]</sup>.

Frey and Amikura have recently reported a surgical modification that combines removing part of the anterior segment of the pancreatic head with longitudinal duct anastomosis to the jejunum<sup>[86]</sup>. A randomized trial<sup>[87]</sup> found little difference between the Frey procedure and the duodenum-preserving resection of the pancreatic head as described by Beger and Buchler<sup>[95]</sup>.

Noteworthy in recent years has been the very low operative morbidity and mortality of pancreatic resection, which may be one reason for the larger numbers of patients with benign disease being referred for surgical treatment. In a recent series of 231 pancreatic resections, the most frequent indication being chronic pancreatitis, the operative mortality was 0.4%<sup>[66]</sup>. McLeod *et al*<sup>[96]</sup> studied the morbidity of the Whipple operation. Although the study focused on resections for neoplasms, the observations pertain as well to those for chronic pancreatitis and show satisfactory digestion, weight maintenance, and activity level in the great majority of patients. A study of quality of life after pancreatic resections found that diabetes and its complications had the greatest negative influence on everyday well-being<sup>[97]</sup>.

Distal pancreatectomy<sup>[98]</sup> has a very limited role in management of pain, and only in patients with non-dilated pancreatic duct and pseudocysts involving the tail of the pancreas does this procedure seem to be associated with a good outcome<sup>[99]</sup>. Keith *et al*<sup>[100]</sup>, analyzed the results of 80% distal pancreatectomy, pancreaticoduodenectomy and total pancreatectomy. After an average follow-up of 5 years, 9 years, and 6 years, respectively, he found that four of five patients after pancreaticoduodenectomy required narcotics. Thirteen of 32 patients had complete pain relief after 80% distal pancreatectomy. Finally total pancreatectomy is usually reserved as a last resort following a failed partial pancreatic resection.

Resection of pancreatic tissue results in the loss of

some exocrine and endocrine function and increases the possibility or hastens the onset of fat malabsorption and diabetes. Whereas only 20% of normal pancreatic tissue is required for clinically adequate function, the pancreas already damaged by chronic pancreatitis may have substantially reduced reserves even before resection. Because of the complete lack of insulin and glucagon after total pancreatectomy, very brittle diabetes may ensue and can be the source of considerable morbidity and even mortality. In an attempt to lessen these adversities, autotransplantation of either part of the organ<sup>[101]</sup> or of islet tissue<sup>[102]</sup> has been described. In the latter study, Farney *et al* obtained insulin independence in 20% of 24 patients at a mean follow-up of 5.5 years. A more extensive experience with islet cell autotransplantation was reported by the Minnesota group in 1995 comprising 48 patients<sup>[103]</sup>. Forty-seven of the 48 patients had small duct chronic pancreatitis. Only one postoperative death resulted, but 25% of patient's encountered complications. There were 8 deaths in the follow-up period, none apparently attributable to the operation. In follow-up, from 1 mo to 17 years, 39% of patients reported that pain was resolved, and 61% still had some degree of pain. Twenty of 39 evaluable patients (51%) had initial (less than 1 mo) insulin independence, but this dropped to 15 patients (38%) beyond 1 mo. A more recent European experience of 13 patients indicated sustained insulin independence in 5 of 9 surviving patients (4 late deaths) from 9 to 48 mo after surgery<sup>[104]</sup>. The latest studies suggest improvement in both the areas of brittle diabetes and in pain control. Rodriguez *et al*<sup>[105]</sup> recruited 22 patients who underwent pancreatectomy and autologous islet cell transplantation. All patients demonstrated C-peptide and insulin production indicating graft function. Forty-one percent were insulin dependent, and 27% required minimal amount of insulin or a sliding scale. Eighty-two percent no longer required analgesics postoperatively and 14% experienced a decrease in need for narcotics. Their success was attributed due to the provision of pancreatectomy and islet cell transplantation earlier in the course of the disease. Clayton *et al*<sup>[106]</sup> followed 40 patients who had pancreatectomy followed by islet cell transplantation. At 2 years post-transplant, 18 patients had a median HbA1c of 6.6% (5.2%-19.3%), fasting C-peptide of 0.66 ng/mL (0.26-2.65 ng/mL), and required a median of 12 (0-45) units of insulin per day. At 6 years, these figures were 8% (6.1%-11.1%), 1.68 ng/mL (0.9-2.78 ng/mL) and 43 U/d (6-86 U/d), respectively. The majority of patients (68%) no longer require opiate analgesia. Finally, Gruessner *et al*<sup>[107]</sup> performed 112 islet autotransplants at the time of total pancreatectomy. They found that islet autotransplants, at the time of total pancreatectomy in patients who had not had previous operations on the body and tail of the pancreas, were associated with > 70% of the recipients achieving complete insulin independence. In contrast, a previous distal pancreatectomy or a Puestow drainage procedure was associated with complete insulin independence in < 20%. Islet autotransplantation offers a valuable addition to surgical resection of the

pancreas, as a treatment for chronic pancreatitis; and even in cases in which insulin independence is not achieved, the potential beneficial effects of C-peptide make the procedure worthwhile, particularly in early disease.

Many studies on pancreatic resection and even those on drainage procedures show that up to 15% of patients undergoing these surgical treatments for treatment of pain due to chronic pancreatitis will be found to have pancreatic cancer<sup>[7,79,99,108]</sup> and it has been shown that a chronic pancreatitis is in fact, a small, but real risk factor in the development of pancreatic cancer<sup>[109]</sup>. This is an important consideration to keep in mind during the diagnostic work-up and choice of operation. The morphology of the pancreas by CT imaging and by cholangiopancreatography may fail to discriminate between cancer and chronic pancreatitis. Cytological confirmation by fine-needle aspiration is helpful when positive, but the true diagnosis may become known only with resection (10% of cases). This consideration in some cases may determine the treatment strategy.

**Surgical denervation:** Most of the sensory nerves returning from the pancreas pass through the celiac ganglion and splanchnic nerves. It is hypothesized that interruption of these fibers may lessen pain. Mallet-Guy<sup>[110]</sup> reported an experience with 215 patients over 30 years whose principal treatment for pain was by sensory denervation. These patients first underwent abdominal exploration to document the absence of pancreatic ductal dilation or pseudocysts and to correct any associated biliary pathology; this was immediately followed by resection of the greater splanchnic nerve and celiac ganglion through a left translumbar approach. Although excellent long-term results are reported (90% of patients were pain-free, with 60% followed for more than five years), the heterogeneity of the patient population and the simultaneous use of biliary diversion procedures in many cases precludes meaningful conclusions. This treatment has not been widely accepted.

The celiac block can be done during laparotomy or percutaneously, usually from the back. The placement of the injection can be done simply by using anatomical landmarks or by checking the position with an imaging modality: fluoroscopy, scout X-ray films, ultrasonography, computed tomography, or at angiography. A nerve block with 25 mL of 50% alcohol on each side should be preceded by a positive diagnostic block with long acting local anesthesia, carried out at least 1 d earlier. The method aims at blockage of the splanchnic nerves before they reach the celiac plexus<sup>[111]</sup>.

Stone and Chauvin reported on 15 patients with chronic pancreatitis who had previous unsuccessful operative procedures for pain<sup>[112]</sup>. Denervation was accomplished with a transthoracic left splanchnicectomy with concomitant vagotomy, and all 15 patients had immediate pain control. Five later suffered recurrent pain, but were successfully treated with a right splanchnicectomy. The long-term outcomes are not

known. The advent of thoracoscopic surgery has made this procedure more attractive, and a few small series have reported its feasibility and early results<sup>[113,114]</sup>. Maher *et al* recently reported on 15 patients with chronic pancreatitis, mostly idiopathic, with chronic pain measured by visual analogue pain scale<sup>[115]</sup>. Unilateral thoracoscopic splanchnic nerve resection in eight patients and bilateral in seven patients resulted in significant decreases in pain frequency and intensity, as well as in narcotic consumption. Overall, 80% of patients had good results or were improved, with a mean follow-up of 16 mo. A controlled trial comparing this procedure to other surgical options or to medical treatment is needed. Of note, pancreaticoduodenectomy and duodenum-preserving resection of the pancreatic head may well confer pain relief at least in part through denervation.

## CONCLUSION

Pain is the most difficult to treat symptom of chronic pancreatitis. The current approach is largely based on data from studies of suboptimal quality and expert opinions. At present, a step wise strategy is recommended starting with life style modifications such as alcohol abstinence and low fat diet, then moving to high dose non-coated pancreatic enzymes and oral analgesic therapy. In patients with dilated main pancreatic duct unresponsive to medical therapy, endoscopy or decompressive surgery should be considered. Patients with debilitating pain, non-dilated pancreatic duct and inflammatory masses may be candidates for resective surgery. The role of pain modifying agents (antidepressants, gabapentin, pregabalin), celiac plexus block, antioxidants, octreotide and total pancreatectomy with islet cell auto transplantation remains to be determined.

## REFERENCES

- 1 **Feldman M**, Friedman L, Brandt L. Sleisenger and Fordtran's Gastrointestinal and Liver Disease: Pathophysiology, Diagnosis, Management. 8th ed. Philadelphia: Saunders, 2006: 1271-1300
- 2 **Sarles H**, Sahel J, Staub JL, Bourry J, Laugier R. Chronic Pancreatitis. In: Howat HT, Sarles H, editors. The exocrine pancreas. Philadelphia: WB Saunders, 1979: 402-439
- 3 **Ammann RW**, Buehler H, Muench R, Freiburghaus AW, Siegenthaler W. Differences in the natural history of idiopathic (nonalcoholic) and alcoholic chronic pancreatitis. A comparative long-term study of 287 patients. *Pancreas* 1987; **2**: 368-377
- 4 **Bradley EL 3rd**. Pancreatic duct pressure in chronic pancreatitis. *Am J Surg* 1982; **144**: 313-316
- 5 **Makrauer FL**, Antonioli DA, Banks PA. Duodenal stenosis in chronic pancreatitis: clinicopathological correlations. *Dig Dis Sci* 1982; **27**: 525-532
- 6 **Andren-Sandberg A**. Pain relief in pancreatic disease. *Br J Surg* 1997; **84**: 1041-1042
- 7 **Ammann RW**, Akovbiantz A, Largiader F, Schueler G. Course and outcome of chronic pancreatitis. Longitudinal study of a mixed medical-surgical series of 245 patients. *Gastroenterology* 1984; **86**: 820-828
- 8 **Lankisch PG**, Seidensticker F, Lohr-Happe A, Otto J, Creutzfeldt W. The course of pain is the same in alcohol- and nonalcohol-induced chronic pancreatitis. *Pancreas* 1995; **10**: 338-341
- 9 **Warsaw AL**. Pain in chronic pancreatitis. Patients, patience, and the impatient surgeon. *Gastroenterology* 1984; **86**: 987-989
- 10 **Owyang C**, Louie DS, Tatum D. Feedback regulation of pancreatic enzyme secretion. Suppression of cholecystokinin release by trypsin. *J Clin Invest* 1986; **77**: 2042-2047
- 11 **Lager P**, Jansen JB, Cherian L, Lamers CB, Goebell H. Feedback regulation of human pancreatic secretion. Effects of protease inhibition on duodenal delivery and small intestinal transit of pancreatic enzymes. *Gastroenterology* 1990; **98**: 1311-1319
- 12 **Dobrilla G**. Management of chronic pancreatitis. Focus on enzyme replacement therapy. *Int J Pancreatol* 1989; **5** Suppl: 17-29
- 13 **Andren-Sandberg A**. Theory and practice in the individualization of oral pancreatic enzyme administration for chronic pancreatitis. *Int J Pancreatol* 1989; **5** Suppl: 51-62
- 14 **Mossner J**, Secknus R, Meyer J, Niederau C, Adler G. Treatment of pain with pancreatic extracts in chronic pancreatitis: results of a prospective placebo-controlled multicenter trial. *Digestion* 1992; **53**: 54-66
- 15 **Slaff J**, Jacobson D, Tillman CR, Curington C, Toskes P. Protease-specific suppression of pancreatic exocrine secretion. *Gastroenterology* 1984; **87**: 44-52
- 16 **Isaksson G**, Ihse I. Pain reduction by an oral pancreatic enzyme preparation in chronic pancreatitis. *Dig Dis Sci* 1983; **28**: 97-102
- 17 **Halgreen H**, Pedersen NT, Worning H. Symptomatic effect of pancreatic enzyme therapy in patients with chronic pancreatitis. *Scand J Gastroenterol* 1986; **21**: 104-108
- 18 **Mossner J**, Wresky HP, Kestel W, Zeeh J, Regner U, Fischbach W. Influence of treatment with pancreatic extracts on pancreatic enzyme secretion. *Gut* 1989; **30**: 1143-1149
- 19 **Brown A**, Hughes M, Tenner S, Banks PA. Does pancreatic enzyme supplementation reduce pain in patients with chronic pancreatitis: a meta-analysis. *Am J Gastroenterol* 1997; **92**: 2032-2035
- 20 **Uhl W**, Anghelacopoulos SE, Friess H, Buchler MW. The role of octreotide and somatostatin in acute and chronic pancreatitis. *Digestion* 1999; **60** Suppl 2: 23-31
- 21 **Toskes PP**, Forsmark CE, Demeo MT. A multicenter controlled trial of octreotide for the pain of chronic pancreatitis. *Pancreas* 1993; **8**: 774
- 22 **Malfertheiner P**, Mayer D, Buchler M, Dominguez-Munoz JE, Schiefer B, Ditschuneit H. Treatment of pain in chronic pancreatitis by inhibition of pancreatic secretion with octreotide. *Gut* 1995; **36**: 450-454
- 23 **Scmalz MJ**, Soerger KH, Johanson JF. The effect of octrotide acetate, sandostatin, on the pain of chronic pancreatitis. *Gastroenterology* 1992; **102**: A290
- 24 **Toskes PP**. Medical management of chronic pancreatitis. *Scand J Gastroenterol Suppl* 1995; **208**: 74-80
- 25 **Bhardwaj P**, Thareja S, Prakash S, Saraya A. Micronutrient antioxidant intake in patients with chronic pancreatitis. *Trop Gastroenterol* 2004; **25**: 69-72
- 26 **Morris-Stiff GJ**, Bowrey DJ, Oleesky D, Davies M, Clark GW, Puntis MC. The antioxidant profiles of patients with recurrent acute and chronic pancreatitis. *Am J Gastroenterol* 1999; **94**: 2135-2140
- 27 **Cullen JJ**, Mitros FA, Oberley LW. Expression of antioxidant enzymes in diseases of the human pancreas: another link between chronic pancreatitis and pancreatic cancer. *Pancreas* 2003; **26**: 23-27
- 28 **Quilliot D**, Walters E, Bonte JP, Fruchart JC, Duriez P, Ziegler O. Diabetes mellitus worsens antioxidant status in patients with chronic pancreatitis. *Am J Clin Nutr* 2005; **81**: 1117-1125
- 29 **De las Heras Castano G**, Garcia de la Paz A, Fernandez MD, Fernandez Forcelledo JL. Use of antioxidants to treat

- pain in chronic pancreatitis. *Rev Esp Enferm Dig* 2000; **92**: 375-385
- 30 **Salim AS**. Role of oxygen-derived free radical scavengers in the treatment of recurrent pain produced by chronic pancreatitis. A new approach. *Arch Surg* 1991; **126**: 1109-1114
  - 31 **Banks PA**, Hughes M, Ferrante M, Noordhoek EC, Ramagopal V, Slivka A. Does allopurinol reduce pain of chronic pancreatitis? *Int J Pancreatol* 1997; **22**: 171-176
  - 32 **McCloy R**. Chronic pancreatitis at Manchester, UK. Focus on antioxidant therapy. *Digestion* 1998; **59** Suppl 4: 36-48
  - 33 **Bhardwaj P**, Grag PK, Saray A, Acharya S. Antioxidant Supplementation for Pain Relief in Chronic Pancreatitis: A Randomized Placebo Controlled Double Blind Trial. *Gastroenterology* 2007; **132**: A51
  - 34 **Dreiling DA**, Greenstein RJ. The sphincter of Oddi, sphincterotomy and biliopancreatic disease. *Am J Gastroenterol* 1979; **72**: 665-670
  - 35 **Bagley FH**, Braasch JW, Taylor RH, Warren KW. Sphincterotomy or sphincteroplasty in the treatment of pathologically mild chronic pancreatitis. *Am J Surg* 1981; **141**: 418-422
  - 36 **Smits ME**, Badiga SM, Rauws EA, Tytgat GN, Huibregtse K. Long-term results of pancreatic stents in chronic pancreatitis. *Gastrointest Endosc* 1995; **42**: 461-467
  - 37 **Ashby K**, Lo SK. The role of pancreatic stenting in obstructive ductal disorders other than pancreas divisum. *Gastrointest Endosc* 1995; **42**: 306-311
  - 38 **Kozarek RA**, Ball TJ, Patterson DJ, Brandabur JJ, Traverso LW, Raltz S. Endoscopic pancreatic duct sphincterotomy: indications, technique, and analysis of results. *Gastrointest Endosc* 1994; **40**: 592-598
  - 39 **Delhaye M**, Vandermeeren A, Baize M, Cremer M. Extracorporeal shock-wave lithotripsy of pancreatic calculi. *Gastroenterology* 1992; **102**: 610-620
  - 40 **Neuhaus H**. Fragmentation of pancreatic stones by extracorporeal shock wave lithotripsy. *Endoscopy* 1991; **23**: 161-165
  - 41 **Dumonceau JM**, Costamagna G, Tringali A, Vahedi K, Delhaye M, Hittlet A, Spera G, Giostra E, Mutignani M, De Maertelaer V, Deviere J. Treatment for painful calcified chronic pancreatitis: extracorporeal shock wave lithotripsy versus endoscopic treatment: a randomised controlled trial. *Gut* 2007; **56**: 545-552
  - 42 **Cremer M**, Deviere J, Delhaye M, Baize M, Vandermeeren A. Stenting in severe chronic pancreatitis: results of medium-term follow-up in seventy-six patients. *Endoscopy* 1991; **23**: 171-176
  - 43 **Grimm H**, Meyer WH, Nam VC, Soehendra N. New modalities for treating chronic pancreatitis. *Endoscopy* 1989; **21**: 70-74
  - 44 **Huibregtse K**, Schneider B, Vrij AA, Tytgat GN. Endoscopic pancreatic drainage in chronic pancreatitis. *Gastrointest Endosc* 1988; **34**: 9-15
  - 45 **Geenen JE**, Rolny P. Endoscopic therapy of acute and chronic pancreatitis. *Gastrointest Endosc* 1991; **37**: 377-382
  - 46 **Burdick JS**, Hogan WJ. Chronic pancreatitis: selection of patients for endoscopic therapy. *Endoscopy* 1991; **23**: 155-159
  - 47 **Kozarek RA**. Pancreatic stents can induce ductal changes consistent with chronic pancreatitis. *Gastrointest Endosc* 1990; **36**: 93-95
  - 48 **Gulliver DJ**, Edmunds S, Baker ME, Paine S, Baillie J, Cotton PB, Rice RP. Stent placement for benign pancreatic diseases: correlation between ERCP findings and clinical response. *AJR Am J Roentgenol* 1992; **159**: 751-755
  - 49 **Freedman SF**, Venu RP. Can pancreatic stent placement lead to chronic pancreatitis. *Gastroenterology* 1993; **104**: A304
  - 50 **Dite P**, Ruzicka M, Zboril V, Novotny I. A prospective, randomized trial comparing endoscopic and surgical therapy for chronic pancreatitis. *Endoscopy* 2003; **35**: 553-558
  - 51 **Cahen DL**, Gouma DJ, Nio Y, Rauws EA, Boermeester MA, Busch OR, Stoker J, Lameris JS, Dijkgraaf MG, Huibregtse K, Bruno MJ. Endoscopic versus surgical drainage of the pancreatic duct in chronic pancreatitis. *N Engl J Med* 2007; **356**: 676-684
  - 52 **Siegel JH**, Ben-Zvi JS, Pullano W, Cooperman A. Effectiveness of endoscopic drainage for pancreas divisum: endoscopic and surgical results in 31 patients. *Endoscopy* 1990; **22**: 129-133
  - 53 **Kozarek RA**, Traverso LW. Endoscopic treatment of chronic pancreatitis: An alternative to surgery? *Dig Surg* 1996; **13**: 90
  - 54 **Leung JW**, Bowen-Wright M, Aveling W, Shorvon PJ, Cotton PB. Coeliac plexus block for pain in pancreatic cancer and chronic pancreatitis. *Br J Surg* 1983; **70**: 730-732
  - 55 **Pap A**, Nauss LA, Di Magno EP. Is percutaneous celiac plexus block PCPB associated with pain relief in chronic pancreatitis? A comparison among analgesic, alcohol and steroid PCPB. *Pancreas* 1990; **5**: 725
  - 56 **Wallace MB**, Hawes RH. Endoscopic ultrasound in the evaluation and treatment of chronic pancreatitis. *Pancreas* 2001; **23**: 26-35
  - 57 **Busch EH**, Atchison SR. Steroid celiac plexus block for chronic pancreatitis: results in 16 cases. *J Clin Anesth* 1989; **1**: 431-433
  - 58 **Gress F**, Schmitt C, Sherman S, Ikenberry S, Lehman G. A prospective randomized comparison of endoscopic ultrasound- and computed tomography-guided celiac plexus block for managing chronic pancreatitis pain. *Am J Gastroenterol* 1999; **94**: 900-905
  - 59 **Gress F**, Schmitt C, Sherman S, Ciaccia D, Ikenberry S, Lehman G. Endoscopic ultrasound-guided celiac plexus block for managing abdominal pain associated with chronic pancreatitis: a prospective single center experience. *Am J Gastroenterol* 2001; **96**: 409-416
  - 60 **Duval MK Jr**. Caudal pancreatico-jejunostomy for chronic relapsing pancreatitis. *Ann Surg* 1954; **140**: 775-785
  - 61 **Puestow CB**, Gillesby WJ. Retrograde surgical drainage of pancreas for chronic relapsing pancreatitis. *AMA Arch Surg* 1958; **76**: 898-907
  - 62 **Partington PF**, Rochelle RE. Modified Puestow procedure for retrograde drainage of the pancreatic duct. *Ann Surg* 1960; **152**: 1037-1043
  - 63 **Frey CF**, Child CG, Fry W. Pancreatectomy for chronic pancreatitis. *Ann Surg* 1976; **184**: 403-413
  - 64 **Grace PA**, Pitt HA, Tompkins RK, DenBesten L, Longmire WP Jr. Decreased morbidity and mortality after pancreatoduodenectomy. *Am J Surg* 1986; **151**: 141-149
  - 65 **Crist DW**, Sitzmann JV, Cameron JL. Improved hospital morbidity, mortality, and survival after the Whipple procedure. *Ann Surg* 1987; **206**: 358-365
  - 66 **Fernandez-del Castillo C**, Rattner DW, Warshaw AL. Standards for pancreatic resection in the 1990s. *Arch Surg* 1995; **130**: 295-299; discussion 299-300
  - 67 **Frey CF**. Why and when to drain the pancreatic ductal system. In: Beger HG, Buchler M, Ditschuneit H, Malfetheriner P, editors. Chronic pancreatitis: research and clinical management. Berlin: Springer-Verlag, 1990: 415-425
  - 68 **Nagata A**, Homma T, Tamai K, Ueno K, Shimakura K, Oguchi H, Furuta S, Oda M. A study of chronic pancreatitis by serial endoscopic pancreatography. *Gastroenterology* 1981; **81**: 884-891
  - 69 **Nealon WH**, Townsend CM Jr, Thompson JC. Operative drainage of the pancreatic duct delays functional impairment in patients with chronic pancreatitis. A prospective analysis. *Ann Surg* 1988; **208**: 321-329
  - 70 **Nealon WH**, Thompson JC. Progressive loss of pancreatic function in chronic pancreatitis is delayed by main pancreatic duct decompression. A longitudinal prospective analysis of the modified puestow procedure. *Ann Surg* 1993; **217**: 458-466; discussion 466-468
  - 71 **Eckhauser FE**, Knol JA, Mulholland MW, Colletti LM. Pancreatic surgery. *Curr Opinion Gastroenterol* 1996; **12**: 448-456
  - 72 **Sharma AK**, Pande GK, Sahni P, Nundy S. Surgery for



- nonalcoholic chronic pancreatitis. *World J Surg* 1998; **22**: 236-239; discussion 239-240
- 73 **Mannell A**, Adson MA, McIlrath DC, Ilstrup DM. Surgical management of chronic pancreatitis: long-term results in 141 patients. *Br J Surg* 1988; **75**: 467-472
  - 74 **Sato T**, Miyashita E, Yamauchi H, Matsuno S. The role of surgical treatment for chronic pancreatitis. *Ann Surg* 1986; **203**: 266-271
  - 75 **Jalleh RP**, Aslam M, Williamson RC. Pancreatic tissue and ductal pressures in chronic pancreatitis. *Br J Surg* 1991; **78**: 1235-1237
  - 76 **Ebbehoj N**, Borly L, Bulow J, Rasmussen SG, Madsen P. Evaluation of pancreatic tissue fluid pressure and pain in chronic pancreatitis. A longitudinal study. *Scand J Gastroenterol* 1990; **25**: 462-466
  - 77 **Holmberg JT**, Isaksson G, Ihse I. Long term results of pancreaticojejunostomy in chronic pancreatitis. *Surg Gynecol Obstet* 1985; **160**: 339-346
  - 78 **Bradley EL 3rd**. Long-term results of pancreaticojejunostomy in patients with chronic pancreatitis. *Am J Surg* 1987; **153**: 207-213
  - 79 **Markowitz JS**, Rattner DW, Warshaw AL. Failure of symptomatic relief after pancreaticojejunal decompression for chronic pancreatitis. Strategies for salvage. *Arch Surg* 1994; **129**: 374-379; discussion 379-380
  - 80 **Prinz RA**, Aranha GV, Greenlee HB. Combined pancreatic duct and upper gastrointestinal and biliary tract drainage in chronic pancreatitis. *Arch Surg* 1985; **120**: 361-366
  - 81 **Warshaw AL**. Conservation of pancreatic tissue by combined gastric, biliary, and pancreatic duct drainage for pain from chronic pancreatitis. *Am J Surg* 1985; **149**: 563-569
  - 82 **Munn JS**, Aranha GV, Greenlee HB, Prinz RA. Simultaneous treatment of chronic pancreatitis and pancreatic pseudocyst. *Arch Surg* 1987; **122**: 662-667
  - 83 **Warshaw AL**, Rattner DW. Timing of surgical drainage for pancreatic pseudocyst. Clinical and chemical criteria. *Ann Surg* 1985; **202**: 720-724
  - 84 **Lohr-Happe A**, Peiper M, Lankisch PG. Natural course of operated pseudocysts in chronic pancreatitis. *Gut* 1994; **35**: 1479-1482
  - 85 **Frey CF**, Smith GJ. Description and rationale of a new operation for chronic pancreatitis. *Pancreas* 1987; **2**: 701-707
  - 86 **Frey CF**, Amikura K. Local resection of the head of the pancreas combined with longitudinal pancreaticojejunostomy in the management of patients with chronic pancreatitis. *Ann Surg* 1994; **220**: 492-504; discussion 504-507
  - 87 **Izbicki JR**, Bloechle C, Knoefel WT, Kuechler T, Binmoeller KF, Broelsch CE. Duodenum-preserving resection of the head of the pancreas in chronic pancreatitis. A prospective, randomized trial. *Ann Surg* 1995; **221**: 350-358
  - 88 **Buchler MW**, Friess H, Bittner R, Roscher R, Krautzberger W, Muller MW, Malfertheiner P, Beger HG. Duodenum-preserving pancreatic head resection: Long-term results. *J Gastrointest Surg* 1997; **1**: 13-19
  - 89 **Beger HG**, Krautzberger W, Bittner R, Buchler M, Limmer J. Duodenum-preserving resection of the head of the pancreas in patients with severe chronic pancreatitis. *Surgery* 1985; **97**: 467-473
  - 90 **Beger HG**, Muchler M. Duodenum-preserving resection of the head of the pancreas in chronic pancreatitis with inflammatory mass in the head. *World J Surg* 1990; **14**: 83-87
  - 91 **Traverso LW**, Longmire WP Jr. Preservation of the pylorus in pancreaticoduodenectomy. *Surg Gynecol Obstet* 1978; **146**: 959-962
  - 92 **Russel RCG**. Indications for surgical treatment in chronic pancreatitis. In: Standards in Pancreatic Surgery. Berger HG, Cuchler M, Malfertheiner P, editors. Berlin: Springer-Verlag, 1983: 350-357
  - 93 **Buchler MW**, Friess H, Muller MW, Wheatley AM, Beger HG. Randomized trial of duodenum-preserving pancreatic head resection versus pylorus-preserving Whipple in chronic pancreatitis. *Am J Surg* 1995; **169**: 65-69; discussion 69-70
  - 94 **Klempa I**, Spatny M, Menzel J, Baca I, Nustede R, Stockmann F, Arnold W. Pancreatic function and quality of life after resection of the head of the pancreas in chronic pancreatitis. A prospective, randomized comparative study after duodenum preserving resection of the head of the pancreas versus Whipple's operation. *Chirurg* 1995; **66**: 350-359
  - 95 **Bloechle C**, Izbicki JR, Knoefel WT, Kuechler T, Broelsch CE. Quality of life in chronic pancreatitis--results after duodenum-preserving resection of the head of the pancreas. *Pancreas* 1995; **11**: 77-85
  - 96 **McLeod RS**, Taylor BR, O'Connor BI, Greenberg GR, Jeejeebhoy KN, Royall D, Langer B. Quality of life, nutritional status, and gastrointestinal hormone profile following the Whipple procedure. *Am J Surg* 1995; **169**: 179-185
  - 97 **Petrin P**, Andreoli A, Antoniutti M, Delina Z, Corrado DL, Bruno B, Luigi G, Sergio P. Surgery for chronic pancreatitis: What quality of life ahead? *World J Surg* 1995; **19**: 398
  - 98 **Andren-Sandberg A**, Wagner M, Tihanyi T, Lofgren P, Friess H. Technical aspects of left-sided pancreatic resection for cancer. *Dig Surg* 1999; **16**: 305-312
  - 99 **Rattner DW**, Fernandez-del Castillo C, Warshaw AL. Pitfalls of distal pancreatectomy for relief of pain in chronic pancreatitis. *Am J Surg* 1996; **171**: 142-145; discussion 145-146
  - 100 **Keith RG**, Saibil FG, Sheppard RH. Treatment of chronic alcoholic pancreatitis by pancreatic resection. *Am J Surg* 1989; **157**: 156-162
  - 101 **Rossi RL**, Soeldner JS, Braasch JW, Heiss FW, Shea JA, Watkins E Jr, Silverman ML. Long-term results of pancreatic resection and segmental pancreatic autotransplantation for chronic pancreatitis. *Am J Surg* 1990; **159**: 51-57; discussion 57-58
  - 102 **Farney AC**, Najarian JS, Nakhleh RE, Lloveras G, Field MJ, Gores PF, Sutherland DE. Autotransplantation of dispersed pancreatic islet tissue combined with total or near-total pancreatectomy for treatment of chronic pancreatitis. *Surgery* 1991; **110**: 427-437; discussion 437-439
  - 103 **Wahoff DC**, Papalois BE, Najarian JS, Kendall DM, Farney AC, Leone JP, Jessurun J, Dunn DL, Robertson RP, Sutherland DE. Autologous islet transplantation to prevent diabetes after pancreatic resection. *Ann Surg* 1995; **222**: 562-575; discussion 575-579
  - 104 **Oberholzer J**, Triponez F, Mage R, Anderegg E, Buhler L, Cretin N, Fournier B, Goumaz C, Lou J, Philippe J, Morel P. Human islet transplantation: lessons from 13 autologous and 13 allogeneic transplantations. *Transplantation* 2000; **69**: 1115-1123
  - 105 **Rodriguez Rilo HL**, Ahmad SA, D'Alessio D, Iwanaga Y, Kim J, Choe KA, Moulton JS, Martin J, Pennington LJ, Soldano DA, Biliter J, Martin SP, Ulrich CD, Somogyi L, Welge J, Matthews JB, Lowy AM. Total pancreatectomy and autologous islet cell transplantation as a means to treat severe chronic pancreatitis. *J Gastrointest Surg* 2003; **7**: 978-989
  - 106 **Clayton HA**, Davies JE, Pollard CA, White SA, Musto PP, Dennison AR. Pancreatectomy with islet autotransplantation for the treatment of severe chronic pancreatitis: the first 40 patients at the leicester general hospital. *Transplantation* 2003; **76**: 92-98
  - 107 **Gruessner RW**, Sutherland DE, Dunn DL, Najarian JS, Jie T, Hering BJ, Gruessner AC. Transplant options for patients undergoing total pancreatectomy for chronic pancreatitis. *J Am Coll Surg* 2004; **198**: 559-567; discussion 568-569
  - 108 **Lankisch PG**, Lohr-Happe A, Otto J, Creutzfeldt W. Natural course in chronic pancreatitis. Pain, exocrine and endocrine pancreatic insufficiency and prognosis of the disease. *Digestion* 1993; **54**: 148-155
  - 109 **Lowenfels AB**, Maisonneuve P, Cavallini G, Ammann RW, Lankisch PG, Andersen JR, Dimagno EP, Andren-Sandberg A, Domellof L. Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. *N Engl J Med* 1993;

- 328: 1433-1437
- 110 **Mallet-Guy PA**. Late and very late results of resections of the nervous system in the treatment of chronic relapsing pancreatitis. *Am J Surg* 1983; **145**: 234-238
- 111 **Ischia S**, Ischia A, Polati E, Finco G. Three posterior percutaneous celiac plexus block techniques. A prospective, randomized study in 61 patients with pancreatic cancer pain. *Anesthesiology* 1992; **76**: 534-540
- 112 **Stone HH**, Chauvin EJ. Pancreatic denervation for pain relief in chronic alcohol associated pancreatitis. *Br J Surg* 1990; **77**: 303-305
- 113 **Worsey J**, Ferson PF, Keenan RJ, Julian TB, Landreneau RJ. Thoracoscopic pancreatic denervation for pain control in irresectable pancreatic cancer. *Br J Surg* 1993; **80**: 1051-1052
- 114 **Cuschieri A**, Shimi SM, Crosthwaite G, Joypaul V. Bilateral endoscopic splanchnicectomy through a posterior thoracoscopic approach. *J R Coll Surg Edinb* 1994; **39**: 44-47
- 115 **Maher JW**, Johlin FC, Pearson D. Thoracoscopic splanchnicectomy for chronic pancreatitis pain. *Surgery* 1996; **120**: 603-609; discussion 609-610

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Peter V Draganov, Dr, Series Editor

## Pancreatic function testing: Here to stay for the 21st century

John G Lieb II, Peter V Draganov

John G Lieb II, Peter V Draganov, University of Florida, Florida 32610, United States

**Author contributions:** Lieb JG did the literature search and compiled the first draft of the manuscript; Draganov PV contributed to providing the idea for the manuscript and performing editing for content and guiding the overall direction of the manuscript.

**Correspondence to:** Peter V Draganov, University of Florida, 1600 SW Archer Rd Room HD 602, PO Box 100214 Gainesville, Florida 32610,

United States. [dragapv@medicine.ufl.edu](mailto:dragapv@medicine.ufl.edu)

Telephone: +1-352-3922877 Fax: +1-352-3923618

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### Abstract

The diagnosis of Chronic Pancreatitis (CP) is based on the detection of abnormal structure or function of the diseased pancreas. The pancreatic function tests more accurately determine the presence of CP than tests of structure, especially for early stage disease. The function tests can be divided into two categories: non-invasive and invasive. The invasive "tube" tests can reliably detect mild, early CP, but are only available at a few referral centers and tend to be poorly tolerated by patients. The non-invasive tests are easy to obtain, but tend to perform poorly in patients with early, mild disease. Therefore, no one test is useful in all clinical situations, and a detailed understanding of the rational, pathophysiologic basis, strengths, and limitations of various tests is needed. This review highlights the role of various pancreatic function tests in the diagnosis of CP including fecal fat analysis, fecal elastase, fecal chymotrypsin, serum trypsin, the secretin stimulation test, the cholecystokinin (CCK) stimulation test, the combined secretin-CCK stimulation test, the intraductal and endoscopic secretin stimulation tests, and the functional magnetic resonance imaging of the pancreas after secretin stimulation.

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**Key words:** Pancreatic function testing; Secretin stimulation test; CCK stimulation test; Fecal elastase; Endoscopic secretin stimulation test; Chronic pancreatitis

**Peer reviewer:** Chris E Forsmark, Professor, Division of Gastroenterology, Hepatology, and Nutrition, University of

### INTRODUCTION

Gastroenterologists frequently encounter patients with Chronic Pancreatitis (CP), which is responsible for 86 000 annual admissions in the United States alone<sup>[1]</sup>. Even more frequently encountered is the patient with chronic abdominal pain and suspected CP based on equivocal imaging or laboratory findings. Although defined by irreversible histologic damage to the pancreas, histologic specimens are difficult and morbid to obtain. Therefore, in practice, the diagnosis of CP is based on the detection of abnormal structure or function (endocrine and exocrine) of the diseased pancreas. However, gross radiographic and endoscopic structural changes are insensitive and can be nonspecific - especially for early stage disease. Therefore, gastroenterologists are often forced to rely on tests of pancreatic function, the so-called, pancreatic function tests (PFT's), to diagnose CP. Arguably, these more accurately determine the presence of CP than tests of structure. Unfortunately, many of these PFT's themselves have significant drawbacks.

Several new PFT's have been introduced in the last 5-10 years, such as fecal elastase, Secretin-stimulated Magnetic Resonance Cholangio-pancreatography (S-MRCP), and endoscopic Pancreatic Function Testing (ePFT).

A few key points in using and interpreting PFT's are: first, they can be falsely positive for at least a few months after an attack of acute pancreatitis; second, negative PFT's do not exclude acute relapsing pancreatitis in patients who do not yet have structural or functional pancreas damage; third, although the best PFT's, especially the secretin-based stimulation tests, are more sensitive in the detection of CP than nonfunctional tests, rarely they still can miss early stage CP.

### NORMAL PANCREATIC PHYSIOLOGY

In order to appreciate the utility of pancreatic function

testing, one has to understand the normal functioning of the pancreas. In the basal or fasting state, the pancreas excretes small amounts of protein - rich and mildly alkaline fluid. During a meal, gastric distension and acid production stimulate the duodenal S cells to release secretin into the blood, which signals the ductal cells of the small ducts of the pancreas to secrete a large volume of bicarbonate-rich, clear, watery fluid (so called *hydraulic secretion*). Similarly, the postprandial increase in amino acids and fatty acids in the duodenal fluid stimulates the I cells of the duodenum to secrete cholecystokinin (CCK, aka pancreozymin). CCK, in turn, signals the acinar cells of the pancreas to release enzyme-rich fluid into the pancreatic duct. This is so called *ecbolic secretion*<sup>[2]</sup>. For completeness, vago-vagal pathways also stimulate pancreatic secretion and modulate hormone release. These are primarily responsible for an increase in pancreatic secretion during the cephalic phase of digestion. The effects of these two hormonal systems (Secretin and CCK) are measurable and are abnormal in CP. For example, CCK levels are elevated in patients with early CP compared to controls, and these levels are often low in advanced disease<sup>[3]</sup>. In general terms, the chronically damaged pancreas produces decreased volume, bicarbonate, and enzymes in pancreatic juice in response to a stimulus than the normal pancreas. These decrements can be exploited during pancreatic function testing.

## NATURAL HISTORY OF CP

Pancreatic function testing is clinically important for a number of reasons. First, CP is a heterogenous disease. Patients lie on a spectrum ranging from early, painful disease (so called minimal change, or small duct CP) with relatively preserved physiology to end stage disease with very little endocrine or exocrine function. Patients with early stage CP are very difficult to diagnose and distinguish from other causes of chronic abdominal pain. For example, conventional testing, such as pancreas-protocol computed tomography (CT) scans, Magnetic Resonance Imaging (MRI), and Magnetic Resonance Cholangio-Pancreatography (MRCP), generally detects patients with late stage CP, typically when 50% or more of the gland is fibrotic and has been essentially destroyed. Some experts suggest that the traditional pancreatic function tests may detect patients with as little as 30% damage to the pancreas<sup>[4]</sup>.

Another reason that PFT's are useful is that clinical assessment of steatorrhea (exocrine dysfunction) is unreliable. Many patients can have steatorrhea with only a single formed bowel movement a day. Further complicating the prediction of steatorrhea is the often long course of acute pain relapses or early CP that occurs for many years before the development of steatorrhea. In natural history studies, the time to the development of steatorrhea is quite long, about 20 years. Part of this lag time is explained by the pancreas' extensive reserve of lipase secreting capacity. The pancreas has to lose ninety percent of its lipase

production before steatorrhea is measurable by fecal fat testing. Yet, lipase depletion occurs earlier and is more profound than protease and amylase deficiency<sup>[5]</sup>. This fact can be exploited during pancreatic function testing. Part of lipase's vulnerability is its dependence on bicarbonate secretion by the pancreas to ensure a high duodenal pH - up to 7.5-9.0 for optimum activity. Endocrine dysfunction may occur at, or slightly after, the development of steatorrhea<sup>[6]</sup>. Certainly, the time course of exocrine and endocrine dysfunction varies depending on the etiology of the CP. As an extreme example, cystic fibrosis patients can present in infancy with failure to thrive due to exocrine failure<sup>[7]</sup>.

## PROBLEMS WITH STRUCTURAL (NONFUNCTIONAL) TESTS FOR CP

Besides the subtle progression of the natural history of CP as a reason for the utility of pancreatic function testing, many of the conventional tests in the detection of CP have a number of drawbacks.

### CT

CT is fairly sensitive for the detection of advanced CP with calcification, atrophy, fat replacement, and ductal dilation. In some studies as high as 75%<sup>[8]</sup> to 80%<sup>[9]</sup>. However, others have found that when compared to better tests such as ERCP and Secretin-CCK function testing, CT is only 47% sensitive in the diagnosis of CP. The specificity of CT is considerably higher than the sensitivity, around 90%<sup>[10]</sup>. CT carries the additional benefit of evaluating the pancreas for other pathology (e.g. pancreatic cancer), and the whole abdomen for alternative explanation of the patient symptoms.

### MRCP

MRCP is a fairly good test for the detection of advanced CP. However, even compared to the relatively insensitive endoscopic retrograde cholangiopancreatography (ERCP), MRCP is only about 75% sensitive for advanced disease and 25% for small duct CP<sup>[11]</sup>. Generally, MRCP detects many of the same changes that are seen on CT. An added benefit of MRCP is improved detection and characterization of biliary and pancreatic strictures compared to other noninvasive imaging tests. However, the visualization of the pancreatic duct (PD) can be difficult by MRCP, which depends on volume and flow in the pancreatic duct that is already quite low in CP. Non-occluding strictures can make visualization of the PD difficult. Generally, conventional MRCP, like CT, does not detect subtle side branch abnormalities of minimal change CP<sup>[12]</sup>.

### ERCP

This test involves cannulation of the pancreatic and biliary ducts. ERCP is generally considered the gold standard in the diagnosis of structural pancreatic duct diseases. In several studies, ERCP can even detect a very small number of patients with negative PFT's<sup>[13]</sup>.



However, these changes can be seen in the normally aging pancreas, and, overall, the sensitivity of ERCP for small duct CP is significantly less than that of the best pancreatic function tests, even at the quaternary centers most proficient at ERCP<sup>[14,15]</sup>. Overall, ERCP has sensitivity of 66% for detecting minimal change CP and is 93% sensitive for late stage CP, compared to secretin stimulation testing<sup>[5]</sup>. In addition, ERCP is highly operator dependent. Furthermore, it is fairly invasive and carries a risk of up to 20% of acute post-ERCP pancreatitis which is greatest in the patients suspected of having minimal change CP (with non-dilated ducts). Recent preliminary data suggest that even a relatively mild episode of acute post ERCP pancreatitis may lead to CP when evaluated several years after the episode of post-ERCP pancreatitis<sup>[16]</sup>.

### **Endoscopic ultrasound (EUS)**

EUS is an excellent initial test of choice in the diagnosis of minimal change CP. It has relatively few risks, even if fine needle aspiration is used, and is as sensitive as MRCP in the detection of occult choledocholithiasis<sup>[17]</sup>, and is superior to MRCP and transabdominal ultrasound in detecting cholecystolithiasis. However, it does have several drawbacks. It still requires sedation so a full day of work is missed - not only by the patient, but by a driver/chaperone - making it relatively expensive. EUS is highly operator dependent. In addition, even more than ERCP, EUS can be falsely positive due to the echotexture changes of the normal aging pancreas or in diabetics. Therefore, EUS is better at "ruling out" CP than it is at "ruling in" CP. Sensitivities and specificities of EUS vary from 90% and 85% *versus* histology for advanced disease<sup>[18]</sup> to 97% and 60%, respectively for EUS-FNA compared to ERCP<sup>[19]</sup>, to 57% and 64%, respectively for plain EUS compared to secretin stimulation testing<sup>[20]</sup>, to 83% and 80%, respectively in a mixed population of early and advanced disease compared to histology<sup>[21]</sup>. Much controversy surrounds the endosonographic definition of CP, with some groups still using 3 EUS criteria for CP, while most agreeing that 5 or more criteria must be present diffusely<sup>[22]</sup>. Unfortunately, to date no consensus exists on the exact EUS diagnostic criteria for CP.

## **PANCREATIC FUNCTION TESTS FOR CP**

### **Noninvasive "tubeless" pancreatic function tests**

In an effort to discover a sensitive and specific function test for CP that avoids risk and invasive procedures and that can be performed on outpatient basis, several tests have been developed, all of which suffer several severe shortcomings, but may be useful in diagnosing CP in a patient with a long alcohol history or with equivocal imaging findings. Generally, these tests only detect advanced CP with steatorrhea, but are fairly cheap and reliable.

**Seventy-two hour fecal fat:** The 72 h fecal fat collection was once a routine part of the workup for malabsorption,

and it remains the gold standard for quantification of steatorrhea. However, it suffers from many drawbacks, including its nonspecificity for pancreatic disease. For example, bacterial overgrowth, short bowel syndrome, and small bowel mucosal disease (e.g. celiac disease and Crohn's disease) can present with steatorrhea. However, the diarrhea of CP tends to be less voluminous yet fattier than other diarrheal illnesses. In addition, the 72 h fecal fat is inaccurate when performed in the outpatient setting for several reasons. First, it is unrealistic to expect the patient to refrigerate 72 h worth of stool. Second, adherence to a standardized 100 g/24 h fat diet per day for a total of 6 d (the 3 d preceding the test and then the test itself) is difficult. Achieving at least 100 g/d, typical for a large fast food lunch of double cheeseburger and French fries with milkshake, is relatively easy (of course, false negatives can occur in the patient unable to consume that much fat due to pain, though, these are typically early CP patients, who do not yet have steatorrhea). However, quantification of daily fat intake with food diaries as an outpatient is unreliable, making calculation of the coefficient of fat absorption similarly unreliable. Third, for this test, the patient must be off of oral pancreatic enzymes supplements for about a week prior to collection. As a result, some patients have bloating, abdominal discomfort, and gas from malabsorption or are otherwise unwilling to stop the enzymes.

In our institution, for the above reasons, we reserve the 72 h stool collection for research purposes, during which time the patient is admitted to a metabolic ward with a dietician familiar with the protocol to monitor consumption and adjust later meals to account for what has not been consumed. A 72 h stool collection during a high fat diet showing more than 7 g/d fat in the stool is abnormal<sup>[23]</sup>. The levels of steatorrhea seen in CP tend to be much higher (often > 20 g/d). For practicality, most pancreatologists have abandoned this test. However, a modified 24 h protocol can be used for clinical purposes to monitor response to enzyme therapy in patients experiencing an unexplained increase in steatorrhea, especially in growing children with cystic fibrosis, despite alleged compliance with enzymes.

**Spot fecal fat:** Sudan staining of a random stool sample for fecal fat is relatively insensitive for fat malabsorption. Generally, it detects steatorrhea only at 25 g/d or more. As a stool collection, it suffers many of the drawbacks of the 72 h fecal fat, including patient embarrassment, need to stop pancreatic enzyme supplements, need to be on a high fat diet for several days before the collection, *etc.* Greater than 6 droplets of fat per high power field are indicative of steatorrhea. As in the case of the 72 h fecal fat analysis, fat substitutes in foods such as Olestra<sup>®</sup>/Olean<sup>®</sup> or drugs such as orlistat or ezetimibe can give false positive results.

**Fecal chymotrypsin:** In advanced CP, lower concentrations of pancreatic proteases reach the stool than in controls. Trypsin is the principal protease secreted by the

pancreas, however, it undergoes degradation in the distal small bowel so is not a good fecal marker for pancreas enzyme output<sup>[24]</sup>. On the other hand, several other proteases made by the pancreas, such as chymotrypsin are useful stool markers. As with all fecal protease assays, the fecal chymotrypsin should be thought of as a surrogate for the 72 h fecal fat rather than for the conventional, “tube,” pancreatic function tests. Chymotrypsin evades degradation in stool by binding to insoluble debris in stool and is stable for several days at room temperature, enabling a sample to be shipped to a reference lab. A fecal chymotrypsin below 3 U/g of stool suggests advanced CP. This test is altered by exogenous pancreatic enzyme supplementation so is useful to monitor for compliance, but is not available in the United States<sup>[25]</sup>. The fecal chymotrypsin assay is of little clinical value to detect early stage CP, but it has a reasonable sensitivity for advanced disease of from 50% to 80%, increasing to 80%-90% in cystic fibrosis<sup>[26]</sup>, with a specificity of 50%-100%<sup>[27-29]</sup>. As in all fecal protease assays, watery diarrhea, such as from short bowel syndrome, can give false positive results (low fecal chymotrypsin) by diluting the sample.

**Fecal elastase (FE):** Pancreatic elastase-1 is a pancreas-specific protease that is minimally degraded during intestinal transit. In fact, it is concentrated 6-fold in stool compared to duodenal juice<sup>[30,31]</sup>. The concentration of fecal elastase in stool measured by Enzyme Linked Immunosorbant Assay (ELISA) correlates well with duodenal amylase, lipase, and trypsin in both CP patients and controls<sup>[32]</sup>. Typically, a fecal elastase less than 100 mcg/g of stool indicates severe pancreatic insufficiency. A value between 100-200 mcg is indeterminate, but in the face of other evidence, is suggestive of CP. Values over 200 mcg are normal.

FE suffers from many of the same limitations of the fecal chymotrypsin assay, notably that it only detects patients with steatorrhea and severe CP that likely could have been detected by other means. In various studies, compared to conventional pancreatic function testing and ERCP, the sensitivity of FE varies from between 0%-65% for mild disease to 33%-100% for severe CP, with generally good specificity (from 29% to 95%)<sup>[33-37]</sup>. FE may be superior to fecal chymotrypsin. For example, in one small study the FE had a sensitivity of 64% for detecting CP compared to 25% for fecal chymotrypsin<sup>[38]</sup>. Also, like fecal chymotrypsin only a spot stool sample is required rather than a 24 h or 72 h collection. FE also does not cross react to exogenous porcine enzymes so patients can remain on therapy for the test. However, FE is more expensive than fecal chymotrypsin.

**Serum trypsin:** The serum trypsinogen (a.k.a. trypsin) assay is unique among pancreatic function tests in being a serum sample, making it convenient and relatively cheap. Low levels, less than 20 ng/mL, are specific for CP, but are sensitive only for advanced disease. Levels from 20-29 are indeterminate, but sometimes represent early CP<sup>[39]</sup>. Sensitivities for mild to severe CP patients

combined range from 33%-65%, but specificity is quite high<sup>[40]</sup>. Sensitivity for exocrine dysfunction is quite high, at about 95%<sup>[39]</sup>. One added benefit is that trypsin levels over 150 ng/mL are indicative of pancreatic inflammation. For example, the trypsin can be positive for a relapse of CP even when amylase and lipase levels are normal. Conversely, it can help differentiate benign, chronically elevated amylase and lipase from pancreatic inflammation<sup>[41]</sup>. The test used in our institution is a Radio-Immune Assay (RIA), so it has the disadvantage of requiring several days to obtain a result. We typically obtain this test along with the fecal elastase and pancreatic protocol CT as an initial battery in all patients suspected of having CP referred to our clinic. However, like the fecal assays, it is basically a marker of advanced disease and steatorrhea.

### ***Invasive, traditional, “tube” pancreatic function tests***

Since first described in the 1930s and 1940s, several techniques have been developed to measure pancreatic function after physiologic or supraphysiologic stimuli<sup>[42,43]</sup>. The central theme of these tests is to collect and quantitate the quality of pancreatic secretions to determine pancreatic secretory capacity.

**Secretin stimulation test (SST):** In a technique more widely publicized by Dreiling<sup>[44]</sup>, a double lumen, 26 Fr, oro-duodenal tube with both gastric and duodenal ports is introduced fluoroscopically, stiffened with a guidewire, with only topical anesthesia (benzocaine spray and viscous lidocaine) applied to the posterior pharynx. The weighted tip should be advanced close to the ligament of Treitz and the tapered radiopaque portion of the tube should be positioned at the pylorus. Placement can be hampered by multiple factors including patient discomfort, nausea, gastroparesis, and pyloric stenosis.

We place both the gastric and duodenal ports to low constant suction by an electric flywheel pump whose gauge measures 2-5 inches Hg (51-127 mmHg). However, the suction produced by these pumps may be lower than the gauge suggests: our lab has found that standard wall units are too strong and inconsistent and may result in adherence of the tube to the duodenal wall with clogging of the ports. Constant vigilance is required to prevent clogging of ports which decreases yield of duodenal fluid. During experiments with Polyethylene Glycol (PEG) labeled with carbon 14 (<sup>14</sup>C), 85% or more of duodenal fluid can be collected with this double lumen “Dreiling” tube with relatively little reflux of duodenal contents into the stomach<sup>[45,46]</sup>. We then measure basal duodenal and basal gastric pH and volume over 15 min. Next, we give a bolus of intravenous (IV) secretin, because bolus administration has been shown to be equivalent<sup>[47]</sup> or superior<sup>[48]</sup> to continuous infusion. The typical dose of porcine secretin is a 1 U/kg IV bolus. This is a supraphysiologic dose, but is usually well tolerated other than some flushing. However, the cost of secretin is fairly high. One study showed that an even higher dose of secretin (4-5 U/kg) might be more sensitive<sup>[49]</sup>. We now use synthetic human secretin at

dose 0.2 mg/kg which has been shown to be equivalent to porcine secretin<sup>[50]</sup>. We then measure three parameters of the duodenal fluid collected over one hour in four 15 min aliquots: volume, pH, and bicarbonate concentration in mEq/L measured by back titration with hydrochloric acid. Others have found that automated analyzers are almost as good as the standard labor intense back titration<sup>[51]</sup>. The gastric pH and volume at the end of the study are also recorded. The highest concentration of bicarbonate obtained among the four 15 min aliquots is the peak bicarbonate concentration. For completeness, a microscopic exam is performed on the duodenal aspirate for Giardia, Gram stain, and Crystals. Then, the bicarbonate output (the product of bicarbonate concentration and volume) for that hour long post-stimulation period is calculated. The tube is then removed and the patient can resume normal activities and can drive home. Standardized ranges are 80-130 mEq/L for the peak bicarbonate, 1.5-5.7 mL/kg for the volume/kg of patient weight, and 10.1 to 37.0 mEq/h for the bicarbonate output. If the peak bicarbonate is less than 80 mEq/L, the patient is very likely to have CP. If the volume is low and proper position of the collecting tube is reconfirmed, we typically state that the patient should be evaluated further for a pancreatic duct obstruction.

The SST is arguably the most sensitive test for CP. Classically, bicarbonate is thought to be produced by small pancreatic ducts<sup>[2]</sup>. Consequently, one might anticipate that the SST would be the most sensitive test to diagnose small duct, minimal change CP. This hypothesis was upheld in several studies. The SST, when compared with histology, is 75% sensitive in detecting early stage CP, and up to 97% for late stage disease with a specificity of 90%<sup>[52,53]</sup>. Compared to SST, ERCP has about a 66% sensitivity for early disease, though it comes close to SST for late stage disease<sup>[4,54]</sup>.

In addition, several histologic studies suggest bicarbonate production may be the best way to diagnose early CP. A study in dogs indicates that the maximal bicarbonate output is closely related to functional pancreatic mass<sup>[55]</sup>. In addition, an early study by Dr. Dreiling found an excellent correlation between findings on histology and findings of the SST. The SST picked up 20/24 patients (83%) who had CP by pathology whereas ERCP was only 17/24 (71%) sensitive. All underwent SST first, followed by ERCP, and 24 went on to exploratory laparotomy<sup>[54]</sup>.

However, the SST does have some shortcomings, notably difficulty with tube placement and that false positives can be seen for several months after an attack of acute pancreatitis. This is the reason we delay EUS, S-MRCP, fecal elastase testing, fecal fat testing, and SST for several months after an attack of acute pancreatitis.

**CCK stimulation testing:** In use almost as long as the SST, the classical CCK stimulation test is a useful test, developed and used primarily at the Mayo Clinic in Rochester, Minnesota. Because this test measures ecboic (enzyme) output, it is, in theory, a measure of

the processes that lead to steatorrhea, and could be less sensitive than SST. However, it is still one of the most sensitive tests for the presence of CP. One study of normal controls in Japan found no differences between the SST and the CCK stimulation test<sup>[56]</sup>. The CCK stimulation test has a number of drawbacks including the need for placement of two specialized 2-lumen tubes with simultaneous gastric and duodenal aspiration and duodenal perfusion of a solution containing mannitol and PEG. CCK is also administered under constant infusion at 40 ng/kg per hour, but it can be given as a bolus<sup>[57,58]</sup>. Caerulein, which is found on the skin of tree frogs and can be produced synthetically, can substitute for CCK. Caerulein is, in fact, many times more potent a secretagogue than CCK<sup>[59]</sup>. Bombesin can also substitute for CCK<sup>[60]</sup>.

In the classical CCK stimulation test, as in most tube tests, the basal 20 min aspiration of duodenal and gastric contents is discarded. The gastric and duodenal ports are continually withdrawn under low intermittent suctioning and duodenal fluid is collected over 80 min into four 20 min aliquots. Also during the first 20-40 min, the contraction of the gallbladder by CCK (and resultant flow of bile into the duodenum) affects the measurement of pancreatic output. In addition, as CCK can delay gastric emptying<sup>[61]</sup>, and is thought to cross the blood brain barrier and mediate central pain mechanisms<sup>[62]</sup>, symptoms of nausea and vomiting are common during infusion and more common than symptoms from secretin infusion<sup>[63]</sup>. The classic CCK stimulation test also requires measurement, and constant intestinal perfusion, of a nonabsorbable marker, and recovery rates vary significantly<sup>[64]</sup>. If the illustration in the *Gastroenterology* article which first described it is still in use today, it has fewer aspiration ports in the duodenum than the conventional Dreiling tube, and uses pressure suctioning of 40 mmHg<sup>[65]</sup> which, as mentioned above, may be somewhat different than the suction used at University of Florida with the conventional Dreiling tube.

A modified version of this test using a conventional Dreiling tube, placed under light sedation, and measuring only lipase by a hospital based lab assay was found to be very sensitive in patients with both early (Cambridge 2) and late stage CP by ERCP (Cambridge 3 and 4)<sup>[66]</sup>. However, no one has compared this test directly to the SST. In addition, as we shall discuss later in the section on endoscopic secretin stimulation testing, use of sedation may affect recovery of secretions and cost.

**Combined secretin-CCK (secretin-pancreozymin) stimulation testing:** This test is used mostly in Europe and Japan and allows measurement of both bicarbonate and enzyme production by the pancreas. In theory, the simultaneous administration of Secretin with CCK has the potential to dilute the measurement of enzyme activity by watery, bicarbonate solution. However, CCK can also be given before<sup>[67]</sup> or after<sup>[57]</sup> Secretin. It also shares one of the drawbacks of the CCK stimulation test: increased bile secretion into the duodenum.

In one study of the Secretin-CCK test, the peak bicarbonate - rather than CCK-related parameters - was correlated nearly linearly to the severity of histologic changes in CP. Also in this study, the second and third best measures of histologic damage were the amylase activity and the total volume, respectively. In that study, the secretin-CCK test was 67% sensitive for various stages of CP, which is somewhat less than other studies of the SST. However, this study used stringent requirements for the diagnosis of CP. All 3 parameters (peak bicarbonate, volume of duodenal secretions, and amylase output) had to be decreased in order to qualify as CP. Applying our cutoffs for peak bicarbonate, only, to this data would give greater sensitivity with only some loss of specificity<sup>[4,68]</sup>.

Another study found that the trypsin activity in pancreatic fluid was not as sensitive a measure of CP as the peak bicarbonate during Secretin-CCK testing<sup>[69]</sup>. A recent, and probably the largest, study of Secretin-CCK stimulation testing supported this finding, mostly in cystic fibrosis patients. In this study, 336 CCK-Secretin tests were reviewed. Using enzyme (trypsin) activity alone (cutoff < 50 U/kg per hour) would have had 25% false positives if enzyme recovery were not corrected for losses (if a marker had not been used); i.e. 25% of patients with good enzyme activity would have been falsely classified as pancreas-insufficient<sup>[70]</sup>.

A third study of 19 alcoholic CP patients and 6 patients with idiopathic CP who underwent CCK-secretin testing and went on to surgery for refractory symptoms, 18/18 of whom had an abnormal ERCP, found that the peak bicarbonate concentration and output were the best measures of small duct dilation seen on histology. In addition, peak bicarbonate output was the best measure of acinar atrophy with a Spearman correlation coefficient of -0.71 ( $P$  between 0.001 and 0.01) and the chymotrypsin output was also significantly correlated (Spearman, -0.57), but with a higher  $P$  of between 0.01 and 0.02. Peak volume also correlated fairly well with acinar atrophy (Spearman -0.44,  $P$  between 0.02 and 0.05), but peak bicarbonate concentration was weaker (Spearman -0.17,  $P > 0.05$ ). In summary, this study found that the hydraulic parameters (volume, peak bicarbonate concentration, peak bicarbonate output) were overall better predictors of abnormal histology than the ebolic parameters (chymotrypsin)<sup>[2]</sup>.

These studies indicate that the CCK portion of the Secretin-CCK stimulation test adds little information in the diagnosis of CP that the secretin stimulation portion alone (or perhaps the classic SST) already provides. However, the Secretin-CCK test is certainly a more sensitive measure of pancreatic enzyme production than the bentiromide test, which tests primarily protease production by the pancreas<sup>[71]</sup>.

**Perfusion testing:** Researchers in the Gastroenterology Division at the University of Florida in Gainesville over the last 25 years have developed and implemented a method of measuring endogenous and exogenous pancreatic enzyme activity in the duodenum of patients

with CP analogous to the Mayo clinic CCK methodology. This “perfusion test” enables quantification of delivery of exogenous pancreatic enzymes to the duodenum. Some notable differences between this perfusion test and the CCK stimulation test include use of a standardized meal rather than CCK to stimulate the pancreas, use of a modified Dreiling tube attached to a 7 Fr Dobhoff tube, placed without sedation under fluoroscopy, and the perfusion of radiolabeled Carbon-14 Polyethylene Glycol (PEG) to enhance assessments of recovery. This perfusion test measures endogenous enzyme production in the fasted and fed states with a standardized Ensure<sup>®</sup> meal. Volume of both gastric and duodenal collections, pH, and enzyme activity are recorded over a 3 h period. The test is then repeated immediately after intake of an exogenous pancreatic enzyme<sup>[72]</sup>. The inconvenience and time required for this test render it useful only for the research setting.

**Intraductal SST:** In the intraductal secretin stimulation test, typically the main pancreatic duct is cannulated using ERCP techniques and then pancreatic fluid is collected, after the administration of secretin alone, or secretin followed by CCK. The patient is sedated without anticholinergic medications such as diphenhydramine (Benadryl<sup>®</sup>) or opiates, usually with benzodiazepines. Typically, the pancreatic fluid collected in this manner has a higher bicarbonate concentration than in the classical SST, around 130 mEq/L for controls, and less than 105 mEq/L for CP patients, owing to lack of contamination by bile and duodenal content. Some of the disadvantages of this test include the complication rate of ERCP, the need for sedation, and the relatively short time periods of collection (usually 15 min, as limited by sedation and fluoroscopy room time). An advantage of the intraductal test is that pure pancreatic juice is collected without contamination with bile or duodeno-gastric contents and that it can be used in patients with Billroth I and II gastric resections.

One group showed that the intraductal test could not reliably differentiate between 19 CP patients, 14 “early CP” patients, and 14 controls<sup>[73]</sup>, despite a long intraductal collection period of 60 min. The investigators used extra CCK with secretin after the initial secretin boluses in 15 patients. They used only 70 U maximum of secretin and did not adjust for weight of the patients. In addition, their aspiration catheter was prefilled with a dye to assist in identifying the start of the collection, which may have been problematic due to mixing. Also their “early CP” patients had only acute relapsing pancreatitis with no evidence of chronicity by imaging or conventional pancreatic function testing.

For the analyses, it appears they combined the patients with “early CP” and those with CP. They found that this combined group of CP patients produced significantly less volume of pancreatic secretions than controls after stimulation with 1 CU of secretin and 70 CU of secretin but not after 4 CU of secretin. CP patients also had significantly less bicarbonate concentrations only after 4 CU of secretin compared to



controls. Bicarbonate output was decreased significantly at all time points for CP patients compared to controls. Interestingly, at only the first minute time point, in patients with CP, the protein content of fluid was higher but not significantly so, than controls, perhaps due to concretions of inspissated enzymes in this group from PD stasis. However, after 70 CU of secretin, the protein output of CP patients was significantly less than controls.

A second, larger study of 12 patients with CP and 33 controls (22 normals and 11 with other nonpancreatic GI disorders), which used only a 20 min collection time, found that the sensitivity of the intraductal test peak bicarbonate compared to SST was 100% with a specificity of only 66%. Volume had an 88% sensitivity and a 91% specificity for CP by SST<sup>[74]</sup>.

The most recent study of the intraductal secretin stimulation test was less favorable. In this comparison of the intraductal secretin stimulation test and SST, in which 19 patients served as their own control, the sensitivity of the intraductal secretin stimulation test compared to the conventional SST was only 80%, with a very poor specificity of 20%. Against pancreatogram, the intraductal test was 100% sensitive but only 55% specific<sup>[75]</sup>. This group used three 5-min collections (as is customary for most intraductal secretin stimulation tests) and the first was discarded. Based on these results, we do not recommend the use of the intraductal secretin stimulation test for routine diagnostic or research purposes.

#### **Endoscopic secretin stimulation testing (eSST):**

An alternative to traditional pancreatic function testing is to sedate the patient, and collect duodenal juice under endoscopic guidance from a polyethylene tube passed through the biopsy channel of a standard upper endoscope after stimulation with secretin<sup>[51,76]</sup> or the combination of secretin-CCK<sup>[77]</sup>. This offers the advantage of patient comfort and sedation. The eSST has been extensively studied by a group of investigators from the Cleveland Clinic. The overall impression is that the eSST has the potential to yield results similar to the conventional SST. This comes to no surprise since the two tests are very similar with the main differences being the use of sedation in the eSST and the use of the endoscope to collect duodenal secretions rather than a Dreiling tube for the conventional SST. However, it should be noted that the eSST and the SST have only been directly compared in one small cross over study of healthy controls only, without any CP patients, in which the SST group also received sedation, which we do not do and could have confounded the results in favor of the eSST<sup>[78]</sup>.

Unfortunately, the eSST has several disadvantages. Although the eSST is technically easy to perform, it is impractical, and to date it has not gained acceptance. The main problem appears to be that occupying an endoscopy room and keeping the patient sedated for more than one hour are cost-prohibitive. Although the Cleveland Clinic group has shown that a 45 min

endoscopic collection is reasonable with good sensitivity with some loss of specificity<sup>[79]</sup>, we have shown that a full 60 min is necessary for full sensitivity and specificity of the classical secretin stimulation test<sup>[80]</sup>. Furthermore, patients and their escorts will also have to miss a whole day of work. In addition, medications used for sedation may have effects on pancreatic secretions<sup>[81]</sup>. Opiates may constrict the sphincter of Oddi, and propofol contains 5% triglyceride which may have effects on pancreatic secretion. Although one small study of normal subjects did not find an effect of light sedation on secretion during endoscopic secretin stimulation testing, it used fairly low doses - 2.5 mg of midazolam and 62.5 mg of meperidine<sup>[82]</sup>. However, we have found that greater amounts of sedatives are required in most patients with chronic abdominal pain who are referred for evaluation of possible CP.

#### **“Enhanced imaging” pancreatic function tests (S-MRCP):**

Because of some of the shortcomings of conventional MRCP in the diagnosis of pancreatic disorders, some have investigated the use of MRI with secretin stimulation to increase the flow and volume in the pancreatic duct. The filling of the duodenum can be semi-quantitated to assess for CP. One possible problem with this technique is that it measures volume of pancreatic flow rather than bicarbonate concentration. In theory, obstructive lesions, or sphincter of Oddi spasm could give positive results in the absence of true CP<sup>[83]</sup>. In addition, MR images are acquired over at most 30 min, which is often an insufficient length of time during secretin stimulation and which may lead to reduced sensitivity.

One German study of 18 CP patients, defined by ERCP, many of whom had previously undergone pancreatic duct stenting and removal, and 5 diseased controls exemplified some of these issues with S-MRCP. This study, even on these patients with obviously advanced CP, showed a 69% sensitivity of S-MRCP with 1 CU/kg of secretin and 90% specificity as compared to relatively insensitive pancreatic function tests, such as the fecal elastase and <sup>13</sup>C Mixed Chain Triglyceride Breath Test (MCT-BT)<sup>[83]</sup>.

Another method that S-MRCP uses to assess for CP is parenchymal enhancement during gadolinium infusion (also used during conventional MRI, but which is not used during conventional MRCP). To assess for parenchymal enhancement, T1-weighted sequences with fat suppression are crucial. Also important is the pattern of gadolinium enhancement of the parenchyma: CP patients show decreased enhancement in the arterial phase and increased enhancement in the early venous phase, which are thought to be due to decreased pancreatic blood flow. On T2 imaging, enhancement is seen in CP patients compared to controls, indicating fatty or fibrous replacement of the parenchyma. After 0.5 IU/kg of secretin, the reduced T2 signal changes showed a good correlation with the Lundh test, a pancreatic function test using meal based stimulation. This study also showed a good correlation between

duodenal diameter after S-MRCP and the Lundh test. Patients with severe CP had an average increase in duodenal diameter of 1.7 mm. In mild CP the increase was 4.7 mm, and in controls, 14 mm. However, in this study, the patient population was not well defined. They did have a cohort with mild pancreatitis but again we do not know the criteria used to establish this<sup>[84]</sup>.

Another group, this time from Japan, has distinguished S-MRCP, which they reserve to look for duct changes, from "Secretin-Stimulated, Diffusion Weighted MRI" which focuses on secretin-induced changes within the parenchyma of the gland. This new type of MRI calculates the Apparent Diffusion Coefficient (ADC) which measures diffusion of water molecules in the microcirculation. They claim that this type of MRI is even more sensitive than S-MRCP and that it evaluates local and regional pancreatic exocrine function. They also measured changes in alcoholic patients, known not to have structural pancreatic disease by conventional CT. Notably these patients did not undergo pancreatic function testing or ERCP<sup>[11]</sup>.

## CONCLUSION

Most pancreatic function tests have high sensitivity and specificity to accurately diagnose patients with advanced CP. The noninvasive tests tend to perform poorly in patients with early, mild disease. Some specialized invasive "tube" tests can reliably detect mild, early CP but are only available at a few quaternary referral centers. S-MRCP and Diffusion Weighted, Secretin Stimulated MR are promising technologies but, for the near future, are not likely to provide the same discriminating power as the best "tube" tests. The quest for a simple, noninvasive, cheap, and accurate pancreatic function test continues.

## REFERENCES

- Kozak LJ, Owings MF, Hall MJ. National Hospital Discharge Survey: 2002 annual summary with detailed diagnosis and procedure data. *Vital Health Stat* 13 2005; 1-199
- Heij HA, Obertop H, van Blankenstein M, ten Kate FW, Westbroek DL. Relationship between functional and histological changes in chronic pancreatitis. *Dig Dis Sci* 1986; **31**: 1009-1013
- Slaff JI, Wolfe MM, Toskes PP. Elevated fasting cholecystokinin levels in pancreatic exocrine impairment: evidence to support feedback regulation. *J Lab Clin Med* 1985; **105**: 282-285
- Chowdhury RS, Forsmark CE. Review article: Pancreatic function testing. *Aliment Pharmacol Ther* 2003; **17**: 733-750
- Forsmark CE. Chronic pancreatitis. In Sleisenger and Fordtran's Gastrointestinal and Liver Disease, 7th ed. Philadelphia: Saunders, 2002: 949
- Layer P, Yamamoto H, Kalthoff L, Clain JE, Bakken LJ, DiMaggio EP. The different courses of early- and late-onset idiopathic and alcoholic chronic pancreatitis. *Gastroenterology* 1994; **107**: 1481-1487
- Durie PR. Pancreatic aspects of cystic fibrosis and other inherited causes of pancreatic dysfunction. *Med Clin North Am* 2000; **84**: 609-620, ix
- Manfredi R, Brizi MG, Masselli G, Gui B, Vecchioli A, Marano P. Imaging of chronic pancreatitis. *Rays* 2001; **26**: 143-149
- Luetmer PH, Stephens DH, Ward EM. Chronic pancreatitis: reassessment with current CT. *Radiology* 1989; **171**: 353-357
- Malfertheiner P, Buchler M, Stanescu A, Ditschuneit H. Exocrine pancreatic function in correlation to ductal and parenchymal morphology in chronic pancreatitis. *Hepatogastroenterology* 1986; **33**: 110-114
- Sugiyama M, Haradome H, Atomi Y. Magnetic resonance imaging for diagnosing chronic pancreatitis. *J Gastroenterol* 2007; **42** Suppl 17: 108-112
- Akisik MF, Sandrasegaran K, Aisen AA, Maglinte DD, Sherman S, Lehman GA. Dynamic secretin-enhanced MR cholangiopancreatography. *Radiographics* 2006; **26**: 665-677
- Gupta V, Toskes PP. Diagnosis and management of chronic pancreatitis. *Postgrad Med J* 2005; **81**: 491-497
- Forsmark CE, Toskes PP. What does an abnormal pancreatogram mean? *Gastrointest Endosc Clin N Am* 1995; **5**: 105-123
- Toskes PP. Update on diagnosis and management of chronic pancreatitis. *Curr Gastroenterol Rep* 1999; **1**: 145-153
- Symersky T, van Hoorn B, Masclee AA. The outcome of a long-term follow-up of pancreatic function after recovery from acute pancreatitis. *JOP* 2006; **7**: 447-453
- Ledro-Cano D. Suspected choledocholithiasis: endoscopic ultrasound or magnetic resonance cholangio-pancreatography? A systematic review. *Eur J Gastroenterol Hepatol* 2007; **19**: 1007-1011
- Varadarajulu S, Eltoun I, Tamhane A, Eloubeidi MA. Histopathologic correlates of noncalcific chronic pancreatitis by EUS: a prospective tissue characterization study. *Gastrointest Endosc* 2007; **66**: 501-509
- Hollerbach S, Klamann A, Topalidis T, Schmigel WH. Endoscopic ultrasonography (EUS) and fine-needle aspiration (FNA) cytology for diagnosis of chronic pancreatitis. *Endoscopy* 2001; **33**: 824-831
- Chowdhury RS, Bhutani MS, Mishra G, Toskes PP, Forsmark CE. Comparative analysis of pancreatic function testing versus morphologic assessment (by EUS) for the evaluation of chronic unexplained abdominal pain. *Gastroenterology* 2001; **120**: A647
- Chong AK, Hawes RH, Hoffman BJ, Adams DB, Lewin DN, Romagnuolo J. Diagnostic performance of EUS for chronic pancreatitis: a comparison with histopathology. *Gastrointest Endosc* 2007; **65**: 808-814
- Raimondo M, Wallace MB. Diagnosis of early chronic pancreatitis by endoscopic ultrasound. Are we there yet? *JOP* 2004; **5**: 1-7
- Safdi M, Bekal PK, Martin S, Saeed ZA, Burton F, Toskes PP. The effects of oral pancreatic enzymes (Creon 10 capsule) on steatorrhea: a multicenter, placebo-controlled, parallel group trial in subjects with chronic pancreatitis. *Pancreas* 2006; **33**: 156-162
- Adham NF, Dyce BJ, Geokas MC, Haverback BJ. Stool chymotrypsin and trypsin determinations. *Am J Dig Dis* 1967; **12**: 1272-1276
- Goldberg DM. Proteases in the evaluation of pancreatic function and pancreatic disease. *Clin Chim Acta* 2000; **291**: 201-221
- Scotta MS, Marzani MD, Maggiore G, De Giacomo C, Melzi D'Eril GV, Moratti R. Fecal chymotrypsin: a new diagnostic test for exocrine pancreatic insufficiency in children with cystic fibrosis. *Clin Biochem* 1985; **18**: 233-234
- Dominguez-Munoz JE, Hieronymus C, Sauerbruch T, Malfertheiner P. Fecal elastase test: evaluation of a new noninvasive pancreatic function test. *Am J Gastroenterol* 1995; **90**: 1834-1837
- Loser C, Mollgaard A, Folsch UR. Faecal elastase 1: a novel, highly sensitive, and specific tubeless pancreatic function test. *Gut* 1996; **39**: 580-586
- Durr HK, Otte M, Forell MM, Bode JC. Fecal chymotrypsin: a study on its diagnostic value by comparison with the secretin-cholecystokinin test. *Digestion* 1978; **17**: 404-409
- Sziegoleit A, Krause E, Klor HU, Kanacher L, Linder D.

- Elastase 1 and chymotrypsin B in pancreatic juice and feces. *Clin Biochem* 1989; **22**: 85-89
- 31 **Sziegoleit A**, Linder D. Studies on the sterol-binding capacity of human pancreatic elastase 1. *Gastroenterology* 1991; **100**: 768-774
  - 32 **Stein J**, Jung M, Sziegoleit A, Zeuzem S, Caspary WF, Lembcke B. Immunoreactive elastase I: clinical evaluation of a new noninvasive test of pancreatic function. *Clin Chem* 1996; **42**: 222-226
  - 33 **Dominguez-Munoz JE**, Hieronymus C, Sauerbruch T, Malfertheiner P. Fecal elastase test: evaluation of a new noninvasive pancreatic function test. *Am J Gastroenterol* 1995; **90**: 1834-1837
  - 34 **Gullo L**, Ventrucci M, Tomassetti P, Migliori M, Pezzilli R. Fecal elastase 1 determination in chronic pancreatitis. *Dig Dis Sci* 1999; **44**: 210-213
  - 35 **Lankisch PG**, Schmidt I, König H, Lehnich D, Knollmann R, Lohr M, Liebe S. Faecal elastase 1: not helpful in diagnosing chronic pancreatitis associated with mild to moderate exocrine pancreatic insufficiency. *Gut* 1998; **42**: 551-554
  - 36 **Loser C**, Mollgaard A, Folsch UR. Faecal elastase 1: a novel, highly sensitive, and specific tubeless pancreatic function test. *Gut* 1996; **39**: 580-586
  - 37 **Amann ST**, Bishop M, Curington C, Toskes PP. Fecal pancreatic elastase 1 is inaccurate in the diagnosis of chronic pancreatitis. *Pancreas* 1996; **13**: 226-230
  - 38 **Katschinski M**, Schirra J, Bross A, Goke B, Arnold R. Duodenal secretion and fecal excretion of pancreatic elastase-1 in healthy humans and patients with chronic pancreatitis. *Pancreas* 1997; **15**: 191-200
  - 39 **Jacobson DG**, Curington C, Connery K, Toskes PP. Trypsin-like immunoreactivity as a test for pancreatic insufficiency. *N Engl J Med* 1984; **310**: 1307-1309
  - 40 **Borgstrom A**, Wehlin L. Correlation between serum concentrations of three specific exocrine pancreatic proteins and pancreatic duct morphology at ERCP examinations. *Scand J Gastroenterol* 1984; **19**: 220-227
  - 41 **Lake-Bakaar G**, McKavanagh S, Gatus B, Summerfield JA. The relative values of serum immuno-reactive trypsin concentration and total amylase activity in the diagnosis of mumps, chronic renal failure, and pancreatic disease. *Scand J Gastroenterol* 1980; **15**: 97-101
  - 42 **Chiray M**, Jeandel A, Salmon A. L'exploration clinique du pancreas et l'injection intraveineuse de secretine purifiée. *Presse Med* 1930; **38**: 977
  - 43 **Lagerlof HO**. Pancreatic function and pancreatic disease: studied by means of secretin. *Acta Med Scand* 1942; **128**: 1
  - 44 **Dreiling DA**, Hollander F. Studies in pancreatic function; preliminary series of clinical studies with the secretin test. *Gastroenterology* 1948; **11**: 714-729
  - 45 **Go VL**, Hofmann AF, Summerskill WH. Simultaneous measurements of total pancreatic, biliary, and gastric outputs in man using a perfusion technique. *Gastroenterology* 1970; **58**: 321-328
  - 46 **Lagerlof HO**, Schutz HB, Holmer S. A secretin test with high doses of secretin and correction for incomplete recovery of duodenal juice. *Gastroenterology* 1967; **52**: 67-77
  - 47 **Takebe T**, Koike D, Yokoyama Y. Modern trends in biochemical tests of the pancreas: CS test and S test. *Biliary Tract Pancreas* 1986; **7**: 583-588
  - 48 **Farini R**, Del Favero G, Adorati M, Pedrazzoli S, Fabris G, Giordano P, D'Angelo A, Zotti E, Lise M, Chiaramonte M, Salvagnini M, Naccarato R. Comparison between bolus injection and infusion of secretin and pancreozymin in the diagnosis of chronic pancreatic disease (one hour test). *Acta Hepatogastroenterol (Stuttg)* 1977; **24**: 462-468
  - 49 **Bordalo O**, Noronha M, Lamy J, Dreiling D. The secretin test with the standard dose and high dose in chronic pancreatitis. *Minerva Med* 1976; **67**: 3599-3606
  - 50 **Somogyi L**, Ross SO, Cintron M, Toskes PP. Comparison of biologic porcine secretin, synthetic porcine secretin, and synthetic human secretin in pancreatic function testing. *Pancreas* 2003; **27**: 230-234
  - 51 **Ceryak S**, Steinberg WM, Marks ZH, Ruiz A. Feasibility of an endoscopic secretin test: preliminary results. *Pancreas* 2001; **23**: 216-218
  - 52 **Steer ML**, Waxman I, Freedman S. Chronic pancreatitis. *N Engl J Med* 1995; **332**: 1482-1490
  - 53 **Kitagawa M**, Naruse S, Ishiguro H, Nakae Y, Kondo T, Hayakawa T. Evaluating exocrine function tests for diagnosing chronic pancreatitis. *Pancreas* 1997; **15**: 402-408
  - 54 **Waye JD**, Adler M, Dreiling DA. The pancreas: a correlation of function and structure. *Am J Gastroenterol* 1978; **69**: 176-181
  - 55 **Hansky J**, Tiscornia OM, Dreiling DA, Janowitz HD. Relationship between maximal secretory output and weight of the pancreas in the dog. *Proc Soc Exp Biol Med* 1963; **114**: 654-656
  - 56 **Suzuki T**, Suzuki K, Kobayashi E, Ogawa Y, Kawamura Y, Nakai T, Suzuki S, Hayakawa T, Noda A, Kondo T. Comparative study of the secretin test and pancreozymin secretin test in chronic pancreatitis. *Nippon Shokakibyo Gakkai Zasshi* 1986; **83**: 2209-2215
  - 57 **Bank S**, Marks IN, Moshal MG, Efron G, Silber R. The pancreatic-function test--method and normal values. *S Afr Med J* 1963; **37**: 1061-1066
  - 58 **Zieve L**, Silvis SE, Mulford B, Blackwood WD. Secretion of pancreatic enzymes. I. Response to secretin and pancreozymin. *Am J Dig Dis* 1966; **11**: 671-684
  - 59 **Gullo L**, Costa PL, Fontana G, Labo G. Investigation of exocrine pancreatic function by continuous infusion of caerulein and secretin in normal subjects and in chronic pancreatitis. *Digestion* 1976; **14**: 97-107
  - 60 **Basso N**, Giri S, Improta G, Lezoche E, Melchiorri P, Percoco M, Speranza V. External pancreatic secretion after bombesin infusion in man. *Gut* 1975; **16**: 994-998
  - 61 **Liddle RA**, Morita ET, Conrad CK, Williams JA. Regulation of gastric emptying in humans by cholecystokinin. *J Clin Invest* 1986; **77**: 992-996
  - 62 **Xie JY**, Herman DS, Stiller CO, Gardell LR, Ossipov MH, Lai J, Porreca F, Vanderah TW. Cholecystokinin in the rostral ventromedial medulla mediates opioid-induced hyperalgesia and antinociceptive tolerance. *J Neurosci* 2005; **25**: 409-416
  - 63 **Akisik MF**, Sandrasegaran K, Aisen AA, Maglinte DD, Sherman S, Lehman GA. Dynamic secretin-enhanced MR cholangiopancreatography. *Radiographics* 2006; **26**: 665-677
  - 64 **Conwell DL**, Zuccaro G, Morrow JB, Van Lente F, O'Laughlin C, Vargo JJ, Dumot JA. Analysis of duodenal drainage fluid after cholecystokinin (CCK) stimulation in healthy volunteers. *Pancreas* 2002; **25**: 350-354
  - 65 **Go VL**, Hofmann AF, Summerskill WH. Simultaneous measurements of total pancreatic, biliary, and gastric outputs in man using a perfusion technique. *Gastroenterology* 1970; **58**: 321-328
  - 66 **Conwell DL**, Zuccaro G, Morrow JB, Van Lente F, Obuchowski N, Vargo JJ, Dumot JA, Trolli P, Shay SS. Cholecystokinin-stimulated peak lipase concentration in duodenal drainage fluid: a new pancreatic function test. *Am J Gastroenterol* 2002; **97**: 1392-1397
  - 67 **Sun DC**. Normal values for pancreozymin-secretin test. *Gastroenterology* 1963; **44**: 602-606
  - 68 **Hayakawa T**, Kondo T, Shibata T, Noda A, Suzuki T, Nakano S. Relationship between pancreatic exocrine function and histological changes in chronic pancreatitis. *Am J Gastroenterol* 1992; **87**: 1170-1174
  - 69 **Rolny P**, Jagenburg R. The secretin-CCK test and a modified Lundh test. A comparative study. *Scand J Gastroenterol* 1978; **13**: 927-931
  - 70 **Schibli S**, Corey M, Gaskin KJ, Ellis L, Durie PR. Towards the ideal quantitative pancreatic function test: analysis of test variables that influence validity. *Clin Gastroenterol Hepatol* 2006; **4**: 90-97
  - 71 **Lankisch PG**, Schreiber A, Otto J. Pancreolauryl test.

- Evaluation of a tubeless pancreatic function test in comparison with other indirect and direct tests for exocrine pancreatic function. *Dig Dis Sci* 1983; **28**: 490-493
- 72 **Slaff J**, Jacobson D, Tillman CR, Curington C, Toskes P. Protease-specific suppression of pancreatic exocrine secretion. *Gastroenterology* 1984; **87**: 44-52
- 73 **Denyer ME**, Cotton PB. Pure pancreatic juice studies in normal subjects and patients with chronic pancreatitis. *Gut* 1979; **20**: 89-97
- 74 **Ochi K**, Harada H, Mizushima T, Tanaka J, Matsumoto S. Intraductal secretin test is as useful as duodenal secretin test in assessing exocrine pancreatic function. *Dig Dis Sci* 1997; **42**: 492-496
- 75 **Draganov P**, Patel A, Fazel A, Toskes P, Forsmark C. Prospective evaluation of the accuracy of the intraductal secretin stimulation test in the diagnosis of chronic pancreatitis. *Clin Gastroenterol Hepatol* 2005; **3**: 695-699
- 76 **Conwell DL**, Zuccaro G Jr, Vargo JJ, Trolli PA, Vanlente F, Obuchowski N, Dumot JA, O'Laughlin C. An endoscopic pancreatic function test with synthetic porcine secretin for the evaluation of chronic abdominal pain and suspected chronic pancreatitis. *Gastrointest Endosc* 2003; **57**: 37-40
- 77 **Bornschein W**. A fast endoscopic test of pancreatic secretion (endoscopic secretin-caerulein-test) (author's transl). *Z Gastroenterol* 1978; **16**: 582-592
- 78 **Stevens T**, Conwell DL, Zuccaro G Jr, Van Lente F, Purich E, Khandwala F, Fein S. A randomized crossover study of secretin-stimulated endoscopic and dreiling tube pancreatic function test methods in healthy subjects. *Am J Gastroenterol* 2006; **101**: 351-355
- 79 **Stevens T**, Conwell DL, Zuccaro G Jr, Lewis SA, Love TE. The efficiency of endoscopic pancreatic function testing is optimized using duodenal aspirates at 30 and 45 minutes after intravenous secretin. *Am J Gastroenterol* 2007; **102**: 297-301
- 80 **Draganov P**, George S, Toskes PP, Forsmark CE. Is a 15-minute collection of duodenal secretions after secretin stimulation sufficient to diagnose chronic pancreatitis? *Pancreas* 2004; **28**: 89-92
- 81 **Konturek SJ**. Opiates and the gastrointestinal tract. *Am J Gastroenterol* 1980; **74**: 285-291
- 82 **Conwell DL**, Zuccaro G, Purich E, Fein S, Vanlente F, Vargo J, Dumot J, O'Laughlin C, Trolli P. The effect of moderate sedation on exocrine pancreas function in normal healthy subjects: a prospective, randomized, cross-over trial using the synthetic porcine secretin stimulated Endoscopic Pancreatic Function Test (ePFT). *Am J Gastroenterol* 2005; **100**: 1161-1166
- 83 **Schneider AR**, Hammerstingl R, Heller M, Povse N, Murzynski L, Vogl TJ, Caspary WF, Stein J. Does secretin-stimulated MRCP predict exocrine pancreatic insufficiency?: A comparison with noninvasive exocrine pancreatic function tests. *J Clin Gastroenterol* 2006; **40**: 851-855
- 84 **Czako L**. Diagnosis of early-stage chronic pancreatitis by secretin-enhanced magnetic resonance cholangiopancreatography. *J Gastroenterol* 2007; **42** Suppl 17: 113-117

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# Analysis of surgical and perioperative complications in seventy-five right hepatectomies for living donor liver transplantation

Salvatore Gruttadauria, James Wallis Marsh, Giovan Battista Vizzini, Fabrizio di Francesco, Angelo Luca, Riccardo Volpes, Amadeo Marcos, Bruno Gridelli

Salvatore Gruttadauria, Giovan Battista Vizzini, Fabrizio di Francesco, Angelo Luca, Riccardo Volpes, Bruno Gridelli, Department of Abdominal Transplant Surgery, Istituto Mediterraneo Trapianti e Terapie ad Alta Specializzazione, Palermo 90127, Italy

Salvatore Gruttadauria, James Wallis Marsh, Amadeo Marcos, Bruno Gridelli, Department of Abdominal Transplant Surgery, University of Pittsburgh Medical Center, Palermo 90127, Italy

**Author contributions:** Gruttadauria S, Marsh JW, Marcos A and Gridelli B contributed equally to perform all surgical cases; Gruttadauria S, Vizzini GB, di Francesco F, Volpes R and Luca A designed research; Gruttadauria S and di Francesco F performed research; Gruttadauria S, Marsh JW, Marcos A and Gridelli B analyzed data; Gruttadauria S and di Francesco F wrote the paper. **Correspondence to:** Salvatore Gruttadauria, MD, Department of Abdominal Transplant Surgery, Istituto Mediterraneo Trapianti e Terapie ad Alta Specializzazione, Palermo 90127, Italy. [sgruttadauria@ismett.edu](mailto:sgruttadauria@ismett.edu)

Telephone: +39-91-2192111 Fax: +39-91-2192400

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**CONCLUSION:** The need to define, categorize and record complications when healthy individuals, such as living donors, undergo a major surgical procedure, such as a right hepatectomy, reflects the need for prompt and detailed reports of complications arising in this particular category of patient. Perioperative complications and post resection liver regeneration are not influenced by anatomic variations or patient demographic.

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**Key words:** Right hepatectomy; Surgery; Living-related liver transplantation; Surgical complications

**Peer reviewers:** Paulo Ney Aguiar Martins, MD, PhD, Surgery Department, Transplantation Division, Harvard Medical School, Massachusetts General Hospital, Boston 02129, United States; Dr. Adam G Testro, Department of Gastroenterology and Liver Transplantation, Austin Health Institution, Heidelberg 3032, Australia

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## Abstract

**AIM:** To present an analysis of the surgical and perioperative complications in a series of seventy-five right hepatectomies for living-donation (RHLD) performed in our center.

**METHODS:** From January 2002 to September 2007, we performed 75 RHLD, defined as removal of a portion of the liver corresponding to Couinaud segments 5-8, in order to obtain a graft for adult to adult living-related liver transplantation (ALRLT). Surgical complications were stratified according to the most recent version of the Clavien classification of postoperative surgical complications. The perioperative period was defined as within 90 d of surgery.

**RESULTS:** No living donor mortality was present in this series, no donor operation was aborted and no donors received any blood transfusion. Twenty-three (30.6%) living donors presented one or more episodes of complication in the perioperative period. Seven patients (9.33%) out of 75 developed biliary complications, which were the most common complications in our series.

## INTRODUCTION

Lortat-Jacob reported the first anatomic right hepatectomy in 1952<sup>[1]</sup>. Since then, and particularly in the past two decades, hepatic surgery has achieved important technical breakthroughs, such as intermittent portal triad clamping, total vascular exclusion, preoperative portal vein embolization with two-stage hepatectomy, and sophisticated methods of parenchymal transection.

An increased interest in the outcomes of right hepatectomy for adult to adult living-related liver transplantation (ALRLT) has likely contributed to these breakthroughs<sup>[2]</sup>.

Although surgical techniques of excellence and major improvements in perioperative management are now a

reality in referral centers for liver surgery, there are still several issues that make this major surgical procedure extremely worrisome when performed in healthy individuals, such as living donors.

In particular, there is still no definite consensus regarding the amount of liver that can be safely resected<sup>[3]</sup>, a crucial point for the recipient and perhaps more important for the healthy donor.

Recent studies have emphasized that in living-related liver transplantation, results and survival appear to correlate with stratification in the volume of the liver allograft transplanted, expressed either as a graft-to-body weight ratio or as a percentage of the standard liver volume of the recipient<sup>[4]</sup>.

Clearly, living-related liver transplantation (LRLT) represents the natural evolution of other surgical procedures, namely reduced-size liver transplantation and split-liver transplantation<sup>[5]</sup>, and is based on the segmental anatomy of the liver and on its peculiar capacity to regenerate.

This procedure represents a major challenge for the centers involved, though it has been widely reported that it is a valuable option for decreasing mortality rates and drop out from waiting lists<sup>[6,7]</sup>.

However, potential risk for the donor makes this procedure unique, and when complications in the healthy donor arise, the implications for the medical community are potentially devastating<sup>[8]</sup>. A recent systematic review<sup>[9]</sup> that focused on adult donor outcomes concluded that there are small but real risks when using the right lobe for living donors, though it also claimed that nearly all donors returned to normal function within 6 mo. Moreover, due to the short history of ALRLT, the long-term risks for the living donor are still largely unknown.

Numerous single-institution series have reported their complications for liver living-related donors<sup>[10-15]</sup> and a recent large study from the U.S. reported an analysis of administrative data on a group of 433 right- and left-lobe living donors identified as those at risk for perioperative complications<sup>[16]</sup>.

The ethical debate over the potential risk to the donor<sup>[17]</sup> renders this field of surgery controversial and, as a result, we believe, worthy of reports on all single center experiences.

The aim of this study is, in fact, to present an analysis of the surgical and perioperative complications in a series of seventy-five consecutive right hepatectomies for living related liver transplantation (RHLD) performed in our center.

## MATERIALS AND METHODS

From January 2002 to September 2007, we performed 75 RHLD, defined as removal of a portion of liver corresponding to Couinaud segments 5-8, in order to obtain a graft for ALRLT. Two left-hepatectomies, corresponding to Couinaud segments 2-4, were performed for the same purpose during the initial phase of our experience, but are not reported in this study. The number of cases per year has been progressively increasing, with

a peak reached in 2006, when 24 RHLD were performed. The trend has continued through this year (2007), with 17 RHLD performed so far. ALRLT represented the 20% of our total liver transplant activity over the last 2 years.

### Donor selection and characteristics

All living donors went through a complete evaluation process, managed by a multidisciplinary team consisting of clinical psychologists, hepatologists, anesthesiologists, transplant surgeons, referring physicians and family doctors. The evaluation process was completed in 3 d, with blood work, ultrasound and consults on the first day; Volumetric Angio computed tomography (CT) Scan and Cholangio nuclear magnetic resonance imaging (MRI) on the second day; and liver biopsy on the third day.

Initially, the work-up included endoscopic retrograde cholangiography; this has since been replaced by Cholangio MRI.

Beginning in 2002, a total of 254 potential living donors were evaluated; 165 (65%) were excluded, and of those accepted for living donation, 12 (5%) are still undergoing work-up. At first we were more restrictive; as a result, all patients with aberrant vascular or biliary anatomy, or steatosis greater than 10%, were rejected. Then 20% macrovesicular steatosis was categorized as the upper limit.

Of the 75 living donors accepted, the ages ranged between 18 and 54, and all were biologically or emotionally related to the recipients. There were 46 ABO identical couples and 29 compatible couples (Table 1). These demographic data are quite similar to those reported online by the European Liver Transplant Registry concerning the activity of 135 institutions in 35 European countries in the period 1991-2005.

The CT-scan-calculated graft to recipient body-weight ratio was always above 0.8%, and all anatomic anomalies of the vascular and biliary system were detected by preoperative imaging (Table 1).

Two anti-hepatitis B core positive donors were immediately accepted<sup>[6]</sup> in accordance with the far-eastern experience, and were transplanted in two recipients with end stage liver disease secondary to hepatitis C virus, treated after transplant with lamivudine 100 mg/d.

Seven other donors were initially excluded because of their elevated body weight, which was a body mass index (BMI) of > 30. After nutritional assessment (nutritional and dietary past history, and life-style evaluation) the dietician arranged a personal diet, moderately hypocaloric (carbohydrates 55%-57%, proteins 17%-19%, and lipids 24%-27%) and encouraged the donor to perform physical activity. Acceptable monthly weight loss was considered approximately 2-4 kg, with a final BMI of < 30 kg/m<sup>2</sup>. After 3 mo of a low calorie diet all seven living donors had a protocol liver biopsy that showed hepatic steatosis of < 20%, and were therefore considered eligible for donation.

Surgical complications were stratified according to the Clavien classification of postoperative surgical complications<sup>[18]</sup> (Table 2).

The perioperative period was defined as within 90 d

**Table 1** Demographic, anatomic and surgical characteristics of 75 RHL D (mean  $\pm$  SD)

Characteristics	n	Percent (%)
Age		32.27 $\pm$ 9.29
Range		[18;54]
Classes		
0-20	6	8
21-40	53	70.67
41-60	16	21.33
Sex		
Male	35	46.67
Female	40	53.33
Height (cm)		169.05 $\pm$ 8.86
Weight (kg)		68.19 $\pm$ 11.79
Donor relationship		
Biologically related	65	86.67
Sibling	10	13.33
Child	51	68.00
Parent	4	5.33
Not biologically related	10	13.33
Spouse	5	6.67
Other nonbiological	5	6.67
Donors		
ICU length of stay (d)		
Average		1.66
Range		[1;5]
Total length of stay (d)		
Average		8.26
Range		[6;14]
Length of donor surgery (h)		7.90 $\pm$ 1.75
Graft weight (g)		784.57 $\pm$ 158.15
GRBWR		1.43 $\pm$ 0.44
Bile ducts		
Double bile ducts	50	67
Single bile duct	25	33
Hepatic veins		
1	58	77
2	17	23
Hepatic arteries		
1	73	97
2	2	3
Portal veins		
1	65	87
2	10	13

GRBWR: Graft to recipient body weight ratio; ICU: Intensive care unit.

of surgery. Detailed descriptions of this surgical technique have been previously reported elsewhere<sup>[6,19]</sup>.

### Postoperative management and follow-up

After surgery, all donors were extubated before leaving the theater, and transferred to the intensive care unit (ICU) for at least 24-h monitoring. Deep venous thrombosis prophylaxis was based on early administration of low molecular heparin, started as soon as the prothrombin activity reached 50%, together with compression devices and early mobilization. Liver function tests were checked daily for at least 7 d, and then weekly for the first 2 mo. The subcutaneous administration of low molecular heparin was discontinued 14 d after surgery.

In order to guarantee optimal analgesia and early mobilization, all but two donors underwent epidural catheter placement immediately before surgery. Catheter removal was performed after 72 h, and after having normalized the coagulation parameters. Antimicrobial

prophylaxis changed over time: the first 13 donors received piperacillin tazobactam for the first 72 h, after which prophylaxis consisted of ceftriaxone.

A CT scan of the abdomen was performed 2 mo after surgery, with volumetric analysis of the liver.

Three months after surgery all the donors were seen at the outpatient clinic for check up.

### Statistical analysis

Data are expressed in mean  $\pm$  SD for continuous variables and as percentage for categorical variables. Data were compared with chi-square test or Fisher's exact test 2 tailed for categorical variables and Student's *t*-test for continuous variables; *P* < 0.05 were considered significant.

All statistical analyses were performed using SPSS (SPSS Inc., Chicago, Ill, United States).

## RESULTS

None of patients manifested any complications from pre-operative liver biopsy.

No living donor mortality was present in this series. No donor operation was aborted and no donors received any blood transfusion.

After the first 9 cases, we started to reinfuse the blood aspirated during surgery with the Cell Saver System (median: 250 mL; min: 0; max 1680).

Length of surgery, length of stay in the ICU, and total hospitalization are reported in Table 1, while all complications, codified according to the Clavien system, and their management, are reported in Table 2.

Twenty-three (30.6%) living donors presented one or more episodes of complication in the perioperative period. All these complications were resolved within the perioperative period.

Two donors (I.D. 1 and 12) had a small re-laparotomy because the intra-abdominal drain could not be removed.

One donor (I.D. 6) experienced a transient partial portal vein thrombosis, asymptotically detected by ultrasound and completely resolved with low molecular heparin.

Two donors (I.D. 17 and 18) developed complications graded IV by the Clavien system. They were both admitted to the ICU: in one case for monitoring of an acute pancreatitis following an endoscopic retrograde cholangiopancreatography (ERCP) performed because of a biliary leak, and in the second case for monitoring of a pulmonary embolism with no cardiac derangements.

Five patients (I.D. 13, 14, 17, 20, 21) presented complications that required multiple treatments: i.e. percutaneous drainage and stent placement.

Two patients (I.D. 19 and 21) presented two discrete, unrelated complications each: pleural effusion plus intra abdominal collection in one case and pleural effusion plus biliary leak in the other case.

Seven patients (9.33%) (I.D. 5, 9, 13, 14, 17, 20, 21) out of 75 developed biliary complications, which were the most common complications in our series. However, all of them were successfully treated by interventional procedures with removal of all stents or catheters within 6 mo

Table 2 Classification of surgical complications in RHL

Patients ID	Complications/Treatment	Classification of surgical complications Clavien annals of surgery 2004	Frequency (%) of complication for every classification grade
1	JP retained in the abdomen/Relaparotomy	Grade IIIb	13.04
2	Edema, ascites/None	Grade I	21.74
3	Prolonged hyperbilirubinemia/None	Grade I	
4	Fluid collection/Percutaneous drainage	Grade IIIa	17.39
5	Biliary leak /ERCP with stent placement	Grade IIIb, d	21.74
6	Transient portal vein thrombosis/Enoxaparin	Grade II	13.04
7	Bilateral massive pleural effusion/Percutaneous drainage	Grade IIIa	
8	Colitis by CD/Metronidazole	Grade II	
9	Biliary leak/ERCP (stent placement)	Grade IIIb, d	
10	Mild pleural effusion/None	Grade I	
11	Intraabdominal fluid collection/Percutaneous drainage	Grade IIIa	
12	JP retained in the abdomen/Relaparotomy	Grade IIIb	
13	Intraabdominal fluid collection; biliary leak/Percutaneous drainage; ERCP (sphincterotomy)	Grade IIIb	
14	Intraabdominal fluid collection; biliary leak/Percutaneous drainage; ERCP (sphincterotomy and stent placement)	Grade IIIb, d	
15	Intraabdominal fluid collection/Percutaneous drainage	Grade IIIa	
16	Prolonged hyperbilirubinemia/None	Grade I	
17	Intraabdominal fluid collection; Biliary leak/Percutaneous drainage, ERCP (sphincterotomy, stent placement X 3, acute pancreatitis, PTBD placement)	Grade IIIb, d/ Grade IV	4.35
18	Pulmonary embolism and iliac vein thrombosis/Anticoagulation	Grade IV	4.35
19	Moderate pleural effusion; intraabdominal fluid collection/Percutaneous drainage; percutaneous drainage	Grade IIIa- Grade IIIa, d	4.35
20	Biliary leak/ERCP (Sphincterotomy, endoscopic stent placement failure); PTBD	Grade IIIb, d	
21	Intraabdominal fluid collection; biliary leak, moderate pleural effusion/Percutaneous drainage; ERCP (sphincterotomy, endoscopic stent placement); percutaneous drainage	Grade IIIb, d	
22	Prolonged hyperbilirubinemia/None	Grade I	
23	Fever/Antibiotic treatment	Grade II	

Table 3 CT scan calculated donors mean liver volume (mean  $\pm$  SD)

Total liver volume	CT scan calculated	
	Right lobe volume	Remnant liver volume
1538.94 $\pm$ 277	954.67 $\pm$ 219.6	584.28 $\pm$ 121.67
	CT scan calculated right lobe volume 2 mo after surgery into the recipient	CT scan calculated remnant liver volume 2 mo after surgery
	1511.60 $\pm$ 257.88	1065.08 $\pm$ 195.24
	98% regeneration	69% regeneration

from surgery.

CT-scan-calculated donor mean total liver volume, mean right lobe volume, mean remnant liver volume, plus mean liver volume 2 mo after surgery in the donor and in the recipients, are reported in Table 3.

Mean value of donor liver volume was restored to 98% of the preoperative mean volume within 2 mo of surgery in the recipient and to 69% of the preoperative mean volume in the donor.

There was an 18% difference ( $P = 0.0001$ ) between CT-scan-calculated right lobe donor mean volume (954.67) and right lobe weight mean value (784.56) on the back table.

There were no differences in distribution of anatomical variations in the groups of complicated and uncomplicated RHL (Table 4). In addition, there were no differences between the complicated and uncomplicated RHL regarding the baseline and post regeneration mean value of calculated liver volumes (Table 5).

Table 4 Distribution of anatomic variations in the complicated and uncomplicated groups of RHL,  $n$  (%)

Anatomic variations	23 complicated RHL	54 RHL without complications	$P$ value
Double bile ducts	15 (65.21)	36 (66.67)	0.78
Single bile duct	8 (34.78)	18 (33.33)	
Hepatic veins			0.09
Single	21 (91.30)	39 (72.22)	
Double	2 (8.69)	15 (27.78)	0.82
Hepatic arteries			
Single	22 (95.65)	53 (98.15)	0.54
Double	1 (4.34)	2 (1.85)	
Portal veins			0.54
Single	20 (86.95)	46 (85.19)	
Double	3 (13.04)	8 (14.81)	

All patients returned to their own activity after this perioperative period.

## DISCUSSION

Donor safety has to be the first priority during the entire process of living-related transplantation, from the first day of evaluation through the entire follow-up period.

Therefore, an accurate and comprehensive step-by-step work-up protocol for donor evaluation has been designed in our center in order to ensure donor safety and, additionally, to confirm that the donor is capable of providing a suitable graft for the recipient.

In our experience, use of routine liver biopsy, though not generally accepted in all centers, allowed the exclu-



**Table 5** CT scan calculated donors mean liver volume in the complicated and uncomplicated groups of RHL D (mean  $\pm$  SD)

	23 complicated RHL D	54 RHL D without complications	P value
CT scan calculated total liver volume	1517.7 $\pm$ 292.4	1547.20 $\pm$ 292.43	0.68
CT scan calculated right lobe volume	957.67 $\pm$ 226.37	953.50 $\pm$ 219.16	0.94
CT scan calculated remnant liver volume	560.05 $\pm$ 89.19	593.70 $\pm$ 131.69	0.28
CT scan calculated remnant liver volume 2 mo after surgery	1078.6 $\pm$ 201.65	1059.57 $\pm$ 194.42	0.72

sion of potential donors who otherwise would have been considered fit to donate based on other tests<sup>[20]</sup>.

On the other hand, the biopsy allowed us to enroll donors who were anti-hepatitis B core positive.

Moreover, the routine use of liver biopsy as a screening tool in the living donor work-up allowed us to explore more safely the very common problem of steatosis.

The usefulness of steatotic livers depends on the percentage of fat, as livers with moderate to severe steatosis decrease graft and patient survival (with an additional unpredictable risk for liver donor regeneration). A BMI of  $> 30$  may reliably predict a higher degree of steatosis in most donors. In order to enlarge the pool of living donor livers, but also to improve post-transplant outcomes, we made an attempt to lower the percentage of steatosis, rather than to turn down such overweight donors, by applying a short-term treatment of diet and exercise in all living-donor candidates with hepatic steatosis. After RHL D, no such donors experienced life-threatening complications or died. No long-term clinical impairment of treated donors has been observed and, after donation, all of them have returned to previous activity.

A strategy of careful evaluation of the living donor performed by an interdisciplinary team cannot be over-emphasized.

A wide range of living donor complication rates are reported in the literature, with an estimated risk of mortality and morbidity after RHL D of 0.4% and 35%, respectively.

Overall, the complication rates range from 0% to 67%, with an overall crude complication rate of 31%<sup>[21]</sup>. The literature has reported 11 deaths, and 2 liver transplants in donors who have undergone RHL D. Additionally, one donor is in a persistent vegetative state after donation<sup>[22]</sup>.

Organ shortage is a dramatic problem which can be limited by the rational use of ALRLT. So, based on our previous experience with liver resection<sup>[2]</sup> and use of partial livers from deceased donors<sup>[23]</sup>, we began the living-related liver transplant program. Moreover, our partnership with one of the most active living-related liver transplant programs in the world<sup>[24]</sup> has allowed us to gain experience rapidly in this controversial field of surgery.

In our series, 30.6% of living donors developed a complication in the perioperative period, this not different from data recently reported in the literature<sup>[24]</sup>. In this group, RHL D with complications, there was no major incidence of anatomic variants, or difference in terms of liver regeneration after surgery, when compared with patients who did not develop any complications.

Additionally, our data regarding CT-scan-calculated liver volume confirmed that volumetric imaging may

overestimate the actual liver volume<sup>[24]</sup>.

Biliary complications (9.33%) were the most common complications after RHL D in our series, though no patients had to undergo repeated laparotomy for this reason. In two cases, after the failure of the endoscopic treatment, we were able to resolve the biliary leak due to a combined “rendezvous” procedure between endoscopist and interventional radiologist, who were able to pass an internal external transhepatic biliary drainage.

None of the 75 live donors in this series, regardless of their post-operative course, manifested any regrets about live donation.

In conclusion, this study reports the largest Italian experience with RHL D, focused on perioperative complications and on donor safety, which must be the first priority in right-lobe living-related donation.

Strict donor selection, detailed informed consent validated by the Italian law, together with a growing volume of cases performed every year, have allowed us to safely perform right hepatectomies for living donation in our center.

The need to define, categorize and record complications when healthy individuals, such as living donors, undergo a major surgical procedure like a right hepatectomy, reflects the need for prompt and detailed reports of complications arising in this particular category of patient.

Perioperative complications and post resection liver regeneration are not influenced by anatomic variations or patient demographic.

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## COMMENTS

### Background

Adult to Adult living related liver donors play an essential role in filling the gap of transplants needed due to a heavy shortage of cadaveric donations. Considering that living related donors are healthy individuals at baseline, it is imperative to ensure good outcomes and return to quality of life.

### Research frontiers

The study improved measures to assure safety in the healthy donor, improved overall diagnostic capability by non-invasive tools in the donor work-up, and provided possibility of expanding Milan Criteria for recipient of living-related liver transplantation (LRLT). It indicated improvements in prevention of biliary complications and small-for-size syndrome.

### Innovations and breakthroughs

Authors designed an accurate, comprehensive step-by-step work-up protocol for donor evaluation to ensure donor safety and to confirm that the donor is capable of providing a suitable graft. Their research has proven the necessity of evaluat-

ing the overall health of both the donor and recipient at many different levels from biopsy to body mass index. It indicated liver biopsy in the exclusion of potential donors otherwise considered fit to donate. These biopsies assess the quality of the donation to ensure the likelihood of success of the transplant and the health of both the donor and the recipient. These biopsies also confirm the true donor status of Hepatitis B, thereby allowing us to enroll donors who had false positive serum tests. They also test body mass index in order to prescribe a diet and exercise program to heavier donors to allow their inclusion. Their experience shows that heavier donors, when subjected to an exercise and diet program, all return to previous activity. In fact, no life threatening complications, long term impairments, or deaths have occurred in these donors. It indicated improvements in prevention of biliary complications and small-for-size syndrome.

### Applications

It would be applied in improving in prevention of biliary complications and measures to assure safety in the healthy donor.

### Peer review

This is an important issue that needs reporting. The authors performed a single institution series-report study of 75 patients stratifying them into two groups, complicated (23) and uncomplicated resections (54) to try to identify factors that might have influenced outcome. For this purposed, they analyzed anatomical variations and the liver remnant volume. With high wait list mortality and rather static donor levels, ALRLT is an option that needs serious consideration and historically the high rate of donor complications has held units back from moving forward with this procedure.

## REFERENCES

- Fortner JG, Blumgart LH. A historic perspective of liver surgery for tumors at the end of the millennium. *J Am Coll Surg* 2001; **193**: 210-222
- Gruttadauria S, Vasta F, Minervini MI, Piazza T, Arcadipane A, Marcos A, Gridelli B. Significance of the effective remnant liver volume in major hepatectomies. *Am Surg* 2005; **71**: 235-240
- Kadry Z, Furukawa H, Todo S, Clavien PA. Assessment of liver function and mass in cirrhotic and noncirrhotic livers. In: Clavien PA FY, Lysterly HK, Morse MA, Venook AP. *Malignant Liver Tumors: Current and Emerging Therapies*, 2nd ed. Sudbury: Jones and Bartlett, 2003: 73-78
- Kiuchi T, Kasahara M, Uryuhara K, Inomata Y, Uemoto S, Asonuma K, Egawa H, Fujita S, Hayashi M, Tanaka K. Impact of graft size mismatching on graft prognosis in liver transplantation from living donors. *Transplantation* 1999; **67**: 321-327
- Heffron TG, Gruttadauria S, Campi O, Cavanna JM. Surgical innovations in pediatric liver transplantation: reduced-size, split, and living-related transplantation. *Prob Gen Surg* 1998; **15**: 104
- Gruttadauria S, Marsh JW, Cintonino D, Biondo D, Luca A, Arcadipane A, Vizzini G, Volpes R, Marcos A, Gridelli B. Adult to adult living-related liver transplant: report on an initial experience in Italy. *Dig Liver Dis* 2007; **39**: 342-350
- Olthoff KM, Merion RM, Ghobrial RM, Abecassis MM, Fair JH, Fisher RA, Freise CE, Kam I, Pruett TL, Everhart JE, Hulbert-Shearon TE, Gillespie BW, Emond JC. Outcomes of 385 adult-to-adult living donor liver transplant recipients: a report from the A2ALL Consortium. *Ann Surg* 2005; **242**: 314-323, discussion 323-325
- Miller C, Florman S, Kim-Schluger L, Lento P, De La Garza J, Wu J, Xie B, Zhang W, Bottone E, Zhang D, Schwartz M. Fulminant and fatal gas gangrene of the stomach in a healthy live liver donor. *Liver Transpl* 2004; **10**: 1315-1319
- Middleton PF, Duffield M, Lynch SV, Padbury RT, House T, Stanton P, Verran D, Maddern G. Living donor liver transplantation--adult donor outcomes: a systematic review. *Liver Transpl* 2006; **12**: 24-30
- Beavers KL, Sandler RS, Fair JH, Johnson MW, Shrestha R. The living donor experience: donor health assessment and outcomes after living donor liver transplantation. *Liver Transpl* 2001; **7**: 943-947
- Broelsch CE, Malago M, Testa G, Valentin Gamazo C. Living donor liver transplantation in adults: outcome in Europe. *Liver Transpl* 2000; **6**: S64-S65
- Broering DC, Wilms C, Bok P, Fischer L, Mueller L, Hillert C, Lenk C, Kim JS, Sterneck M, Schulz KH, Krupski G, Nierhaus A, Ameis D, Burdelski M, Rogiers X. Evolution of donor morbidity in living related liver transplantation: a single-center analysis of 165 cases. *Ann Surg* 2004; **240**: 1013-1024; discussions 1024-1026
- Chan SC, Fan ST, Lo CM, Liu CL, Wong J. Toward current standards of donor right hepatectomy for adult-to-adult live donor liver transplantation through the experience of 200 cases. *Ann Surg* 2007; **245**: 110-117
- Fan ST, Lo CM, Liu CL, Yong BH, Chan JK, Ng IO. Safety of donors in live donor liver transplantation using right lobe grafts. *Arch Surg* 2000; **135**: 336-340
- Fujita S, Kim ID, Uryuhara K, Asonuma K, Egawa H, Kiuchi T, Hayashi M, Uemoto S, Inomata Y, Tanaka K. Hepatic grafts from live donors: donor morbidity for 470 cases of live donation. *Transpl Int* 2000; **13**: 333-339
- Patel S, Orloff M, Tsoulfas G, Kashyap R, Jain A, Bozorgzadeh A, Abt P. Living-donor liver transplantation in the United States: identifying donors at risk for perioperative complications. *Am J Transplant* 2007; **7**: 2344-2349
- Pomposelli JJ, Verbesey J, Simpson MA, Lewis WD, Gordon FD, Khettry U, Wald C, Ata S, Morin D, Garrigan K, Jenkins RL, Pomfret EA. Improved survival after live donor adult liver transplantation (LDALT) using right lobe grafts: program experience and lessons learned. *Am J Transplant* 2006; **6**: 589-598
- Dindo D, Demartines N, Clavien PA. Classification of surgical complications: a new proposal with evaluation in a cohort of 6336 patients and results of a survey. *Ann Surg* 2004; **240**: 205-213
- Gruttadauria S, Mandala L, Vasta F, Cintonino D, Musumeci A, Marsh W, Marcos A, Gridelli B. Improvements in hepatic parenchymal transection for living related liver donor. *Transplant Proc* 2005; **37**: 2589-2591
- Hasegawa Y, Kawachi S, Shimazu M, Hoshino K, Tanabe M, Fuchimoto Y, Obara H, Shinoda M, Shimizu H, Yamada Y, Akatsu T, Irie R, Sakamoto M, Morikawa Y, Kitajima M. Discontinuation of living donor liver transplantation for PSC due to histological abnormalities in intraoperative donor liver biopsy. *Am J Transplant* 2007; **7**: 2204-2207
- Surman OS. The ethics of partial-liver donation. *N Engl J Med* 2002; **346**: 1038
- Barr ML, Belghiti J, Villamil FG, Pomfret EA, Sutherland DS, Gruessner RW, Langnas AN, Delmonico FL. A report of the Vancouver Forum on the care of the live organ donor: lung, liver, pancreas, and intestine data and medical guidelines. *Transplantation* 2006; **81**: 1373-1385
- Gridelli B, Remuzzi G. Strategies for making more organs available for transplantation. *N Engl J Med* 2000; **343**: 404-410
- Tan HP, Patel-Tom K, Marcos A. Adult living donor liver transplantation: who is the ideal donor and recipient? *J Hepatol* 2005; **43**: 13-17

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# Treatment and survival in a population-based sample of patients diagnosed with gastroesophageal adenocarcinoma

Deirdre P Cronin-Fenton, Margaret M Mooney, Limin X Clegg, Linda C Harlan

Deirdre P Cronin-Fenton, Limin X Clegg, Surveillance Research Program, DCCPS, National Cancer Institute, Bethesda MD 20892-7344, United States

Deirdre P Cronin-Fenton, Department of Clinical Epidemiology, Aarhus University Hospital, Olof Palmes Alle 43-45, 8200 Aarhus N, Denmark

Margaret M Mooney, CTEP, DCTD, National Cancer Institute, Bethesda

Linda C Harlan, Applied Research Program, DCCPS, National Cancer Institute, Bethesda MD 20892-7344, United States

Limin X Clegg, Office of Healthcare Inspections, Office of Inspector General, Department of Veterans Affairs, Washington DC 20420, United States

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**Correspondence to:** Dr. Linda C Harlan, Applied Research Program, DCCPS, National Cancer Institute, 6130 Executive Blvd MSC 7344, Bethesda MD 20892-7344, United States. [lh50w@nih.gov](mailto:lh50w@nih.gov)

Telephone: +1-301-4967085 Fax: +1-301-4353710

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## Abstract

**AIM:** To examine the extent of use of specific therapies in clinical practice, and their relationship to therapies validated in clinical trials.

**METHODS:** The US National Cancer Institutes' Patterns of Care study was used to examine therapies and survival of patients diagnosed in 2001 with histologically-confirmed gastroesophageal adenocarcinoma ( $n = 1356$ ). The study re-abstracted data and verified therapy with treating physicians for a population-based stratified random sample.

**RESULTS:** Approximately 62% of patients had stomach adenocarcinoma (SAC), while 22% had gastric-cardia adenocarcinoma (GCA), and 16% lower esophageal adenocarcinoma (EAC). Stage IV/unstaged esophageal cancer patients were most likely and stage I - III stomach cancer patients least likely to receive chemotherapy as all or part of their therapy; gastric-cardia patients received chemotherapy at a

rate between these two. In multivariable analysis by anatomic site, patients 70 years and older were significantly less likely than younger patients to receive chemotherapy alone or chemoradiation for all three anatomic sites. Among esophageal and stomach cancer patients, receipt of chemotherapy was associated with lower mortality; but no association was found among gastric-cardia patients.

**CONCLUSION:** This study highlights the relatively low use of clinical trials-validated anti-cancer therapies in community practice. Use of chemotherapy-based treatment was associated with lower mortality, dependent on anatomic site. Findings suggest that physicians treat lower esophageal and SAC as two distinct entities, while gastric-cardia patients receive a mix of the treatment strategies employed for the two other sites.

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**Key words:** Adenocarcinoma; Esophageal adenocarcinoma; Gastroesophageal; Gastric adenocarcinoma; Survival; Chemotherapy; Radiotherapy

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## INTRODUCTION

The incidence and mortality of esophageal and gastric-cardia adenocarcinoma (GCA) has increased dramatically since the 1970s in western countries, while that of stomach cancer has decreased<sup>[1-3]</sup>. Gastroesophageal

adenocarcinomas have a poor prognosis<sup>[4-8]</sup>. However, numerous randomized clinical trials (RCTs) have evaluated, and continue to evaluate, the survival benefit of various treatment regimens.

Surgery remains standard care for early stage esophageal cancer. The MAGIC trial, a large phase III European RCTs found that patients with resectable lower esophageal or gastric adenocarcinomas treated with peri-operative chemotherapy had better progression-free and overall survival rates compared to surgery only<sup>[9]</sup>. This benefit was supported by the Fédérale Nationale des Centres de Lutte Contre Le Cancer (FNLCC) for patients with gastroesophageal adenocarcinoma who received pre-operative chemotherapy compared to surgery alone<sup>[10]</sup>. However, RCTs evaluating pre-operative chemoradiation compared to surgery alone have had conflicting results; some indicate better survival for esophageal cancer patients<sup>[11,12]</sup>. For locally advanced esophageal cancer, a phase III RCT, RTOG 85-01, demonstrated improved survival in patients who received chemoradiotherapy compared to radiation alone<sup>[13]</sup>, although most of these patients had squamous esophageal cancer. Another small RCT in patients with locally advanced esophageal cancer found chemoradiation superior to radiation alone<sup>[14]</sup>. These trials support the use of definitive chemoradiotherapy for locally advanced disease and its potential use for some patients with resectable disease. Current National Comprehensive Cancer Network (NCCN) guidelines for patients who are medically unfit for surgery or have unresectable disease recommend radiation and concurrent chemotherapy as treatment or best supportive care if patients cannot tolerate chemotherapy<sup>[15]</sup>.

Surgery is also the standard of care for early stage gastric cancer. A US Intergroup phase III trial, INT-0116, demonstrated post-surgical chemoradiation improved overall and disease-free survival in patients with stomach adenocarcinoma (SAC) and GCA<sup>[16]</sup>. The MAGIC and FNLCC trials also included patients with SAC and GCA. RCTs have also evaluated various chemotherapy treatments for patients with advanced or metastatic stomach and gastric-cardia cancer and have demonstrated improved survival for particular regimens<sup>[17,18]</sup>. The current NCCN guidelines for patients with metastatic stomach cancer recommend chemotherapy as treatment or best supportive care for those unable to tolerate chemotherapy<sup>[19]</sup>.

Few studies have examined community-based patterns of care for these cancers. A study on esophageal adenocarcinoma (EAC) and squamous cell carcinoma patients diagnosed between 1996 and 1999 found that chemoradiation was most frequently given although patients with chemoradiation followed by surgery had better survival compared to definitive chemoradiation<sup>[20]</sup>. Research suggests, however, that community-based use of treatment and the observed survival of patients in the community can vary depending on clinical and non-clinical factors<sup>[21-26]</sup>.

We present a population-based study, analyzing the receipt of various treatment strategies among a stratified

random sample of patients with gastroesophageal adenocarcinoma. This study aims to determine whether treatment strategies used in routine community practice are based on anatomic location or cancer origin and to examine community-based use of specific chemotherapy regimens, especially those evaluated in RCTs. Finally, we assess factors that influence treatment receipt and patient survival.

## MATERIALS AND METHODS

We included individuals aged  $\geq 20$ , newly diagnosed during 2001 with histologically-confirmed lower esophageal (EAC), GCA and SAC. Patients were ineligible if diagnosis was by death certificate only, autopsy, if they had a previous cancer diagnosis, other than non-melanoma skin, or were simultaneously diagnosed with another cancer. Patients were sampled from the National Cancer Institute's Surveillance, Epidemiology and End-Results Program (SEER) including Atlanta, Detroit, Seattle, New Mexico, Iowa, Louisiana, New Jersey, Connecticut, Utah, and California (Los Angeles County, San Francisco/Oakland, San Jose/Monterey, and greater California). Individuals were stratified by registry and race/ethnicity, and randomly sampled within strata. Non-Hispanic blacks, Hispanics, Asians/Pacific Islanders and Native Americans were over-sampled to obtain more stable estimates.

Data from medical records were re-abstracted to verify patient demographics, tumor characteristics, and treatment. Abstractors from each registry were centrally trained to ensure consistency of abstracting and coding. Because therapy is frequently provided in an outpatient setting, each patient's physician was contacted to verify treatment received, and provide names of any other physician who may have administered therapy. That physician was then contacted. All co-morbid conditions recorded at the hospitalization for most definitive treatment were abstracted. These were coded centrally by a single Registered Health Information Technologist and analyzed using the Charlson score<sup>[27]</sup>; an index of nineteen conditions, weighted according to the adjusted risk of one-year mortality.

We included 1411 cases. Patients were grouped by anatomic sites based on ICD-O2 codes; EAC (ICD-O: C15.2, C15.5,  $n = 165$ ), GCA (the portion of the stomach surrounding the gastroesophageal junction) (C16.0,  $n = 246$ ), and SAC (C16.1-C16.9,  $n = 1000$ ) and stage, I - III and IV/unstaged. Because of small numbers (18) of IVb EAC, these were grouped with IV/unstaged.

Treatments were defined as the receipt of surgery, radiotherapy, chemotherapy, in any combination. Non-adenocarcinoma cases were excluded from the therapy analyses ( $n = 55$ ); 1356 adenocarcinoma patients were included in the treatment analyses.

Data analyses were performed using Stata 8.0 and SUDAAN (Research Triangle Institute, Research Triangle Park, NC). Analyses were weighted to reflect the SEER population from which the sample was drawn. Multivariable analyses were conducted using logistic



and multinomial logistic regression. Cancer survival was analyzed using Cox regression models with a maximum two-year follow-up (through December 2003). All *P*-values were two-sided.

## RESULTS

Approximately 62% of patients had SAC, 22% GCA, and 16% lower EAC (Table 1). Median age was highest (76 years) for stage I-III SAC patients and lowest (67 years) for stage IV/unstaged GCA (data not shown).

### Lower EAC

Over 12% of stage I-III patients with EAC received surgery alone (Table 2). About 27% of patients with stage I-III EAC received tri-modality therapy (surgery, radiation and chemotherapy), while 36.5% of these patients received chemotherapy plus radiation therapy with no surgery. One-quarter of stage I-III EAC patients received no chemotherapy. The most frequently administered agent was 5-FU, frequently with cisplatin. Few patients with late/unstaged EAC received surgery, in any combination. Chemoradiotherapy, however, was given to nearly 47% of these patients. In multinomial logistic regression, age  $\geq 70$  was associated a 70%-80% decreased use of chemotherapy and chemoradiation in patients with EAC (Table 3).

Non-Hispanic blacks and Asian/Pacific Islanders with EAC had significantly higher hazards of cancer deaths than non-Hispanic white patients (Table 4). Patients age  $< 70$  with a Charlson score of  $\geq 1$  had a significantly increased risk as did those with late/unstaged disease. EAC patients who received chemotherapy had better survival, although not statistically significant. In a separate model, EAC patients who received chemoradiation had decreased hazards (HR = 0.69, 95% CI = 0.43-1.06 model not presented). The prognostic factors in the Cox proportional hazards model containing chemoradiation were otherwise the same as those significantly associated with death in the model which adjusted for chemotherapy.

### GCA

Patients with GCA received therapies at a rate between that of EAC and SAC patients. Surgery alone was provided to 34% of stage I-III GCA patients (Table 2). One-quarter of stage I-III GCA patients received trimodal therapy. Nearly twice as many GCA patients as EAC patients but less GCA than SAC patients received no chemotherapy. Fewer patients received chemoradiotherapy compared to EAC patients. 5-FU was the most frequently used chemotherapeutic agent. Nearly twice as many late/unstaged GCA patients as EAC received no therapy. In multinomial logistic regression, age  $\geq 70$  was associated with a 70%-80% decrease in chemotherapy alone or chemoradiotherapy (Table 3). Women and patients with a Charlson Score of  $\geq 1$  were significantly less likely to receive chemotherapy, but not chemoradiation. In the Cox proportional hazards models patients, with late/unstaged disease or

poorly/undifferentiated tumors had an increased risk of cancer deaths while married individuals had a decreased risk (Table 4).

### SAC

Of the three anatomic sites, patients with SAC were most likely to receive surgery alone (Table 2). Nearly 50% of stage I-III SAC patients received surgery alone. Less than 20% of stage I-III SAC patients received trimodal therapy. Fewer SAC patients than EAC or GCA patients received chemotherapy. As with the other two anatomic sites, 5-FU was most frequently administered. Of the three anatomic sites, late/unstaged SAC patients received no definitive cancer treatment most often. In multinomial regression, age  $\geq 70$  was associated with 80% less chemotherapy alone or chemoradiation (Table 3). Late/unstaged disease was associated with decreased use of chemoradiation but a substantial increased use of chemotherapy alone. Proportional hazards models for cancer deaths showed that in non-surgical patients, late/unstaged disease or a poor/undifferentiated tumor was associated with increased risk of cancer death (Table 4). However, patients receiving chemotherapy had a significantly decreased risk. Among surgical patients, a Charlson Score of  $\geq 1$ , regardless of age and having late/unstaged disease was associated with increased hazards. Lower risks were seen among Asian/Pacific Islanders, and a non-significant decreased risk among patients who received chemotherapy (Table 4). Patients who received chemoradiation had a statistically significant decreased risk both with (HR = 0.56, 95% CI = 0.35-0.89) and without surgery (HR = 0.62, 95% CI = 0.43-0.92) (model not presented) but all other prognostic factors had similar associations with hazard ratios as the Cox models which adjusted for chemotherapy.

## DISCUSSION

RCTs have demonstrated that certain treatment strategies and regimens improve survival for patients with esophageal and gastric cardia adenocarcinoma. Variation in gastroesophageal cancer survival, however, has sometimes been attributed to case mix<sup>[28]</sup>. We therefore selected adenocarcinoma cases only and categorized patients by anatomic site to assess rates of treatment and survival among a population-based sample of patients treated in the community. We found significant differences in treatment and survival by anatomic site, stage, age, and race/ethnicity. This study highlights the considerably varied approach that community physicians take to treat adenocarcinomas at each anatomic site.

### Lower EAC

While there is no consensus definition of the optimal therapy for patients with resectable EAC, clinical trials have indicated survival improvements when surgery is supplemented with additional therapies. Of the three cancer sites investigated in the current study, stage I-III EAC patients had the lowest rates of surgery alone

**Table 1** Percentage distribution (weighted for the sampling fraction) of clinical and non-clinical characteristics for gastroesophageal cancer patients diagnosed in 2001 NCI: Patterns of care study ( $n = 1411$ ) (Wt%)

	Lower esophagus		Gastric cardia		Stomach	
	I-III ( $n = 86$ )	IV-V ( $n = 79$ )	I-III ( $n = 119$ )	IV-V ( $n = 127$ )	I-III ( $n = 491$ )	IV-V ( $n = 509$ )
Age						
< 70	57.5	46.4	57.2	54.0	36.7	42.4
≥ 70	42.5	53.6	42.8	46.0	63.3	57.6
Marital status						
Other	39.9	43.6	37.9	41.0	46.4	48.9
Married	60.1	56.4	62.1	59.0	53.6	51.1
Race						
NH White	93.9	87.8	78.0	75.1	51.0	47.9
NH Black	1.0	3.3	4.3	5.8	13.4	14.2
Hispanic	4.1	7.9	11.6	12.0	15.8	20.5
A/PI	1.0	1.0	6.2	6.8	19.1	17.0
NA/AI	0.0	0.0	0.0	0.4	0.6	0.3
Charlson score						
Zero	72.3	81.0	77.7	86.5	79.5	78.9
1+	27.7	19.0	22.3	13.5	20.5	21.1
Vital status Dec 2003						
Deceased	69.6	88.8	70.0	88.3	53.9	89.9
Histology						
Adeno, NOS	92.8	81.0	79.5	70.4	50.4	50.7
A. intestinal	0.0	3.7	9.1	4.1	14.6	7.2
A. diffuse	0.0	0.0	1.8	4.4	4.0	3.0
Signet	6.0	12.0	8.0	18.9	20.6	28.9
Mucinous	1.2	2.4	0.9	1.0	5.5	5.5
Papillary	0.0	0.9	0.7	0.0	0.9	1.5
Tubular	0.0	0.0	0.0	1.0	2.2	0.6
Linitis plastica	0.0	0.0	0.0	0.2	1.8	2.6
Linitis plastica/Signet ring						
Linitis plastica	3.9	0.0	0.0	4.1	5.3	7.2
Signet	13.9	13.1	15.2	22.4	24.4	33.6
No mention	81.6	86.9	78.3	71.3	69.5	58.5
Unknown	0.6	0.0	6.5	2.2	0.8	0.7
Intestinal metaplasia in resected tumor						
None	31.3	28.5	36.7	17.9	25.4	24.3
Metaplasia	7.9	3.0	18.4	5.9	31.2	9.6
No mention	36.6	29.9	30.6	37.5	30.1	28.0
Unknown	24.2	38.6	14.3	38.8	13.3	38.1
Grade						
Well differentiated	4.1	12.0	2.9	4.9	6.9	2.3
Moderate	18.9	39.6	31.3	33.4	28.1	19.4
Poor/Undif	57.1	28.5	59.0	51.3	56.5	60.7
Unknown	19.8	20.0	6.8	10.4	8.4	17.7
Barrett's esophagus						
No	21.3	27.7	39.1	19.9	43.4	28.4
Yes	33.2	9.3	11.0	3.8	0.0	0.6
Other	3.9	1.1	1.3	0.0	0.0	0.0
No mention	16.8	21.8	27.8	35.2	43.7	31.5
Unknown	24.8	40.1	20.7	41.1	12.9	39.5
History of Barrett's						
No history	19.3	22.6	27.5	25.6	29.4	29.4
History	22.7	14.0	6.0	8.7	1.7	0.5
No mention	54.5	60.0	59.5	61.8	67.2	67.9
Unknown	3.4	3.4	7.0	3.9	1.8	2.1
<i>H. pylori</i>						
Negative	22.6	39.2	32.3	29.6	37.6	33.1
Positive	2.1	13.5	10.0	15.9	18.6	15.9
No mention	73.3	41.9	51.1	52.6	41.4	49.0
Pernicious anemia						
No history	25.9	33.1	26.9	31.5	31.1	28.7
Pernicious	0.6	0.0	8.2	0.4	6.1	3.9
Anemia						
No mention	71.5	62.0	60.4	65.4	59.6	64.9
Unknown	2.0	4.9	4.5	2.7	3.2	2.6

History of ulcers						
No history	23.5	32.6	28.2	29.5	19.6	23.1
Peptic ulcers, NOS	7.9	11.6	5.8	4.7	12	10.5
Duod/pyloric ulcer	2.1	1.7	0.4	1.3	1.1	2.9
Gastric ulcer	4.3	0	8.6	9.6	22.7	13.3
Other	6.5	7.1	0.4	1.4	0.9	0.8
No mention	55.1	44.7	50.1	51	40.6	46.9
Unknown	0.6	2.4	6.6	2.4	3.1	2.5

American Indians/Native American are included in Table 1 for completeness of reporting. Histology groupings were created according to the following: Adenocarcinoma-NOS = 8140, 8210, 8255, 8261 ( $n = 842$ ), Adeno-Intestinal = 8144 ( $n = 122$ ), Adeno-Diffuse = 8145 ( $n = 45$ ), Signet Cell = 8490 ( $n = 295$ ), Mucinous/mucin-producing = 8480 + 8481 ( $n = 48$ ), Papillary/Serous = 8260 + 8460 + 8461 ( $n = 12$ ), Tubular = 8211 ( $n = 17$ ), Linitis plastica = 8142 ( $n = 23$ ).

**Table 2** Percentage distribution (weighted for the sampling fraction) of treatment characteristics and survival for gastroesophageal adenocarcinoma patients diagnosed in 2001 NCI: Patterns of care study ( $n = 1356$ )<sup>1</sup> (Wt%)

	Lower esophagus		Gastric cardia		Stomach	
	I-III	IV-V	I-III	IV-V	I-III	IV-V
Therapy received						
Surgery only	12.3	1.3	34	2.2	49.6	15.7
Radiation only	8.1	3.5	4.7	7.2	2	3.3
Chemotherapy only	0.5	24	1.2	21.9	3.6	22.1
Surgery and radiation	0.8	0	2.6	0.9	3.9	0.4
Surgery and chemo	2.5	1.3	8.6	4.8	3.2	5.6
Surgery, rad, chemo	27	4.6	25	11.2	19.2	6.1
Chemo and radiation	36.5	46.8	9.8	12.4	1.5	3.4
None	12.4	18.7	14.1	39.4	17.1	43.4
Chemotherapy						
No chemo	25.1	21.5	48.7	40.8	64.9	54.5
Single agent	5.4	25.5	11.4	9.9	10.3	8.4
Multi-agent	61.1	50.9	31.4	39.6	17.1	29
Refused	5.9	1.5	4.3	7.2	4	4.3
Rec, unknown if given	1.9	0	1	2.1	1.8	2.3
Unknown	0.6	0.6	3.2	0.4	2	1.5
Chemotherapy agent						
5-FU	58.6	37.9	35.7	34.7	23.2	26.4
Doxorubicin	0.5	0	0.3	1	0.5	3.4
Capecitabine	1.2	0	0.7	7.4	0.6	4
Cisplatin	38.7	37.2	22	17.2	2.8	10.8
Etoposide	1	0	0.7	10.7	1.4	5.8
Irinotecan	0.7	12.9	0.5	5.9	1.6	5.6
Leucovorin	4.1	0.9	6.1	21.8	12.1	15.1
Mitomycin-C	13.7	4.3	0.5	0.6	0.2	1.8
Oxaliplatin		No one				
Epirubicin	0	0	0	1	0	1.2
Paclitaxel	11.8	20.8	5.9	7.3	2.2	4.6
Docetaxel	0	1.6	0.7	2.1	0.6	1.4
Chemotherapy plus surgery (with or without radiation)						
No	70.5	94.2	68.3	84	77.7	87.8
Pre-op	23.7	4.9	16.8	6.4	0.3	0.5
Post-op	5.8	0.9	9	8.5	20.3	11.2
Unknown	0	0	5.9	1.1	1.7	0
Surgery plus chemotherapy and radiation						
No	73	95.4	75	88.5	80.9	93.9
Pre-op	21.9	4.6	13.4	4.3	0.2	0.5
Post-op	5.1	0	8.6	6.9	17.4	5.6
Unknown	0	0	3	0	1.5	0
Median survival time (mo)						
Non-surgical pts	13	8	8	6	5	4
Surgical patients	22	13	19	13	26	6

<sup>1</sup>13 American Indians/Native Americans excluded.

**Table 3** Therapy among patients with gastroesophageal adenocarcinoma by anatomic site, 2001: Multinomial logistic regression for the receipt of chemoradiation (Chemo + RT) and chemotherapy alone

Site Characteristic	Lower esophagus					Gastric-cardia					Stomach				
	Chemo + RT		Chemo		P	Chemo + RT		Chemo		P	Chemo + RT		Chemo		P
	OR	95% CI	OR	95% CI		OR	95% CI	OR	95% CI		OR	95% CI	OR	95% CI	
Age					0.05					< 0.001					< 0.001
< 70	1		1			1		1			1		1		
≥ 70	0.3	0.1-0.96	0.2	0.03-0.9		0.2	0.1-0.4	0.3	0.1-0.7		0.2	0.1-0.4	0.2	0.1-0.3	
Race/ethnicity					0.33					0.64					0.24
Non-hispanic white	1		1			1		1			1		1		
Non-hispanic black	1.1	0.1-10.2	0.9	0.1-14.5		0.5	0.1-1.7	1	0.2-6.7		1.3	0.6-2.5	0.6	0.3-1.0	
Hispanic	0.1	0.01-0.9	0.3	0.02-3.6		0.4	0.1-1.6	1.6	0.3-7.4		1.2	0.6-2.5	0.6	0.3-1.4	
Asian/Pacific Islander						0.5	0.1-1.9	1.4	0.4-4.9		1.7	0.9-3.4	0.8	0.4-1.4	
Gender					0.32					0.01					0.64
Male	1		1			1		1			1		1		
Female	1.2	0.5-3.3	0.3	0.04-2.1		1.2	0.4-3.3	0.3	0.1-0.8		1.3	0.8-2.1	1.1	0.7-1.8	
Marital status					0.28					0.86					0.74
Not married	1		1			1		1			1		1		
Married	2.1	0.7-6.5	1	0.3-3.9		1	0.4-2.6	1.4	0.4-4.6		1.2	0.7-2.0	1.2	0.7-1.9	
Stage					0.12					0.01					< 0.001
I -III	1		1			1		1			1		1		
IV & unknown	1	0.4-2.7	3.6	0.9-13.6		0.7	0.3-1.7	3.8	1.3-11.4		0.6	0.4-0.9	5.2	3.0-8.8	
Differentiation grade					0.57					0.36					0.38
Well/Moderately differentiated	1		1			1		1			1		1		
Poorly/Undifferentiated	1.2	0.4-3.6	0.9	0.2-3.7		0.8	0.3-1.9	1	0.3-3.1		1.3	0.7-2.2	1.3	0.7-2.5	
Unknown	0.6	0.2-1.9	0.2	0.03-1.4		1.5	0.3-7.3	0.2	0.03-1.8		0.7	0.3-2.0	1.6	0.8-3.5	
Charlson score					0.25					0.02					0.35
0	1		1			1		1			1		1		
1+	2	0.6-6.7	0.6	0.1-3.1		0.4	0.1-1.3	0.1	0.02-0.6		0.7	0.4-1.2	0.9	0.5-1.7	
<i>H. pylori</i>					0.02					0.004					0.65
No	1		1			1		1			1		1		
Yes	0.1	0.02-0.8	0.4	0.03-5.6		1	0.2-4.2	0.4	0.1-2.1		1.2	0.6-2.5	1.3	0.6-2.8	
Unknown	0.1	0.03-0.4	0.2	0.03-0.8		0.2	0.1-0.7	0.8	0.3-2.3		1	0.6-1.7	1.5	0.9-2.5	

Model also adjusted for registry.

as their primary treatment, but highest rates of pre-operative chemotherapy and chemoradiation as well as definitive chemoradiation. This may reflect the significant morbidity associated with esophageal surgery<sup>[29-32]</sup>. However, toxicities associated with pre-operative chemotherapy or chemoradiation can preclude a patient from further treatment<sup>[33]</sup>.

RCTs and a meta-analysis have suggested a survival benefit associated with pre-operative and adjuvant chemoradiation compared to surgery alone<sup>[11,33-37]</sup>. The US-Intergroup trial, CALGB-9781, closed early due to poor accrual, but an intent-to-treat analysis on the 56 enrolled patients, demonstrated better median survival in favor of trimodal therapy<sup>[12]</sup>. In our study, over a quarter of stage I -III EAC patients received trimodality therapy. The MAGIC and FNLCC phase III trials support the use of perioperative or preoperative chemotherapy; however, we found that few (2.5%) stage I -III EAC patients received surgery and chemotherapy as primary treatment. Chemoradiation was the treatment strategy received by the largest percentage of patients with stage I -III EAC (36.5%) and late/unstaged EAC patients (47%).

## GCA

Optimal therapy for GCA is not clear. Most RCTs have included patients with this cancer in trials conducted for either or both of the other two anatomic sites<sup>[18,38]</sup>.

Reflective of this, we found that GCA patients seemed to receive treatment at a rate that fell midway between the other two anatomic sites. In the current population-based study, stage I -III GCA patients were most frequently treated with surgery alone (34%) or trimodal therapy (22%). For late/unstaged disease less than 25% received chemotherapy alone and a significant percentage received no therapy (39%).

## SAC

In contrast to EAC and GCA patients, SAC patients received surgery alone most frequently (50% of stage I -III and 16% of stage IV/unstaged disease) and radiotherapy and chemotherapy less frequently than the other two anatomic sites. Although the MAGIC and FNLCC trials demonstrated a survival advantage for patients with gastroesophageal adenocarcinoma<sup>[9]</sup>, this was not evident in the current population-based study, where less than 20% of stage I -III SAC patients received chemoradiation with surgery.

With respect to advanced disease, several RCTs for SAC have demonstrated survival benefits for chemotherapy compared to best supportive care for stage IV (late-stage) disease<sup>[17]</sup>. However, we found that only 22% of patients with late/unstaged SAC received chemotherapy alone, with an additional 15% receiving chemotherapy with surgery, with surgery and radiation, or as chemoradiation. Furthermore, for late/

**Table 4** Cox proportional hazards model for cancer death among lower esophageal and GCA patients overall (Model 1) and among SAC patients who did or did not receive surgery (Model 2)

Characteristic	Model 1						Model 2					
	Lower esophagus (n = 164)			Gastric-cardia (n = 241)			Stomach					
	With & Without surgery			With & Without surgery			No surgery (n = 461)			With surgery (n = 490)		
	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P	HR	95% CI	P
Age & co-morbidity			0.02			0.44			0.97			0.01
< 70, Charlson score = 0	1			1			1			1		
< 70, Charlson score = 1	2.7	1.4-5.2		1	0.4-2.1		0.9	0.6-1.4		2	1.1-3.7	
70+, Charlson score = 0	1.5	0.9-2.6		1.4	0.9-2.3		0.9	0.6-1.4		1.5	0.9-2.5	
70+, Charlson score = 1	1.5	0.7-3.3		1.1	0.5-2.4		1	0.6-1.6		2.4	1.4-4.0	
Race			< 0.001			0.08			0.39			0.003
Non-Hispanic White	1			1			1			1		
Non-Hispanic Black	3.4	1.8-6.7		0.8	0.5-1.2		1.1	0.8-1.5		1.1	0.6-1.7	
Hispanic	1	0.5-2.0		1.3	0.8-2.2		0.8	0.5-1.2		1.3	0.8-2.1	
Asian/Pacific Islander	6.4	2.9-14.4		0.4	0.2-1.0		0.9	0.6-1.2		0.5	0.3-0.9	
Gender			0.78			0.87			0.84			0.18
Male	1			1			1			1		
Female	0.9	0.6-1.5		1	0.6-1.4		1	0.8-1.4		0.8	0.5-1.1	
Marital Status			0.43			0.01			0.39			0.33
Not married	1			1			1			1		
Married	0.8	0.5-1.3		0.6	0.4-0.9		1.2	0.8-1.6		1.2	0.8-1.9	
Stage			< 0.001			< 0.001			0.004			< 0.001
Stage I - III	1			1			1			1		
Stage IV & unknown	2.5	1.5-4.2		2.6	1.6-4.0		1.7	1.2-2.3		5	3.4-7.4	
Differentiation grade			0.11			0.049			0.01			0.14
Well/Moderately	1			1			1			1		
Poorly/Undifferentiated	1.8	1.0-3.3		1.7	1.1-2.6		1.8	1.2-2.6		1.4	0.9-2.2	
Unknown	1.7	0.9-3.2		1.3	0.6-2.8		1.5	1.0-2.3		0.7	0.3-1.7	
Chemotherapy			0.09			0.68			< 0.001			0.12
No	1			1			1			1		
Yes	0.6	0.4-1.1		1.1	0.7-1.8		0.6	0.4-0.8		0.7	0.5-1.1	

unstaged disease, SAC patients received no therapy most frequently (43%).

Overall, our results do suggest that RCT-validated therapies have been incorporated into community practice, albeit at low levels. However, a significant percentage of patients, especially those with stage IV/ unstaged disease, across all anatomic sites received no cancer-directed therapy. Our findings also highlight that the sequence and combination of chemotherapy, radiotherapy and surgery in the adjuvant setting was distinct for each anatomic site. For example, of stage I - III EAC patients who received surgery plus chemotherapy and radiation, 81% received this therapy pre-operatively [most frequently with 5-fluorouracil (5-FU) and cisplatin], while of stage I - III SAC patients who received surgery plus chemotherapy and radiation, 91% received this therapy post-operatively (most frequently with 5-FU and leucovorin). These sequences of therapy as well as the chemotherapeutic agents selected were also consistent with RCTs conducted in these disease sites<sup>[11]</sup>.

### Chemotherapeutic agents

Overall, the most frequently administered chemotherapeutic agents in our study were 5-FU, cisplatin, and leucovorin. Newer agents (paclitaxel, irinotecan) have been investigated in phase II trials<sup>[39,40]</sup> for use in EAC patients. We found that these drugs were used in community practice (Table 2). Use of these compounds was much lower among patients with SAC and GCA cancers. No

patients received oxaliplatin, possibly because these cases were diagnosed in 2001 and findings advocating oxaliplatin for esophageal cancer were only presented in 2006<sup>[38]</sup>. Specific chemotherapeutic agents used alone or in combination with surgery and radiation are listed in Table 5. Whether patients received chemotherapy alone, chemoradiation, or trimodal therapy, the majority of patients received 5-FU in combination with another chemotherapeutic agent.

### Age disparities

Less frequent treatment of elderly patients has been widely reported<sup>[21-25,41]</sup>. Sabel *et al*<sup>[31]</sup> reported that 50% of patients age < 70 and 32% of those age ≥ 70 were suitable for surgery at diagnosis. Similar to this, we found that in stage I - III EAC, 30% of patients aged ≥ 70 compared to 51% of those age < 70 underwent cancer-directed surgery and 56% of gastric-cardia patients aged ≥ 70 compared to 80% of those age < 70. The age-related treatment decline is likely attributable to a number of factors: (1) Potentially higher morbidity among elderly patients; (2) Compromised treatment options due to delayed presentation by elderly patients; (3) Increased anesthesiological risk<sup>[31,42]</sup>; and (4) A higher prevalence of co-morbidities.

Median age at diagnosis for stomach cancer is approximately 70 years<sup>[43]</sup>, an age when patients have a reasonable life-expectancy<sup>[43]</sup>. Selected medically-fit elderly patients do as well as younger patients after surgical or adjuvant therapy<sup>[44,45]</sup>. Our models indicate



**Table 5** Percentage distribution (weighted for the sampling fraction) of chemotherapy agents by selected therapeutic combinations gastroesophageal adenocarcinoma patients diagnosed in 2001; NCI: Patterns of care study ( $n = 1356$ )<sup>1</sup> (Wt%)

	Lower esophagus		Gastric-cardia		Stomach	
	I-III	IV-V	I-III	IV-V	I-III	IV-V
Chemotherapy only						
Etoposide + Doxorubicin + Cisplatin	1 patient	0	0	0	0	0.6
5-FU only	0	3.2	0	3.3	15.9	5.1
Mitomycin only	0	16.7	0	0	0	0
Paclitaxel only	0	2.1	19.2	3.3	0	0
Capecitabine only	0	0	0	0	0	4.4
Gemcitabine only	0	0	0	3.1	0	1.2
5-FU + 1 agent	0	18	56	16.6	25.5	18.8
5-FU + 2 agents	0	4	24.8	40	26.6	23.3
5-FU + 3 agents	0	0	0	7.9	0	15.6
5-FU + 4 agents	0	0	0	2.6	0	2.2
Irinotecan + Paclitaxel	0	20.6	0	1.2	0	0.6
Irinotecan + Cisplatin	0	15.4	0	1.7	0	8.4
Irinotecan + Cisplatin + Paclitaxel	0	0	0	0	20.4	0
Other	0	20	0	20	11.7	19.7
Radiation and chemotherapy						
5-FU only	2.1	9.2	34.8	29.2	5.6	20.6
Cisplatin only	0	17.1	0	3.3	0	0
5-FU + Leucovorin	0	1.2	0	0	0	14.3
5-FU + Cisplatin	50.6	28	61.5	32.6	7.9	0
5-FU + Mitomycin	25.1	0.7	0	0	0	0
5-FU + Irinotecan	0	0	0	0	30.1	0
Paclitaxel + Carboplatin	0	13.6	0	4.7	0	0
Chemo, NOS	4.6	8.9	0	7.5	20	13.1
Other	17.6	21.3	3.7	22.7	36.4	52
Surgery, radiation & chemotherapy						
5-FU only	5	0	9.4	0	32.4	26.5
5-FU + Leucovorin	13.3	0	17.6	31.7	46.5	40.9
5-FU + Cisplatin	41.1	15.6	27.7	7.3	2.3	0
5-FU + Paclitaxel + Carboplatin	15.3	44.6	1.6	0	0.7	0
Other agents/Combos	25.3	39.8	43.7	61	18.1	32.6

<sup>1</sup>13 American Indians/ Native Americans excluded; NOS: Not otherwise specified.

that in EAC or GCA patients, being age < 70 with a Charlson score of 1+ was significantly and inversely associated with surgery (data not shown). Older patients ( $\geq 70$ ) were also less likely to have surgery even with a Charlson score of 0. In our multinomial models, being age  $\geq 70$  was associated with a decreased use of chemotherapy or chemoradiation even after adjusting for co-morbid conditions. Although we saw this disparity in the use of chemotherapy or chemoradiation by age group, there was no evidence of treatment-related differences by racial/ethnic groups. This suggests that in a community-based setting, age, in addition to co-morbidity, influences whether or not a patient receives surgery, chemotherapy, and chemoradiation.

### Survival

Survival from gastric adenocarcinoma is extremely poor<sup>[46]</sup>. Theuer *et al.*<sup>[46]</sup> reported that patients aged  $\geq 70$  had higher risk of death even after adjusting for clinical, non-clinical and treatment-related factors. In contrast, we observed that in GCA and non-surgical SAC patients,

age and co-morbidity were not significant predictors of survival, perhaps due to the poor prognosis for these patients. We noted higher mortality in non-Hispanic blacks and Asian/Pacific Islanders with EAC. Such racial disparities have been reported in other cancers<sup>[47,48]</sup>, however the underlying cause for this poorer survival is not clear.

A US-based survey of 59 radiotherapy facilities indicated improved survival associated with pre-operative chemoradiation for patients with esophageal cancer<sup>[20]</sup>. In the current study, chemotherapy and chemoradiation were associated with decreased mortality for SAC and EAC patients, although not GCA patients. This suggests that the receipt of chemotherapy and/or radiotherapy may improve outcome in these poor prognosis cancers. This analysis was not a randomized study of therapy and although we adjusted for Charlson comorbidity score and additional potential confounders, patients who had better baseline health and who were selected for chemotherapy, may have been more likely to respond to such treatment or may have had better survival regardless of the use of chemotherapy.

In conclusion, RCTs have demonstrated that specific treatment strategies prolong survival in certain patient groups. We note that the use of these therapies was very low in US community-based practice despite their demonstrated survival benefits. Our study shows lower mortality among patients with EAC and SAC who received chemotherapy and significant disparities in terms of age in treatment receipt. Our findings highlight the distinctly individualized approach taken by community physicians in treating adenocarcinoma at these three anatomic sites. Community physicians appear to differentiate gastroesophageal adenocarcinoma as two distinct entities (i.e. EAC and SAC) and use different treatment strategies and chemotherapeutic agents for each, while patients with GCA are treated with a mixture of those employed for the other two anatomic sites. Improvements in community-based treatment of gastroesophageal adenocarcinoma will require better differentiation of treatments for the different anatomic sites and more extensive incorporation of those treatments proven effective in clinical trials. Future RCTs should be designed and appropriately powered to account for differences related to the anatomic site or origin of the tumor as well as the underlying tumor biology.

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## COMMENTS

### Background

Randomized clinical trials (RCTs) have demonstrated that specific treatment strategies prolong survival in certain patient groups with gastric, gastroesophageal and esophageal adenocarcinomas. However, the extent of use of these treatments in routine clinical practice is not clear. Research suggests that community-based use of treatment and the observed survival of patients in the

community can vary depending on clinical and non-clinical factors.

### Research frontiers

To determine whether treatment strategies used in routine community practice are based on anatomic location or cancer origin. To examine community-based use of specific chemotherapy regimens especially those evaluated in RCTs. To assess factors that influence treatment receipt and patient survival.

### Innovations and breakthroughs

We document relatively low community-based use of treatments tested in RCTs in patients with gastroesophageal adenocarcinoma. The use of these therapies was very low despite their demonstrated survival benefits. Our study shows lower mortality among patients with esophageal adenocarcinoma (EAC) and stomach adenocarcinoma (SAC) who received chemotherapy and significant disparities in terms of age in treatment receipt. Community physicians appear to take an individualized approach in treating adenocarcinoma at these three anatomic sites; differentiating between gastric and EAC and using different treatment strategies and chemotherapeutic agents for each, while patients with gastric cardia adenocarcinoma are treated with a mixture of those employed for the other two anatomic sites.

### Applications

Improvements in community-based treatment of gastroesophageal adenocarcinoma will require better differentiation of treatments for the different anatomic sites and more extensive incorporation of those treatments proven effective in clinical trials. Future RCTs should be designed and appropriately powered to account for differences related to the anatomic site or origin of the tumor as well as the underlying tumor biology.

### Peer review

This is a retrospective study of a large number of patients with gastroesophageal adenocarcinoma focusing on treatment modalities and survival. This is an excellent and relevant study, which was well conducted and presented.

## REFERENCES

- 1 **Levi F**, Lucchini F, Gonzalez JR, Fernandez E, Negri E, La Vecchia C. Monitoring falls in gastric cancer mortality in Europe. *Ann Oncol* 2004; **15**: 338-345
- 2 **Brewster DH**, Fraser LA, McKinney PA, Black RJ. Socioeconomic status and risk of adenocarcinoma of the oesophagus and cancer of the gastric cardia in Scotland. *Br J Cancer* 2000; **83**: 387-390
- 3 **O'Connell JB**, Maggard MA, Liu JH, Etzioni DA, Ko CY. A report card on outcomes for surgically treated gastrointestinal cancers: are we improving? *J Surg Res* 2004; **121**: 214-221
- 4 **Berrino F**, Capocaccia R, Esteve J, Gatta G, Hakulinen T, Micheli A, Sant M, Verdecchia A. Survival of cancer patients in Europe: the EURO-CARE-2 Study. Lyon: IARC Scientific Publications, 1999
- 5 **Ries LA**, Wingo PA, Miller DS, Howe HL, Weir HK, Rosenberg HM, Vernon SW, Cronin K, Edwards BK. The annual report to the nation on the status of cancer, 1973-1997, with a special section on colorectal cancer. *Cancer* 2000; **88**: 2398-2424
- 6 **Hansson LE**, Sparen P, Nyren O. Survival in stomach cancer is improving: results of a nationwide population-based Swedish study. *Ann Surg* 1999; **230**: 162-169
- 7 **Koshy M**, Esiashvili N, Landry JC, Thomas CR Jr, Matthews RH. Multiple management modalities in esophageal cancer: combined modality management approaches. *Oncologist* 2004; **9**: 147-159
- 8 **Macdonald JS**. Role of post-operative chemoradiation in resected gastric cancer. *J Surg Oncol* 2005; **90**: 166-170
- 9 **Cunningham D**, Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, Scarffe JH, Lofts FJ, Falk SJ, Iveson TJ, Smith DB, Langley RE, Verma M, Weeden S, Chua YJ, MAGIC Trial Participants. Perioperative chemotherapy versus surgery alone for resectable gastroesophageal cancer. *N Engl J Med* 2006; **355**: 11-20
- 10 **Boige V**, Pignon J, Saint-Aubert B, Lasser P, Conroy T, Bouché O, Segol P, Bedenne L, Rougier P, Ychou M. Final results of a randomized trial comparing preoperative 5-fluorouracil (F)/cisplatin (P) to surgery alone in adenocarcinoma of stomach and lower esophagus (ASLE): FNLC ACCORD07-FFCD 9703 trial. *J Clin Oncol*, 2007 ASCO Annual Meeting Proceedings Part 1, 2007; **25**: 4510
- 11 **Walsh TN**, Noonan N, Hollywood D, Kelly A, Keeling N, Hennessy TP. A comparison of multimodal therapy and surgery for esophageal adenocarcinoma. *N Engl J Med* 1996; **335**: 462-467
- 12 **Tepper JE**, Krasna M, Niedzwiecki D, Hollis D, Reed C, Goldberg R, Rich T, Kiel K, Mayer R. Superiority of trimodality therapy to surgery alone in esophageal cancer: Results of CALGB 9781. *J Clin Oncol*, 2006 ASCO Annual Meeting Proceedings Part 1, 2006; **24**: 4012
- 13 **Cooper JS**, Guo MD, Herskovic A, Macdonald JS, Martenson JA Jr, Al-Sarraf M, Byhardt R, Russell AH, Beitler JJ, Spencer S, Asbell SO, Graham MV, Leichman LL. Chemoradiotherapy of locally advanced esophageal cancer: long-term follow-up of a prospective randomized trial (RTOG 85-01). Radiation Therapy Oncology Group. *JAMA* 1999; **281**: 1623-1627
- 14 **Herskovic A**, Martz K, al-Sarraf M, Leichman L, Brindle J, Vaitkevicius V, Cooper J, Byhardt R, Davis L, Emami B. Combined chemotherapy and radiotherapy compared with radiotherapy alone in patients with cancer of the esophagus. *N Engl J Med* 1992; **326**: 1593-1598
- 15 **National Comprehensive Cancer Network (NCCN)**. Esophageal Cancer: Professional Treatment Guidelines. 2007. Available from: URL: [http://www.nccn.org/professionals/physician\\_gls/PDF/esophageal.pdf](http://www.nccn.org/professionals/physician_gls/PDF/esophageal.pdf)
- 16 **Macdonald JS**, Smalley SR, Benedetti J, Hundahl SA, Estes NC, Stemmermann GN, Haller DG, Ajani JA, Gunderson LL, Jessup JM, Martenson JA. Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. *N Engl J Med* 2001; **345**: 725-730
- 17 **Sastre J**, Garcia-Saenz JA, Diaz-Rubio E. Chemotherapy for gastric cancer. *World J Gastroenterol* 2006; **12**: 204-213
- 18 **Van Cutsem E**, Moiseyenko VM, Tjulandin S, Majlis A, Constenla M, Boni C, Rodrigues A, Fodor M, Chao Y, Voznyi E, Risse ML, Ajani JA. Phase III study of docetaxel and cisplatin plus fluorouracil compared with cisplatin and fluorouracil as first-line therapy for advanced gastric cancer: a report of the V325 Study Group. *J Clin Oncol* 2006; **24**: 4991-4997
- 19 **National Comprehensive Cancer Network (NCCN)**. Gastric Cancer: Professional Treatment Guidelines. 2007. Available from: URL: [http://www.nccn.org/professionals/physician\\_gls/PDF/gastric.pdf](http://www.nccn.org/professionals/physician_gls/PDF/gastric.pdf)
- 20 **Suntharalingam M**, Moughan J, Coia LR, Krasna MJ, Kachnic L, Haller DG, Willet CG, John MJ, Minsky BD, Owen JB. Outcome results of the 1996-1999 patterns of care survey of the national practice for patients receiving radiation therapy for carcinoma of the esophagus. *J Clin Oncol* 2005; **23**: 2325-2331
- 21 **Edwards BK**, Brown ML, Wingo PA, Howe HL, Ward E, Ries LA, Schrag D, Jamison PM, Jemal A, Wu XC, Friedman C, Harlan L, Warren J, Anderson RN, Pickle LW. Annual report to the nation on the status of cancer, 1975-2002, featuring population-based trends in cancer treatment. *J Natl Cancer Inst* 2005; **97**: 1407-1427
- 22 **Cronin DP**, Harlan LC, Clegg LX, Stevens JL, Yuan G, Davis TA. Patterns of care in a population-based random sample of patients diagnosed with non-Hodgkin's lymphoma. *Hematol Oncol* 2005; **23**: 73-81
- 23 **Harlan LC**, Clegg LX, Trimble EL. Trends in surgery and chemotherapy for women diagnosed with ovarian cancer in the United States. *J Clin Oncol* 2003; **21**: 3488-3494
- 24 **Potosky AL**, Harlan LC, Kaplan RS, Johnson KA, Lynch CF. Age, sex, and racial differences in the use of standard adjuvant therapy for colorectal cancer. *J Clin Oncol* 2002; **20**: 1192-1202
- 25 **van Spronsen DJ**, Janssen-Heijnen ML, Lemmens VE, Peters WG, Coebergh JW. Independent prognostic effect of co-

- morbidity in lymphoma patients: results of the population-based Eindhoven Cancer Registry. *Eur J Cancer* 2005; **41**: 1051-1057
- 26 **Cronin DP**, Harlan LC, Potosky AL, Clegg LX, Stevens JL, Mooney MM. Patterns of care for adjuvant therapy in a random population-based sample of patients diagnosed with colorectal cancer. *Am J Gastroenterol* 2006; **101**: 2308-2318
  - 27 **Charlson ME**, Pompei P, Ales KL, MacKenzie CR. A new method of classifying prognostic comorbidity in longitudinal studies: development and validation. *J Chronic Dis* 1987; **40**: 373-383
  - 28 **Verdecchia A**, Corazziari I, Gatta G, Lisi D, Faivre J, Forman D. Explaining gastric cancer survival differences among European countries. *Int J Cancer* 2004; **109**: 737-741
  - 29 **Wenner J**, Zilling T, Bladstrom A, Alvegard TA. The influence of surgical volume on hospital mortality and 5-year survival for carcinoma of the oesophagus and gastric cardia. *Anticancer Res* 2005; **25**: 419-424
  - 30 **Rizk NP**, Bach PB, Schrag D, Bains MS, Turnbull AD, Karpeh M, Brennan MF, Rusch VW. The impact of complications on outcomes after resection for esophageal and gastroesophageal junction carcinoma. *J Am Coll Surg* 2004; **198**: 42-50
  - 31 **Sabel MS**, Smith JL, Nava HR, Mollen K, Douglass HO, Gibbs JF. Esophageal resection for carcinoma in patients older than 70 years. *Ann Surg Oncol* 2002; **9**: 210-214
  - 32 **Sabharwal T**, Morales JP, Irani FG, Adam A. Quality improvement guidelines for placement of esophageal stents. *Cardiovasc Intervent Radiol* 2005; **28**: 284-288
  - 33 **Geh JI**, Crellin AM, Glynn-Jones R. Preoperative (neoadjuvant) chemoradiotherapy in oesophageal cancer. *Br J Surg* 2001; **88**: 338-356
  - 34 **Urba SG**, Orringer MB, Turrisi A, Iannettoni M, Forastiere A, Strawderman M. Randomized trial of preoperative chemoradiation versus surgery alone in patients with locoregional esophageal carcinoma. *J Clin Oncol* 2001; **19**: 305-313
  - 35 **Urschel JD**, Vasan H. A meta-analysis of randomized controlled trials that compared neoadjuvant chemoradiation and surgery to surgery alone for resectable esophageal cancer. *Am J Surg* 2003; **185**: 538-543
  - 36 **Stahl M**, Stuschke M, Lehmann N, Meyer HJ, Walz MK, Seeber S, Klump B, Budach W, Teichmann R, Schmitt M, Schmitt G, Franke C, Wilke H. Chemoradiation with and without surgery in patients with locally advanced squamous cell carcinoma of the esophagus. *J Clin Oncol* 2005; **23**: 2310-2317
  - 37 **Walsh TN**, Noonan N, Hollywood D, Kelly A, Keeling N, Hennessy TP. A comparison of multimodal therapy and surgery for esophageal adenocarcinoma. *N Engl J Med* 1996; **335**: 462-467
  - 38 **Cunningham D**, Rao S, Starling N, Iveson T, Nicolson M, Coxon F, Middleton G, Daniel F, Oates J, Norman AR, NCRI Upper GI Study Group. Randomised multicentre phase III study comparing capecitabine with fluorouracil and oxaliplatin with cisplatin in patients with advanced oesophagogastric (OG) cancer: The REAL 2 trial. *J Clin Oncol*, 2006 ASCO Annual Meeting Proceedings Part 1, 2006; **24**: LBA4017
  - 39 **Iison DH**, Saltz L, Enzinger P, Huang Y, Kornblith A, Gollub M, O'Reilly E, Schwartz G, DeGroff J, Gonzalez G, Kelsen DP. Phase II trial of weekly irinotecan plus cisplatin in advanced esophageal cancer. *J Clin Oncol* 1999; **17**: 3270-3275
  - 40 **Iison DH**, Forastiere A, Arquette M, Costa F, Heelan R, Huang Y, Kelsen DP. A phase II trial of paclitaxel and cisplatin in patients with advanced carcinoma of the esophagus. *Cancer J* 2000; **6**: 316-323
  - 41 **Jougon JB**, Ballester M, Duffy J, Dubrez J, Delaisement C, Velly JF, Couraud L. Esophagectomy for cancer in the patient aged 70 years and older. *Ann Thorac Surg* 1997; **63**: 1423-1427
  - 42 **Perego D**, Casella G, Bonavina L, Pozzetti U, Soligo D, Incarbone R, Buda CA, Baldini V. Esophageal involvement as an uncommon modality of relapse of Hodgkin lymphoma. *Dis Esophagus* 2003; **16**: 270-272
  - 43 **Rice DC**, Correa AM, Vaporciyan AA, Sodhi N, Smythe WR, Swisher SG, Walsh GL, Putnam JB Jr, Komaki R, Ajani JA, Roth JA. Preoperative chemoradiotherapy prior to esophagectomy in elderly patients is not associated with increased morbidity. *Ann Thorac Surg* 2005; **79**: 391-397; discussion 391-397
  - 44 **Tanisada K**, Teshima T, Ikeda H, Abe M, Owen JB, Hanks GE, Yamashita T, Nishio M, Yamada S, Sakai K, Hiraoka M, Hirokawa Y, Oguchi M, Inoue T. A preliminary outcome analysis of the Patterns of Care Study in Japan for esophageal cancer patients with special reference to age: non surgery group. *Int J Radiat Oncol Biol Phys* 2000; **46**: 1223-1233
  - 45 **Adam DJ**, Craig SR, Sang CT, Cameron EW, Walker WS. Esophagectomy for carcinoma in the octogenarian. *Ann Thorac Surg* 1996; **61**: 190-194
  - 46 **Theuer CP**, Kurosaki T, Taylor TH, Anton-Culver H. Unique features of gastric carcinoma in the young: a population-based analysis. *Cancer* 1998; **83**: 25-33
  - 47 **Zell JA**, Rhee JM, Ziogas A, Lipkin SM, Anton-Culver H. Race, socioeconomic status, treatment, and survival time among pancreatic cancer cases in California. *Cancer Epidemiol Biomarkers Prev* 2007; **16**: 546-552
  - 48 **Crew KD**, Neugut AI, Wang X, Jacobson JS, Grann VR, Raptis G, Hershman DL. Racial disparities in treatment and survival of male breast cancer. *J Clin Oncol* 2007; **25**: 1089-1098

S- Editor Li DL L- Editor Alpini GD E- Editor Ma WH



RAPID COMMUNICATION

## ***In vitro* activity of moxifloxacin and piperacillin/sulbactam against pathogens of acute cholangitis**

Andreas Weber, Wolfgang Huber, Klaus Kamereck, Philipp Winkle, Petra Volland, Hans Weidenbach, Roland M Schmid, Christian Prinz

Andreas Weber, Wolfgang Huber, Philipp Winkle, Petra Volland, Hans Weidenbach, Roland M Schmid, Christian Prinz, Department of Gastroenterology, Klinikum rechts der Isar, Technical University Munich, Munich 81675, Germany  
Klaus Kamereck, Institute of Medical Microbiology, Immunology and Hygiene, Klinikum rechts der Isar, Technical University Munich, Munich 81675, Germany

**Author contributions:** Weber A and Huber W contributed equally to this work; Weber A, Huber W, Schmid RM and Prinz C designed research; Weber A, Huber W, Weidenbach H, Winkle P and Volland P performed research; Weber A, Huber W, Kamereck K analyzed data; Weber A, Huber W, Schmid RM and Prinz C wrote the paper.

**Correspondence to:** Dr. Christian Prinz, Professor, Department of Gastroenterology, Klinikum Rechts der Isar, Technical University Munich, Munich 81675, Germany. [christian.prinz@lrs.tum.de](mailto:christian.prinz@lrs.tum.de)

Telephone: +49-89-41405973 Fax: +49-89-41407366

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**CONCLUSION:** *Enterococcus species*, *E.coli* and *Klebsiella species* were the most common bacteria isolated from bile and/or blood from patients with acute cholangitis. Overall, a mixed infection with several species was observed, and bacteria showed a comparable *in vitro* activity for piperacillin/sulbactam and moxifloxacin.

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**Key words:** Cholangitis; Acute cholangitis; Endoscopy; Antibiotics; Moxifloxacin; Piperacillin; Sulbactam; Biliary stricture; Resistance; Bacterial pathogens

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### **Abstract**

**AIM:** To analyze the *in vitro* activity of moxifloxacin and piperacillin/sulbactam against pathogens isolated from patients with acute cholangitis.

**METHODS:** In this prospective study a total of 65 patients with acute cholangitis due to biliary stone obstruction ( $n = 7$ ), benign biliary stricture ( $n = 16$ ), and malignant biliary stricture ( $n = 42$ ) were investigated with regard to spectrum of bacterial infection and antibiotic resistance. Pathogens were isolated from bile cultures in all study patients. In 22 febrile patients, blood cultures were also obtained. *In vitro* activity of moxifloxacin and piperacillin/sulbactam was determined by agar diffusion.

**RESULTS:** Thirty-one out of 65 patients had positive bile and/or blood cultures. In 31 patients, 63 isolates with 17 different species were identified. The predominant strains were *Enterococcus species* (26/63), *E.coli* (13/63) and *Klebsiella species* (8/63). A comparable *in vitro* activity of moxifloxacin and piperacillin/sulbactam was observed for *E.coli* and *Klebsiella species*. In contrast, *Enterococcus species* had higher resistances towards moxifloxacin. Overall bacteria showed antibiotic resistances *in vitro* of 34.9% for piperacillin/sulbactam and 36.5% for moxifloxacin.

### **INTRODUCTION**

Acute cholangitis, first described by Charcot in 1877 is a frequent and potentially serious complication in patients with bile duct obstruction. Ductal obstruction leads to a raised intrabiliary pressure with cholangiovenous reflux and bacteremia, which may progress to septicemia<sup>[1]</sup>. Ductal stones, benign or malignant biliary strictures are reasons for the obstruction. Biliary decompression by endoscopic or percutaneous transhepatic procedures and selection of appropriate antibiotics are crucial in the therapy of these patients<sup>[2-5]</sup>. The efficacy of antibiotics in the treatment of biliary infections depends on the microbiological activity against the most common pathogens and the excretion of the antibacterial agents in the obstructed biliary tract. In case of complete obstruction of the common bile duct, no significant biliary excretion of the antibiotics occurs, so that biliary



bactericidal concentrations cannot be achieved<sup>[6,7]</sup>. However, recently a sufficient biliary concentration of the fluoroquinolone moxifloxacin in patients with obstructive cholangitis was reported<sup>[8]</sup>. Because bacteremia may progress to septicemia, a high level of serum concentrations of the antibiotic agents is also important for the treatment of biliary tract infections. Although acute cholangitis is a common clinical problem associated with a high level of morbidity and mortality, there is no standardized approach for therapy of this disease. The selection of antibacterial agents is based on the severity of the disease, the expected biliary pathogens or the activity of antibacterial agents against the isolated bacteria from blood or bile cultures. Broad spectrum antibiotics, active against gram negative and gram positive organisms, are the preferred treatment<sup>[2,9-11]</sup>. Therefore, in case of severe cholangitis, the mostly preferred drug is piperacillin, a broad spectrum penicillin. In a prospective randomised trial including patients with acute cholangitis, equal clinical efficacy was observed with piperacillin alone compared to ampicillin plus tobramycin<sup>[12]</sup>. The combination of piperacillin with the  $\beta$ -lactamase inhibitor sulbactam might be an alternative procedure when the resistance pattern shows a relatively high incidence of ureidopenicillin-resistant *E.coli* or *Klebsiella species*<sup>[13]</sup>. Because of increasing resistance and allergic reactions against penicillin, other antibacterial agents for the treatment of acute cholangitis become necessary. Moxifloxacin is characterized by an enhanced activity against gram positive, gram negative and in anaerobic organisms and by a sufficient concentration in the obstructive bile duct. Therefore it may be an alternative antibacterial treatment in patients with acute cholangitis. To address this question, we performed a prospective trial to analyze the *in vitro* activity of moxifloxacin and piperacillin/sulbactam against pathogens isolated from patients with acute cholangitis.

## MATERIALS AND METHODS

### Study population

The study included 65 consecutive patients suffering from acute cholangitis who were treated between February 2004 and November 2005 in the Department of Gastroenterology at the Technical University of Munich. All of the following criteria had to be fulfilled: (1) clinical diagnosis of acute cholangitis, (2) elevated cholestasis parameter (bilirubin > 3 mg/dL), (3) elevated infection parameters (leucocytes > 12 G/L, c-reactive protein > 3 mg/dL) or fever (> 38.5°C), and (4) age 18-90 years. Exclusion criteria were as follows: (1) primary sclerosing cholangitis, (2) liver cirrhosis, (3) liver transplantation, (4) acquired immunodeficiency syndrome (AIDS), (5) primary immunodeficiency syndrome, (6) therapy with glucocorticoids and other immunosuppressant drugs, (7) leucopenia (leucocytes < 1 G/L), and (8) infection focus other than acute cholangitis.

### Isolation of bacteria

From all patients included in this study, bile samples for culture were taken. Bile was obtained by endoscopic retrograde cholangiography (ERC) or by percutaneous transhepatic biliary drainage (PTBD). ERC and biliary drainage were performed with a standard videoduodenoscope OlympusTFJ 160-R. Endoscopic sphincterotomy (EST) was conducted using an Olympus papillotome introduced over a Terumo guide wire. At ERC, intraductal bile was collected before contrast agent injection by passing a sterile standard ERC catheter into the obstructed bile duct and aspirating bile into a sterile 10 mL syringe. In case of PTBD, 2-4 mL bile was collected into a sterile 10 mL syringe after penetration of the bile duct with the puncture needle. Thereafter, a percutaneous transhepatic biliary catheter was inserted by the Seldinger technique. Because of the percutaneous placement of this catheter, bile could be obtained all the time in case of fever, chills and increasing infection parameters (leucocytes, c-reactive protein). In 22 febrile patients (temperature > 38.5°C), blood cultures were also obtained. Typically, 10 mL of blood was obtained and transferred into aerobic and anaerobic culture broth (BacTec system, Becton Dickinson, Heidelberg, Germany).

### Microbiological investigation

In case of positive blood- and/or bile cultures, the *in vitro* activity of moxifloxacin and piperacillin/sulbactam was performed by agar diffusion assay test.

The bile/specimen sampled was examined for aerobic and anaerobic bacteria. In each case, 50-100  $\mu$ L bile/specimen were both transferred into liquid nutrient media (glucose broth, thioglycollate broth) and spread on solid culture media (Columbia sheep blood agar, chocolate agar, McConkey agar, Schädler anaerobic agar, Schädler KV anaerobic agar, and Sabouroud agar). Subsequently, the culture media were incubated at 37°C. The aerobic cultures were incubated for 48 h, with the first readout taken after 24 h. The anaerobic cultures were monitored for the first time after 48 h and processed further as required. To identify bacteria in the blood, one aerobic and one anaerobic blood culture bottle (BacTec system, Becton Dickinson, Heidelberg, Germany) were each inoculated with 10 mL of venous blood. The blood cultures were incubated at 37°C for 5 d. For control purposes and to exclude failure of automatic detection of the BacTec system each flask was subcultivated under aerobic (chocolate agar in 10% CO<sub>2</sub>) and anaerobic conditions (Schädler anaerobic agar) at the end of the incubation period. Cultivable germs were identified using the ATB, API or VITEK system (BioMérieux, Nürtingen, Germany). In order to identify antimicrobial inhibitors approximately 10  $\mu$ L of fluid specimen were placed in the depression of an agar plate containing a suspension of spore forming bacteria. With an antibiotic being present and taking effect in the specimen a clear inhibition zone was to be seen around the point of application. Colony forming units were

**Table 1** Patient characteristics, physical and laboratory parameters on admission

		Standard values	Scale unit
Number of patients	65	-	-
Mean age	68 ± 12.3	-	-
Gender			
Male	32	-	-
Female	33	-	-
Bilirubin	7.9 ± 7.4	< 1.2	mg/dL
Alkaline phosphatase	675 ± 510	40-120	U/L
γ-Glutamyltransferase	697 ± 682	< 66	U/L
Aspartate aminotransferase	193 ± 300	10-50	U/L
Alanine aminotransferase	136 ± 147	10-50	U/L
Leucocytes	16.9 ± 10.7	4-9	G/L
C-reactive protein	17.3 ± 9.5	< 0.5	mg/dL

not determined in this study. Antibiotic susceptibility testing was performed using both the disk diffusion test or the MIC test using the VITEK system (BioMérieux, Nürtingen, Germany) or the Etest system (AB Biodisk, Solna, Sweden) according to the recommendations of the CLSI (Clinical Laboratory Standards Institute; formerly NCCLS/National Committee for Clinical Laboratory Standards).

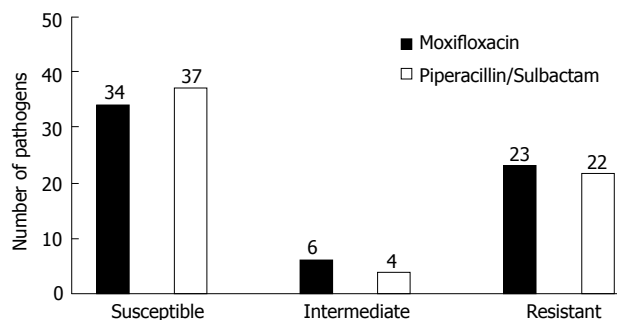
## RESULTS

During the study period from February 2004 to November 2005, a total of 65 consecutive patients with acute cholangitis were included in the current clinical trial. The patients had the following characteristics: mean age 68 ± 12.3 years, 32 male and 33 female, bilirubin 7.9 ± 7.4 mg/dL, alkaline phosphatase 675 ± 510 U/L, γ-glutamyltransferase 697 ± 682 U/L, aspartate aminotransferase 193 ± 300 U/L, alanine aminotransferase 136 ± 147 U/L, leucocytes 16.9 ± 10.7 G/L, c-reactive protein 17.3 ± 9.5 mg/dL (Table 1).

Obstruction of the bile duct was caused by gallstones in 7/65 (10.8%) patients, benign strictures in 16/65 (24.6%) patients and malignant strictures of the biliary tract in 42/65 (64.6%) patients.

Thirty-one out of 65 patients had positive bile- and/or blood cultures. Sixty-three bacterial isolates and 17 different bacterial species were identified from 31 patients. The predominant isolated bacteria were *Enterococcus species* (26/63), *E.coli* (13/63), and *Klebsiella species* (8/63). Thereby, three quarter (74.6%) of the isolated bacteria were obtained from these predominant species, while the remaining quarter (25.4%) consisted of 7 different types. Within the group infected with *Enterococcus species*, *Enterococcus faecium* and *Enterococcus faecalis* were most frequent with 8 and 7 isolates, respectively. Bacteriobilia was documented in 22/65 patients and was polymicrobial in 17 patients (77.3%). Positive blood culture were obtained in 13/65 patients and was polymicrobial in only 1 patient (7.7%).

The resistance pattern of the isolated pathogens was investigated by an *in vitro* activity assay. Table 2 gives an overview of all bacterial pathogens and their resistance patterns regarding moxifloxacin and piperacillin/

**Figure 1** Comparison of *in vitro* activity of moxifloxacin and piperacillin/sulbactam in all isolated bacterial pathogens.

sulbactam. In summary, 34.9% (22/63) of all isolated pathogens were resistant, 6.4% (4/63) were intermediately resistant, and 58.7% (37/63) were susceptible to piperacillin/sulbactam. In comparison to these results 36.5 % (23/63) of all isolated pathogens were resistant, 9.5% (6/63) intermediate resistance, and 54% (34/63) susceptible to moxifloxacin (Figure 1).

## DISCUSSION

Acute cholangitis is an infection of the obstructed biliary tract with a wide spectrum of pathogens. Common microbial populations associated with cholangitis include gram-negative bacteria like *E.coli* and *Klebsiella species*. Gram-positive organisms, mainly *Enterococcus species* and anaerobes, are also found<sup>[14-21]</sup>. While previous works found *E.coli* infection in 20.9% and *Enterococcus species* in 20.9%<sup>[17]</sup>, our current results reveal that the most common isolates are *Enterococcus species* [41.3% (26/63)], *E.coli* [20.6% (13/63)] and *Klebsiella species* [12.7% (8/63)]. In addition to this a lot of other bacterial pathogens were isolated by blood and/or bile cultures (Table 2). Thus, the shift towards the higher rate of *Enterococcus species* and the high prevalence of *Klebsiella* infections might be related to the use of wide-spectrum antibiotics used in the past years.

Establishment of biliary drainage is the mainstay of therapy for patients with acute cholangitis. Endoscopic sphincterotomy with subsequent biliary drainage is the therapy of choice, but in case of therapy failure percutaneous transhepatic bile drainage is an alternative method for biliary drainage<sup>[22-24]</sup>. Nevertheless, once endoscopic and/or percutaneous transhepatic procedures have been performed, the spectrum of bacterial infection might change, and increased frequency of mixed infections has been reported<sup>[17]</sup>. Our current data are in line with this observation and reveal polymicrobial infections of the biliary tract in 17 out of 22 patients.

Overall, our results indicate that bacterial pathogens could only be isolated in 48% of the patients. Antibiotic treatment has to start early during the infectious process. In clinical practice, it is not possible to isolate bacterial pathogens in all patients and the time to receive the resistance pattern creates a delay of several days. Therefore, knowledge of bacterial spectrum and resistance pattern of antimicrobial agents are essential for the treatment of patients suffering from acute cholangitis.

Table 2 Resistance pattern for moxifloxacin and piperacillin/sulbactam in all pathogens

Pathogens	Moxifloxacin			Piperacillin/Sulbactam		
	Susceptible	Intermediate	Resistant	Susceptible	Intermediate	Resistant
<i>Enterococcus species</i>	9	4	13	16	1	9
<i>Enterococcus NS</i>	2	4	2	7		1
<i>Enterococcus faecium</i>	1		7	2	1	5
<i>Enterococcus faecalis</i>	4		3	5		2
<i>Enterococcus casseliflavus</i>	2			2		
<i>Enterococcus gallinarum</i>			1			1
<i>Escherichia coli</i>	8	1	4	11		2
<i>Klebsiella species</i>	5	1	2	4	1	3
<i>Klebsiella pneumoniae</i>	3	1	2	2	1	3
<i>Klebsiella oxytoca</i>	2			2		
<i>Enterobacter species</i>	5			3		2
<i>Enterobacter cloacae</i>	3			3		
<i>Enterobacter NS</i>	2					2
<i>Pseudomonas aeruginosa</i>	2		1		1	2
<i>Aeromonas species</i>	1		1			2
<i>Aeromonas hydrophila/caviae</i>	1					1
<i>Aeromonas NS</i>			1			1
<i>Citrobacter freundii</i>	2			1	1	
Coagulase neg. <i>Staphylococcus</i>			2			2
Gram negative rod NS	1			1		
<i>Streptococcus anginosus</i>	1			1		

NS: Not specified.

Finally, it has to be mentioned that in patients with an obstructed biliary tract, the biliary excretion of several antibiotic agents is limited<sup>[6,25]</sup>. Recently, it was reported that moxifloxacin, a fluoroquinolone, can reach clinically significant concentrations in obstructed biliary tract<sup>[8]</sup>. Therefore it may be a superior treatment in patients with acute cholangitis that suffer from biliary obstruction. Until now, no data about antimicrobial activity of moxifloxacin against pathogens of acute cholangitis exists. Therefore, we isolated pathogens from patients with acute cholangitis and analyzed the *in vitro* activity of moxifloxacin and piperacillin/sulbactam. Our data show a comparable *in vitro* activity of moxifloxacin and piperacillin/sulbactam in patients with acute cholangitis. Kiesslich *et al.*<sup>[26]</sup> reported a resistance rate of 71.8% (28/39) for piperacillin and 76.7% (33/43) for ampicillin (both without  $\beta$ -lactamase inhibitors) in bacteria isolated from obstructed biliary tract during endoscopic retrograde cholangiography. In this study, the resistance rate for other fluoroquinolones ciprofloxacin and levofloxacin was 19.0% (8/42) and 2.2% (1/45), respectively. In agreement with these results, 96% (122/127) sensitivity to ciprofloxacin and 29% (37/127) sensitivity to ampicillin was reported in other studies<sup>[27]</sup>.

The *in vivo* benefit of fluoroquinolones in patients with biliary tract infections was investigated in several clinical trials. Karachlios *et al.*<sup>[28]</sup> performed a prospective, randomized trial with ofloxacin in one, and ceftriaxone in the other group. The clinical cure or improvement of clinical symptoms was the same in both groups. In another prospective randomized trial, an adequate clinical benefit was shown for ciprofloxacin mono therapy in comparison to a triple therapy with ceftazidime, ampicillin and metronidazole<sup>[29]</sup>. Also levofloxacin, a newer enantiomer of ofloxacin showed an adequate clinical effect when compared to ceftriaxone<sup>[30]</sup>. In this

prospective randomized trial, patients of both study groups received metronidazole additionally.

Although, moxifloxacin and piperacillin/sulbactam appears to have a comparable *in vitro* activity against pathogens of acute cholangitis, moxifloxacin may have a clinical benefit due to its extensive biliary excretion in obstructed biliary tract. Randomized clinical trials should be performed to evaluate clinical outcome of moxifloxacin in patients with acute cholangitis.

## COMMENTS

### Background

Cholangitis is a frequent and potentially serious complication in patients with bile duct obstruction. Biliary decompression by endoscopic intervention and selection of appropriate antibiotics are crucial for therapy of these patients. The use of broad-spectrum penicillin is generally accepted. Because of increasing resistance and allergic reactions against penicillin, other antibacterial agents for the treatment of acute cholangitis are essential moxifloxacin is characterized by an enhanced activity against gram-positive and -negative anaerobic organisms as well by a sufficient concentration in the obstructive bile duct. Therefore it may be an alternative antibacterial treatment for acute cholangitis.

### Research frontiers

To our knowledge, no study exists investigating the *in vitro* activity of moxifloxacin against pathogens isolated from patients with acute cholangitis. The current study was designed to analyze the *in vitro* activity of moxifloxacin and piperacillin/sulbactam against pathogens of acute cholangitis.

### Innovations and breakthroughs

The predominant pathogens isolated from patients with acute cholangitis were *Enterococcus species*, *E.coli* and *Klebsiella species*. A comparable *in vitro* activity of moxifloxacin and piperacillin/sulbactam was observed for *E.coli* and *Klebsiella species*. In contrast, *Enterococcus species* had higher resistances towards moxifloxacin. Overall bacteria showed antibiotic resistances of 34.9% for piperacillin/sulbactam and 36.5% for moxifloxacin.

### Applications

These data suggest that moxifloxacin can be used as an alternative antibiotic therapy in patients with cholangitis that show allergic reactions to piperacillin/sulbactam. Additionally, due to the extensive excretion of moxifloxacin in the obstructed biliary tract it may have a clinical advantage compared to the

standard therapy. Randomized controlled trials should be performed to evaluate the clinical outcome of moxifloxacin in patients with acute cholangitis.

### Terminology

Acute cholangitis with the triad of jaundice, fever and abdominal pain: was first described by Charcot in 1877. It is a frequent and potentially serious complication in patients with bile duct obstruction due to ductal stones, benign and malignant bile duct strictures. Bile duct obstruction leads to a raised intrabiliary pressure with cholangiovenous reflux and bacteraemia, which may induce sepsis.

### Peer review

This manuscript evaluates the relative resistance of bacterial cultures isolated from patients suffering acute cholangitis to piperacillin/sulbactam (the current antibiotic therapy) versus moxifloxacin. It is well designed, performed and written. It is of clinical relevance.

## REFERENCES

- 1 Leung JW, Ling TK, Chan RC, Cheung SW, Lai CW, Sung JJ, Chung SC, Cheng AF. Antibiotics, biliary sepsis, and bile duct stones. *Gastrointest Endosc* 1994; **40**: 716-721
- 2 Westphal JF, Brogard JM. Biliary tract infections: a guide to drug treatment. *Drugs* 1999; **57**: 81-91
- 3 Lai EC, Mok FP, Tan ES, Lo CM, Fan ST, You KT, Wong J. Endoscopic biliary drainage for severe acute cholangitis. *N Engl J Med* 1992; **326**: 1582-1586
- 4 Audisio RA, Morosi C, Bozzetti F, Cozzi G, Bellomi M, Pisani P, Pestalozza A, Gennari L, Severini A. The outcome of cholangitis after percutaneous biliary drainage in neoplastic jaundice. *HPB Surg* 1993; **6**: 287-293
- 5 Jain MK, Jain R. Acute bacterial cholangitis. *Curr Treat Options Gastroenterol* 2006; **9**: 113-121
- 6 Leung JW, Chan RC, Cheung SW, Sung JY, Chung SC, French GL. The effect of obstruction on the biliary excretion of cefoperazone and ceftazidime. *J Antimicrob Chemother* 1990; **25**: 399-406
- 7 van den Hazel SJ, de Vries XH, Speelman P, Dankert J, Tytgat GN, Huibregtse K, van Leeuwen DJ. Biliary excretion of ciprofloxacin and piperacillin in the obstructed biliary tract. *Antimicrob Agents Chemother* 1996; **40**: 2658-2660
- 8 Schwab D, Grauer M, Hahn EG, Muhldorfer S. Biliary secretion of moxifloxacin in obstructive cholangitis and the non-obstructed biliary tract. *Aliment Pharmacol Ther* 2005; **22**: 417-422
- 9 Tanaka A, Takada T, Kawarada Y, Nimura Y, Yoshida M, Miura F, Hirota M, Wada K, Mayumi T, Gomi H, Solomkin JS, Strasberg SM, Pitt HA, Belghiti J, de Santibanes E, Padbury R, Chen MF, Belli G, Ker CG, Hilvano SC, Fan ST, Liau KH. Antimicrobial therapy for acute cholangitis: Tokyo Guidelines. *J Hepatobiliary Pancreat Surg* 2007; **14**: 59-67
- 10 van den Hazel SJ, Speelman P, Tytgat GN, Dankert J, van Leeuwen DJ. Role of antibiotics in the treatment and prevention of acute and recurrent cholangitis. *Clin Infect Dis* 1994; **19**: 279-286
- 11 Sinanan MN. Acute cholangitis. *Infect Dis Clin North Am* 1992; **6**: 571-599
- 12 Thompson JE Jr, Pitt HA, Doty JE, Coleman J, Irving C. Broad spectrum penicillin as an adequate therapy for acute cholangitis. *Surg Gynecol Obstet* 1990; **171**: 275-282
- 13 Chamberland S, L'Ecuyer J, Lessard C, Bernier M, Provencher P, Bergeron MG. Antibiotic susceptibility profiles of 941 gram-negative bacteria isolated from septicemic patients throughout Canada. The Canadian Study Group. *Clin Infect Dis* 1992; **15**: 615-628
- 14 Nielsen ML, Justesen T. Anaerobic and aerobic bacteriological studies in biliary tract disease. *Scand J Gastroenterol* 1976; **11**: 437-446
- 15 England DM, Rosenblatt JE. Anaerobes in human biliary tracts. *J Clin Microbiol* 1977; **6**: 494-498
- 16 Maddocks AC, Hilson GR, Taylor R. The bacteriology of the obstructed biliary tree. *Ann R Coll Surg Engl* 1973; **52**: 316-319
- 17 Lorenz R, Herrmann M, Kassem AM, Lehn N, Neuhaus H, Classen M. Microbiological examinations and in-vitro testing of different antibiotics in therapeutic endoscopy of the biliary system. *Endoscopy* 1998; **30**: 708-712
- 18 Brook I. Aerobic and anaerobic microbiology of biliary tract disease. *J Clin Microbiol* 1989; **27**: 2373-2375
- 19 Leung JW, Liu YL, Lau GC, Chan RC, Lai AC, Ling TK, Cheng AF. Bacteriologic analyses of bile and brown pigment stones in patients with acute cholangitis. *Gastrointest Endosc* 2001; **54**: 340-345
- 20 Shimada K, Noro T, Inamatsu T, Urayama K, Adachi K. Bacteriology of acute obstructive suppurative cholangitis of the aged. *J Clin Microbiol* 1981; **14**: 522-526
- 21 Flores C, Maguilnik I, Hadlich E, Goldani LZ. Microbiology of choledochal bile in patients with choledocholithiasis admitted to a tertiary hospital. *J Gastroenterol Hepatol* 2003; **18**: 333-336
- 22 Lai EC, Mok FP, Tan ES, Lo CM, Fan ST, You KT, Wong J. Endoscopic biliary drainage for severe acute cholangitis. *N Engl J Med* 1992; **326**: 1582-1586
- 23 Leung JW, Chung SC, Sung JJ, Banez VP, Li AK. Urgent endoscopic drainage for acute suppurative cholangitis. *Lancet* 1989; **1**: 1307-1309
- 24 Nelsen KM, Kastan DJ, Shetty PC, Burke MW, Sharma RP, Venugopal C. Utilization pattern and efficacy of nonsurgical techniques to establish drainage for high biliary obstruction. *J Vasc Interv Radiol* 1996; **7**: 751-756
- 25 Keighley MR, Drysdale RB, Quoraishi AH, Burdon DW, Alexander-Williams J. Antibiotics in biliary disease: the relative importance of antibiotic concentrations in the bile and serum. *Gut* 1976; **17**: 495-500
- 26 Kiesslich R, Holfelder M, Will D, Hahn M, Nafe B, Genitsariotis R, Daniello S, Maeurer M, Jung M. [Interventional ERCP in patients with cholestasis. Degree of biliary bacterial colonization and antibiotic resistance] *Z Gastroenterol* 2001; **39**: 985-992
- 27 Rerknimitr R, Fogel EL, Kalayci C, Esber E, Lehman GA, Sherman S. Microbiology of bile in patients with cholangitis or cholestasis with and without plastic biliary endoprosthesis. *Gastrointest Endosc* 2002; **56**: 885-889
- 28 Karachalios GN, Nasiopoulou DD, Bourlinou PK, Reppa A. Treatment of acute biliary tract infections with ofloxacin: a randomized, controlled clinical trial. *Int J Clin Pharmacol Ther* 1996; **34**: 555-557
- 29 Sung JJ, Lyon DJ, Suen R, Chung SC, Co AL, Cheng AF, Leung JW, Li AK. Intravenous ciprofloxacin as treatment for patients with acute suppurative cholangitis: a randomized, controlled clinical trial. *J Antimicrob Chemother* 1995; **35**: 855-864
- 30 Kiesslich R, Will D, Hahn M, Nafe B, Genitsariotis R, Maurer M, Jung M. [Ceftriaxone versus Levofloxacin for antibiotic therapy in patients with acute cholangitis] *Z Gastroenterol* 2003; **41**: 5-10

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## Are acute exacerbations of chronic inflammatory appendicitis triggered by coprostasis and/or coproliths?

George Sgourakis, Georgios C Sotiropoulos, Ernesto P Molmenti, Charis Eibl, Stylianos Bonticous, Jurgen Moege, Christoph Berchtold

George Sgourakis, Georgios C Sotiropoulos, Ernesto P Molmenti, Department of General, Visceral, and Transplantation Surgery, Essen 45122, Germany

George Sgourakis, 2nd Surgical Department, Korgialenio-Benakio Red Cross Hospital, Athens 15451, Greece

Georgios C Sotiropoulos, Charis Eibl, Christoph Berchtold, Department of General Surgery, Marien Hospital Bottrop, Bottrop 46236, Germany

Stylianos Bonticous, Jurgen Moege, Institute of Pathology, Marien Hospital Bottrop, Bottrop 46236, Germany

**Author contributions:** Sgourakis G, study conception and design; Sotiropoulos GC, acquisition of data; Sgourakis G, analysis and interpretation of data; Molmenti EP and Eibl C, drafting of manuscript; Bonticous S, independent pathologist; Moege J and Berchtold C, critical revision.

**Correspondence to:** George Sgourakis, MD, PhD, 2nd Surgical Department, Korgialenio-Benakio Red Cross Hospital, 11 Mantzarou street, Neo Psychiko, Athens 15451, Greece. [gsgourakis@yahoo.gr](mailto:gsgourakis@yahoo.gr)

Telephone: +30-210-6716015 Fax: +30-210-6716015

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**Peer reviewers:** Chris JJ Mulder, Professor, Department of Gastroenterology, VU University Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands; Steven D Wexner, MD, Professor of Surgery, The Cleveland Clinic Foundation Health Sciences Center of the Ohio State University, and Clinical Professor, Department of Surgery, Division of General Surgery, University of South Florida College of Medicine, 21st Century Oncology Chair in Colorectal Surgery, Chairman Department of Colorectal Surgery, Chief of Staff, Cleveland Clinic Florida, 2950 Cleveland Clinic Boulevard, Weston, Florida 33331, United States

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### Abstract

**AIM:** To examine the role of coprostasis and coproliths in recurrent appendicitis.

**METHODS:** We evaluated four hundred and twenty seven consecutive pathology reports of all appendectomy specimens from January 2003 to December 2004. Findings were categorised as showing acute appendicitis, acute recurrent appendicitis, subacute recurrent appendicitis, chronic appendicitis, or appendices without inflammation. All patients had presented with acute right lower quadrant pain. In 94 instances, there was a history of recurrent similar episodes in the past.

**RESULTS:** Of the 427 histology reports, 294 were interpreted as showing acute appendicitis, 56 acute recurrent appendicitis, 34 subacute recurrent appendicitis, 28 chronic appendicitis, and 15 non-inflamed appendices. Coprostasis was observed in 58 patients (13.58%) and the presence of coprolith in 6 (1.4%). Coprostasis, and age, were among the predictors in the final model.

**CONCLUSION:** Coprostasis but not coproliths seems to be a contributing factor to acute exacerbations of chronic inflammatory appendicitis.

### INTRODUCTION

Despite the disrepute associated with the term “recurrent appendicitis,” there is evidence to suggest that such appendectomies are associated with improvement of the symptoms that lead to admission of the patients<sup>[1-4]</sup>. The pathophysiology of recurrent inflammation of the appendix is uncertain. Acute appendicitis is thought to be associated with obstruction of the appendiceal lumen, leading to bacterial overgrowth, inflammation, ischaemia, and ultimately perforation. Some authors have thus hypothesized that recurrent lower quadrant pain can be due to either incomplete obstruction of the lumen of the appendix, or a disproportionate production of mucus<sup>[5,6]</sup>. Recurrent symptom alleviation after appendectomy in a proportion of our patients compelled us to examine the potential role of coprostasis and coproliths in recurrent appendicitis.

### MATERIALS AND METHODS

#### Patients

We evaluated prospectively all consecutive pathology reports for appendectomy specimens at the Department

of General Surgery, Marien Hospital Bottrop, Germany from January 2003 to December 2004. Specimens were categorized as acute appendicitis, acute recurrent appendicitis, subacute recurrent appendicitis, chronic appendicitis, and appendices showing no signs of inflammation. The presence of a coprolith (thickened “stone-like” faeces) and coprostasis (appendiceal lumen filled with faeces, completely impacted and not just the presence of a little stool) was noted. Clinical details were supplemented by review of selected case notes.

Specimens were doubly evaluated and classified into five categories by two pathologists separately and the presence of coprostasis and coprolith was also recorded. In those cases with discrepancy in the diagnosis or in the presence of a coprolith or coprostasis specimens ( $n = 4$ ) were reviewed by an experienced independent pathologist.

Patients were divided in 5 groups: Group A for acute appendicitis, group B for subacute recurrent appendicitis, group C for acute recurrent appendicitis, and group D for chronic inflammation of the appendix. Histology reports with normal non-inflamed appendices were classified as group E. Definitions of the pathologic entities were as follows:

**Acute appendicitis (group A):** At early stages the serosa is intensely erythematous due to congestion of the subserosal blood vessels. In advanced stages few intact crypts exist lined with intact mucosal epithelium, lamina propria is hypercellular due to neutrophil infiltration, hemorrhage and ulcers are found at the surface caused by the sloughing off of the inflammatory necrotic tissue.

**Subacute recurrent appendicitis (group B):** This entity is characterized by lympho-follicular hyperplasia, discrete granulocytes, mucocutaneous infiltration and hyperaemic serosa.

**Acute recurrent appendicitis (group C):** In different sections of the appendix, there can be recognized relative diffuse, inflammatory mucocutaneous and appendiceal wall infiltrations. Primarily neutrophils exist that spread out also within the subserosal tissue. Erosive lesions are additionally observed.

**Chronic inflammatory (group D):** This is characterized by the presence of unequivocal mural granulation tissue, with or without frank fibrosis, partial or total obliteration of the lumen by fibrous tissue and hyperplasia or atrophy of the lymphoid tissue.

### Statistical analysis

Statistical methods included nonparametric Yates correction chi-square, Fisher's exact test (two tails) for categorical variables, and Mann-Whitney *U* test for quantitative variables. The Random Forest test (data mining procedure) was used to disclose the variables for use in regression analysis. The General Discriminant analysis model was used to evaluate the discriminating

**Table 1** Incidence of coprostasis and coproliths among groups (%)

Pathology classification	Coprostasis ( $n = 58$ )	Coprolith ( $n = 6$ )
Acute	33/294 (11.22)	3/294 (1.02)
Subacute recurrent	12/34 (35.20)	1/34 (2.94)
Acute recurrent	9/56 (16.07)	1/56 (1.78)
Chronic	3/28 (10.71)	0/28 (0.00)
No inflammation	1/15 (06.66)	1/15 (6.66)

effect of coprostasis and coproliths on the defined groups. A Receiver Operating Characteristic (ROC) curve was used to define the ideal cut-off separator for continuous predictor variables. A significance level of 0.05 was assigned. Statistica release 7 (Statsoft) was used for statistical analysis.

## RESULTS

There were 427 appendectomy pathology reports, corresponding to 265 females and 162 males. Mean patient age was  $24.40 \pm 17.16$  years (range, 4-89 years). All patients were referred for acute right lower quadrant pain. In 94 cases, there was a reported history of recurrent similar episodes in the past. Among these 94 patients 56 had acute recurrent, 34 subacute recurrent and 4 chronic appendicitis.

Of the 427 histology reports, 294 were diagnostic of acute appendicitis, 56 of acute recurrent appendicitis, 34 of subacute recurrent appendicitis, 28 of chronic appendicitis, and 15 of non-inflamed appendices.

Coprostasis was observed in 58 patients (13.58%) and the presence of a coprolith was noted in 6 (1.4%) cases. The incidence and the respective percentages of coprostasis and coprolith among separate histology groups are shown in Table 1.

Associated findings were noted in 6 patients: sigmoid cancer in one, corpus luteum cyst in one, and Meckel's diverticulum in four. Yersinia infection was observed in 1 patient of group B and in one patient of group C. Parasitic infections were diagnosed in 3 patients of group A. Among the 15 cases of group E (non-inflamed appendices), there were four diverticular ruptures, two Meckel's diverticulitis, one carcinoid tumor, one mesenteric arterial embolism, three adnexitis, two tubo-ovarian abscesses, one endometriosis, and one bilateral ovarian biopsy negative for malignancy.

Prominent pathologic findings were encountered more frequently among group A patients. Appendiceal lumen dilatation greater than 10 mm was noticed in 12 patients of group A, one of group B, and 2 of group C. Forty five appendices of group A were gangrenous, and 38 were perforated. This contrasts with the appendices of patients within groups B and C, which were neither gangrenous nor perforated. The incidence of peri-appendicitis was higher in group A (205/294, 69.38%) than in group B (3/34, 8.82%) and group C (28/56, 50%; Table 2). Appendiceal plastrons were documented in 10 patients of group A and one of group D. There were none among patients of groups B and C.

**Table 2** Pathological findings

	Acute (A: <i>n</i> = 294)	Sub-acute recurrent (B: <i>n</i> = 34)	Acute recurrent (C: <i>n</i> = 56)	Chronic (D: <i>n</i> = 28)	<i>P</i>
Gangrenous	45	0	0	0	A vs B, <i>P</i> = 0.0101, A vs C, <i>P</i> = 0.0007
Perforated	38	0	0	0	A vs B, <i>P</i> = 0.0199, A vs C, <i>P</i> = 0.0019
Peri-appendicitis	205	3	28	3	A vs B, <i>P</i> = 0.0001, A vs C, <i>P</i> = 0.0498
Abscess	14	0	0		
Other					
Oxyuriasis	3	0	0	0	
Yersinia	0	1	1	0	

**Table 3** Forward stepwise regression analysis model, only age and coprostasis were among predictors in the final model

	Steps	Degrees of freedom	F to remove	<i>P</i> to remove	F to enter	<i>P</i> to enter	Effect status
Age	Step number 1	3			4472079	0005081	Entered
Gender		3			0880568	0453150	Out
Coprostasis		3			3277140	0023264	Out
Coprolith		3			0681613	0564865	Out
Other pathology		3			1437535	0234958	Out
Age	Step number 2	3	447207	0005081			In
Gender		3			0914989	0435791	Out
Coprostasis		3			3039213	0031532	Entered
Coprolith		3			1140316	0335493	Out
Other pathology		3			1256381	0292311	Out
Age	Step number 3	3	421918	0007020			In
Coprostasis		3	303921	0031532			In
Gender		3			0729353	0536341	Out
Coprolith		3			1119171	0343986	Out
Other pathology		3			1059992	0368677	Out

The presence of coproliths did not discriminate among groups. Summary of stepwise regression; variable appendicitis forward stepwise *P* to enter, < 0.05; *P* to remove, > 0.05.

In order to find the potential role of coprostasis and coprolith as predictors of the various appendicitis classes as described in methods section, we applied Random Forest classification test (this Data Mining technique - Random Forest algorithm developed by Breiman - can be used for classification problems in order to predict a categorical dependent variable). Importance (from high to low) was attributed to Age = 1, Gender = 0.32, Coprostasis = 0.25, Oxyuriasis and Yersinia cases = 0.12 and Appendicolith = 0.05.

Taking into account the potential predictors suggested from the Random Forest test we proceeded for further analysis. Coprostasis, age, gender and oxyuriasis and Yersinia cases were prognostic factors among the four groups (excluded was the “No inflammation” group of patients) by univariate analysis. The presence of a coprolith did not achieve statistical significance. Coprostasis (*P* = 0.0032), age (*P* = 0.0077), and oxyuriasis and Yersinia cases (*P* = 0.0354), but not the presence of coprolith, were also found to be predictive variables by forward stepwise regression analysis. The level of significance for “coprostasis” in each group is reported in Table 3. The null hypothesis was rejected in groups D (*P* = 0.0351) and E (*P* = 0.0496), but substantiated in groups A (*P* = 0.6885), B (*P* = 0.0796) and C (*P* = 0.1311),

implying coprostasis as an etiologic factor in acute, subacute recurrent and acute recurrent appendicitis.

A further Discriminant forward stepwise analysis was employed in order to find the predictive model only for subacute recurrent and acute recurrent cases (Table 3). Only age and coprostasis were among predictors in the final model.

A Receiver Operating Characteristic (ROC) curve was used to select the optimum decision threshold for patient age. Patients less or equal to 40 years had a higher prevalence of subacute (29/5, *P* = 0.0012) and acute recurrent (45/11, *P* = 0.0003) appendicitis. Subacute and acute recurrent appendicitis was also found to be more prevalent in females (27/7, *P* = 0.0083; 39/17, *P* = 0.0241).

## DISCUSSION

The perception that acute appendicitis might subside spontaneously and re-emerge with bouts of right lower quadrant pain (so-called recurrent appendicitis) has met debate and disbelief. Nonetheless, 10% of patients presenting with acute appendicitis report previous similar physical findings that settled without surgery<sup>[7,8]</sup>. Subsequent appendectomy is remedial<sup>[3]</sup>.

It has been assumed that the likely pathophysiologic

mechanism of recurrent appendicitis is either incomplete obstruction of the lumen of the appendix or disproportionate mucus production. Except for two cases of yersiniosis associated with coprostasis, we did not observe any disorder involving the gastrointestinal system (such as inflammatory bowel disease, sarcoidosis, tuberculosis, polyarteritis nodosa, endometriosis, parasitosis, changes in neuroendocrine cells) that could account for the chronic or recurrent inflammation of the appendix.

Our study specifically addressed the presence of coprostasis, as opposed to previous series in which only coproliths were considered as causative of either acute or recurrent disease. A radiographically visualized coprolith was considered by many authors as a specific and unquestionable indicator of appendicitis due to obstruction<sup>[9]</sup>. A growing body of evidence however, suggests that luminal obstruction is not an indispensable factor in the development of appendicitis. Arnbjornsson and Bengmark<sup>[10]</sup> measured intraluminal pressures in acute appendicitis, and concluded that obstruction was the result rather than the cause of the inflammatory process. The incidence of coproliths in our series was 1.21%. The frequency of coprolith in acute appendicitis according to others ranges from 0.02%<sup>[11,12]</sup> up to 65%<sup>[13]</sup>. Our present study also rejects the role of coproliths as causative factors in recurrent appendicitis and imposes the use of high fiber diet after an atypical episode.

The reasons we followed this methodology to trace the relationship of coprostasis to the recurrent appendicitis were: (1) The distinction between subacute and acute recurrent appendicitis helps to better delineate the role of coprostasis as a causative factor since these are two sequential phases of the same entity from its initiation to the well established clinical presentation. (2) The use of data mining procedure disclosed the variables for use in regression analysis. The Random Forest test consists of a collection (ensemble) of simple tree predictors, each capable of producing a response when presented with a set of predictor values. For classification problems, this response takes the form of a class membership, which associates (classifies) a set of independent (predictor) values with one of the categories present in the dependent variable. (3) We had to establish first the predictors of all the patients' cohort and subsequently to insert them in analysis for the recurrent appendicitis (subacute or acute) group of patients only in order to avoid a type II error.

According to previous reports, the cut-off age after which the incidence of acute appendicitis declines is about thirty years. Based on our observations, the cut-off age in recurrent appendicitis is around forty.

In summary, it seems from our data that coprostasis rather than coproliths could be a contributing factor to acute exacerbations of chronic inflammatory appendicitis, and that its clinical and histologic findings are milder than those of acute appendicitis.

## COMMENTS

### Background

According to our experience, pain associated with chronic or recurrent appendicitis decreases after elective appendectomy in selected patients. There is little information about the causal factor. Some authors have hypothesized that recurrent lower quadrant pain can be due to either incomplete obstruction of the lumen of the appendix or to a disproportionate production of mucus.

### Research frontiers

We have recently hypothesized that coprostasis and or coproliths may be the contributing factor to acute exacerbations of chronic inflammatory appendicitis.

### Innovations and breakthroughs

The present data provide circumstantial evidence that adds to the standing of 'chronic or recurrent appendicitis' as a separate clinical entity. Its clinical and histologic findings are milder than those of acute appendicitis and the cut-off age in recurrent appendicitis is around forty. Coprostasis and not coproliths are the contributing factor to acute exacerbations of chronic inflammatory appendicitis.

### Applications

High fiber diet after an atypical recurrent episode is of potential clinical relevance.

### Peer review

This study raises an interesting hypothesis about the role of coprostasis in acute exacerbations of "chronic inflammatory appendicitis" by examining the histologic findings of various manifestations of this entity. It also clarifies the definition and the clinical relevance of this disreputed term.

## REFERENCES

- 1 **Crabbe MM**, Norwood SH, Robertson HD, Silva JS. Recurrent and chronic appendicitis. *Surg Gynecol Obstet* 1986; **163**: 11-13
- 2 **Hawes AS**, Whalen GF. Recurrent and chronic appendicitis: the other inflammatory conditions of the appendix. *Am Surg* 1994; **60**: 217-219
- 3 **Price MR**, Haase GM, Sartorelli KH, Meagher DP Jr. Recurrent appendicitis after initial conservative management of appendiceal abscess. *J Pediatr Surg* 1996; **31**: 291-294
- 4 **Roumen RM**, Groenendijk RP, Sloots CE, Duthoi KE, Scheltinga MR, Bruijninx CM. Randomized clinical trial evaluating elective laparoscopic appendectomy for chronic right lower-quadrant pain. *Br J Surg* 2008; **95**: 169-174
- 5 **Lee AW**, Bell RM, Griffen WO Jr, Hagihara PF. Recurrent appendiceal colic. *Surg Gynecol Obstet* 1985; **161**: 21-24
- 6 **Seidman JD**, Andersen DK, Ulrich S, Hoy GR, Chun B. Recurrent abdominal pain due to chronic appendiceal disease. *South Med J* 1991; **84**: 913-916
- 7 **Heller MB**, Skolnick ML. Ultrasound documentation of spontaneously resolving appendicitis. *Am J Emerg Med* 1993; **11**: 51-53
- 8 **Migraine S**, Atri M, Bret PM, Lough JO, Hinchey JE. Spontaneously resolving acute appendicitis: clinical and sonographic documentation. *Radiology* 1997; **205**: 55-58
- 9 **Fee HJ Jr**, Jones PC, Kadell B, O'Connell TX. Radiologic diagnosis of appendicitis. *Arch Surg* 1977; **112**: 742-744
- 10 **Arnbjornsson E**, Bengmark S. Obstruction of the appendix lumen in relation to pathogenesis of acute appendicitis. *Acta Chir Scand* 1983; **149**: 789-791
- 11 **Lim HK**, Lee WJ, Lee SJ, Namgung S, Lim JH. Focal appendicitis confined to the tip: diagnosis at US. *Radiology* 1996; **200**: 799-801
- 12 **Huwart L**, El Khoury M, Lesavre A, Phan C, Rangheard AS, Bessoud B, Menu Y. [Is appendicolith a reliable sign for acute appendicitis at MDCT?] *J Radiol* 2006; **87**: 383-387
- 13 **Lowe LH**, Penney MW, Scheker LE, Perez R Jr, Stein SM, Heller RM, Shyr Y, Hernanz-Schulman M. Appendicolith revealed on CT in children with suspected appendicitis: how specific is it in the diagnosis of appendicitis? *AJR Am J Roentgenol* 2000; **175**: 981-984





## Retrospective analysis of old-age colitis in the Dutch inflammatory bowel disease population

Muhammed Hadithi, Marcel Cazemier, Gerrit A Meijer, Elisabeth Bloemena, Richel J Felt-Bersma, Chris J Mulder, Stephan GM Meuwissen, Amado Salvador Peña, Adriaan A van Bodegraven

Muhammed Hadithi, Marcel Cazemier, Richel J Felt-Bersma, Chris J Mulder, Stephan GM Meuwissen, Adriaan A van Bodegraven, Department of Gastroenterology, VUmc University Medical Center, Amsterdam, PO Box 7057, Amsterdam 1007 MB, The Netherlands

Gerrit A Meijer, Elisabeth Bloemena, Amado Salvador Peña, Department of Pathology, VUmc University Medical Center, Amsterdam, PO Box 7057, Amsterdam 1007 MB, The Netherlands

Author contributions: All authors contributed equally to this article.

Correspondence to: Adriaan A van Bodegraven, Department of Gastroenterology, VUmc University Medical Center, Amsterdam, PO Box 7057, Amsterdam 1007 MB, The Netherlands. [v.bodegraven@vumc.nl](mailto:v.bodegraven@vumc.nl)

Telephone: +31-20-4440613 Fax: +31-20-4440554

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suggestive of IBD. Extra awareness and extensive biopsy sampling are required in order to avoid an erroneous diagnosis purely based on histological mimicry of changes seen in SCAD, when diagnosing IBD in the presence of diverticulosis coli.

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**Key words:** Inflammatory bowel disease; Old-age colitis; Segmental colitis

**Peer reviewer:** Yoshihide Fujiyama, Professor, Internal Medicine, Division of Gastroenterol, Shiga University of Medical Science, Tsukinowa, Seta, Otsu 520-2192, Japan

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### Abstract

**AIM:** To describe the characteristics of Dutch patients with chronic inflammatory bowel disease (IBD) first diagnosed above 60 years of age—a disease also known as old-age colitis (OAC) and to highlight a condition that has a similar appearance to IBD, namely segmental colitis associated with diverticular disease (SCAD).

**METHODS:** A retrospective longitudinal survey of patient demographic and clinical characteristics, disease characteristics, diagnostic methods, management and course of disease was performed. The median follow-up period was 10 years.

**RESULTS:** Of a total of 1100 IBD patients attending the Department of Gastroenterology, 59 (5%) [median age 82 years (range 64-101); 25 male (42%)] were identified. These patients were diagnosed with ulcerative colitis ( $n = 37$ , 61%), Crohn's disease ( $n = 14$ , 24%), and indeterminate colitis ( $n = 8$ , 15%). Remission was induced in 40 (68%) patients within a median interval of 6 mo (range 1-21) and immunosuppressive therapy was well tolerated. Histological evaluation based on many biopsy samples and the course of the disease led to other diagnosis, namely SCAD instead of IBD in five (8%) patients.

**CONCLUSION:** OAC is not an infrequent problem for the gastroenterologist, and should be considered in the evaluation of older patients with clinical features

### INTRODUCTION

Inflammatory bowel disease (IBD), a lifelong uncontrolled inflammation of the intestinal mucosa, is broadly subdivided into ulcerative colitis, Crohn's disease, and in 10%-15% of patients, indeterminate or unclassified colitis, when a definitive diagnosis of ulcerative colitis or Crohn's disease cannot be made at colonoscopy, colon biopsy or colectomy<sup>[1,2]</sup>.

The pathogenesis of IBD remains obscure. While it is clear that there are genetic, environmental, and immunological factors involved in the pathogenesis of IBD, the exact contribution of each and the sequence of events that culminates in clinically apparent IBD remains the subject of intense investigation.

Although IBD may occur at any age, the peak age of onset is 15-30 years old and approximately 10% of cases occur in individuals < 18 years old<sup>[3]</sup>. Old-age colitis (OAC) refers to patients older than 60 years, who are affected by a broad group of colonic diseases, such as infection, carcinoma, drug-induced disease, vasculitis, microscopic colitis, ischemic colitis, and IBD. Diagnosis of IBD in older patients may be difficult because it can easily be confused

with other forms of colitis commonly occurring at this age.

Earlier reports have indicated that both ulcerative colitis and Crohn's disease have a bimodal age distribution, with a second, smaller peak incidence occurring in individuals aged 50-70 years<sup>[4-7]</sup>. Two recent studies have shown that 21%-23% of ulcerative colitis occurs after the age of 50 years and 5% after 70 years<sup>[8,9]</sup>. This age group comprises around 12% (range 8%-20%) and 16% (range 7%-26%) of all newly diagnosed patients with ulcerative colitis and Crohn's disease, respectively<sup>[10]</sup>.

An additional group of disorders called segmental colitis associated with diverticular disease (SCAD) has been found to masquerade as IBD on both a clinical and histological basis<sup>[11]</sup>, since colonic diverticula, most often involving the sigmoid colon, commonly affect middle-aged and elderly individuals<sup>[12,13]</sup>.

In this retrospective cohort, we attempted to differentiate the broad nomenclature OAC and to describe the characteristics of Dutch IBD patients older than 60 years. In addition, we wanted to highlight one of the conditions that is similar to IBD, namely SCAD, since an overlap of IBD and diverticular disease has long been recognized and is not infrequent in clinical practice<sup>[14]</sup>.

## MATERIALS AND METHODS

An IBD database review of more than 1100 patients, covering the years 1990 to the current time, provided 64 cases with OAC. The diagnosis of IBD was determined according to conventional endoscopic, radiological and histological criteria<sup>[15-17]</sup>. Medical records of each patient in this study were reviewed for the following information: sex, age, diagnosis, duration of disease, presenting symptoms, medications (including non-steroid anti-inflammatory drugs), anatomic location of disease, coexistence of diverticulosis, extraintestinal manifestations, laboratory results, radiological results, histopathological examinations, previous medical and surgical treatment strategies, remission rate and development of refractory disease, postoperative morbidity and mortality, overall outcome, and development of malignancy. Dedicated gastroenterological pathologists revised histological specimens from all subjects. Extraintestinal manifestations included erythema nodosum, pyoderma gangrenosum, peripheral arthritis, sacroiliitis/spondylitis, and episcleritis or uveitis. Refractory disease was defined as patients who were not adequately controlled with conventional therapy or immunosuppressive agents, or who required surgical intervention<sup>[18]</sup>. The diagnosis of colonic diverticular disease was established by colonoscopy, barium enema, or both. SCAD was considered when colitis was restricted to a diverticular segment of the left colon (excluding the rectum); the rectum and proximal colon were endoscopically and histologically normal; and when there was no recurrence of segmental colitis following surgical resection of the affected segment<sup>[11]</sup>.

### Statistical analysis

Descriptive analysis was performed and continuous data

Table 1 General characteristic of patients with OAC

Characteristic	Total (n = 59)
Age, median (range, yr)	82 (64-101)
Men, n (%)	25 (42)
Body mass index, median (range)	21 (17-29)
Patient subsets, n (%)	
Ulcerative colitis	37 (63)
Crohn's disease	14 (24)
Indeterminate colitis	8 (13)
Presenting symptoms, n (%)	
Rectal bleeding	35 (65)
Diarrhea	27 (50)
Abdominal pain	23 (42)
Weight loss	18 (33)
Constipation	7 (13)
Fever	5 (9)
Extra-intestinal symptoms, n (%)	9 (17)
NSAIDs, n (%)	13 (22)
Diverticulosis coli, n (%)	36 (61)

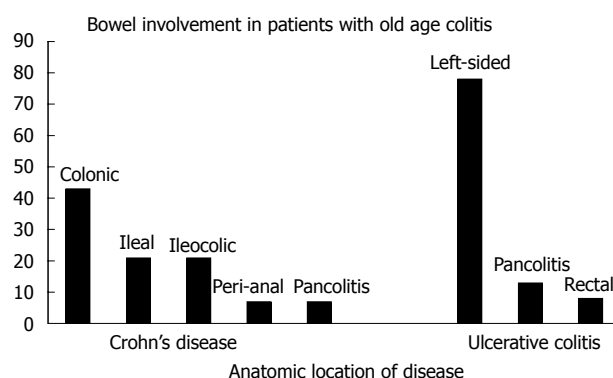


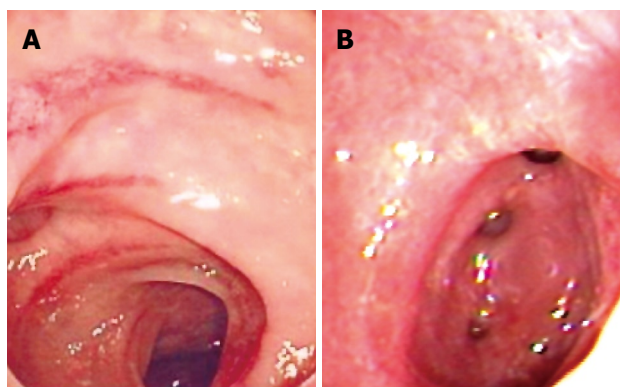
Figure 1 Bowel segment involvement in OAC.

were expressed as the median (range) and categorical data as numbers (percentage). Differences in erythrocyte sedimentation rate, serum albumin and hemoglobin were compared by using one way analysis (ANOVA).  $P \leq 0.05$  was considered statistically significant. Sensitivity and predictive values of radiographic examinations were calculated by using  $2 \times 2$  tables when the diagnosis was based on endoscopic and histological results. Statistical analysis was performed using the Statistical Software Package version 11.0 (SPSS Inc., Chicago, IL, USA).

## RESULTS

### Patients and disease characteristics

A total of 64 patients with OAC were identified. Five patients were excluded because the required histopathological studies were not available, and the remaining 59 patients were included in the analysis. Table 1 summarizes the general and disease-related characteristics and Figure 1 illustrates the bowel involvement in patients with Crohn's disease and ulcerative colitis. Colonic involvement was the rule in all eight patients with indeterminate colitis. Seven patients, including one with indeterminate colitis presented with pancolitis, and one patient had fistulous Crohn's disease. Extra-intestinal



**Figure 2** Endoscopic images showing signs of mucosal inflammation of sigmoid segment affected by diverticulosis coli, the inflammatory signs are stressed along the crests of the colonic folds in (A), and diffusely spread in (B).

manifestations included peripheral arthritis ( $n = 5$ ), uveitis ( $n = 2$ ) axial spondylitis ( $n = 1$ ), and erythema nodosum ( $n = 1$ ).

High erythrocyte sedimentation rate was found in 41 (69%) patients at presentation, exceeding in frequency decreased serum albumin or anemia (30% and 29%, respectively,  $P < 0.001$ ). A total of 51 radiographic examinations were performed including barium enema ( $n = 36$ ), small bowel follow-through ( $n = 8$ ), and abdominal computerized tomography ( $n = 7$ ), and depicted features suggestive of IBD in 17 (29%) patients (sensitivity 0.36 and PPV 0.79). An average of five (range 3-17) biopsies were obtained from separate segments of the colon and usually from affected and normally appearing mucosa on each endoscopic examination. Histology of surgically removed bowel segments was further evaluated in eight patients.

Infiltration of inflammatory cells was confined to the mucosa in the majority of patients [35 (95%) patients with ulcerative colitis, 10 (71%) with Crohn's disease, and seven (87%) with indeterminate colitis], and extended from the mucosa with reactive involvement of muscularis propria in the remaining patients. The inflammatory infiltrate was transmural in one patient with Crohn's disease. Crypt abscesses were identified in 26 (70%) patients with ulcerative colitis, four (29%) with Crohn's disease, and four (50%) with indeterminate colitis. Granulomas on the other hand, were recognized in three (8%) patients with ulcerative colitis, five (36%) with Crohn's disease, and four (50%) with indeterminate colitis.

### Management and course of the disease

Mesalazine preparations, corticosteroids and azathioprine were administered to either induce or maintain remission in a total of 43 (73%), 23 (40%) and seven (12%) patients, respectively. No marked side effects were reported during a median follow-up period of 10 years (range 1-14). Remission was induced in 27 (73%), seven (50%) and six (75%) patients with ulcerative colitis, Crohn's disease and indeterminate colitis, respectively, within a median interval of 6 mo (range 1-21).

Eighteen (30%) patients were considered to

have refractory disease that necessitated surgical intervention, such as sigmoid resection, partial colectomy, pancolectomy, or ileal or ileocecal resection. However, postoperative recurrence was documented in two patients with Crohn's disease and postoperative morbidity and mortality was 66% and 6%, respectively. Two patients died due to terminal cholangiocarcinoma, one patient postoperatively (*Klebsiella pneumonia* sepsis), and three patients due to causes unrelated to IBD, with an overall mortality of 10%.

### SCAD

Diverticulosis coli was present in 61% of patients with OAC. Five (8%) patients showed features that were suggestive of a diagnosis of SCAD. All five had endoscopic as well as histopathological features of colitis that affected the sigmoid colon, with sparing of the rectum and proximal colon. Endoscopic examinations showed either a circumferential localization of erythema, granularity and friability, and sparing of the ostia, as shown in Figure 2A, or diffuse periosteal distribution of erythema, as shown in Figure 2B. Two patients underwent surgery and remained in remission without maintenance treatment. The other three patients were treated initially with a course of mesalazine preparations and were further maintained in remission by increasing daily fluid intake and using fiber-rich laxative preparations. These patients were retrospectively considered to have SCAD and not IBD as initially diagnosed.

### DISCUSSION

This retrospective cohort study showed that 5% of IBD patients who attended our referral center were aged  $> 60$  years old, and could be categorized as having OAC. On the other hand, 8% of patients with OAC have retrospectively non-IBD colitis that the so-called OAC implicates a broader diagnosis than IBD.

With respect to the predominance of ulcerative colitis, anatomic location in Crohn's disease, presenting symptoms, management, and postoperative morbidity and mortality, our findings were in agreement with earlier observations<sup>[10,19-21]</sup>. Unlike our findings, higher incidence rates of IBD in older patients and higher frequency rates of isolated proctitis within the ulcerative colitis subgroup have been reported<sup>[22-24]</sup>. The difference in these rates is very likely related to the retrospective nature of the studies. Noticeably, two patients developed cholangiocarcinoma and none developed colorectal carcinoma during the follow-up period. The available data disallowed further disclosure of underlying sclerosing cholangitis. The use of immunosuppressive agents in this older population appears to warrant broadened application, even if there is little objective data on which to base this practice<sup>[25]</sup>. The use of infliximab as an anti-inflammatory treatment in patients with IBD has been reported to be safe, including in those aged  $> 60$  years old<sup>[26]</sup>. However, there appears to be a significant risk of deleterious and fatal adverse events when infliximab is used in older patients<sup>[27]</sup>. More



safety data about the use of biological agents in older populations are needed, especially when more new agents with proven efficacy are evolving.

Some attribute one-third of the small incidence peak of IBD in this age group to ischemia<sup>[28]</sup>. However, the chronic course of the disease and the emergence of refractory colitis made the diagnosis of ischemic colitis unlikely in our study, along with the histological findings that were also not supportive of a diagnosis of ischemic colitis.

A recognized pitfall in clinical practice appeared in this series; misdiagnosing SCAD as IBD in 8% of patients<sup>[29-33]</sup>. SCAD has long been recognized as an example of the overlap of IBD and diverticular disease<sup>[14,16,29,30]</sup>. The pathogenesis of this apparently distinct form of colitis is unclear<sup>[14]</sup>.

Factors such as age and the high predilection of Crohn's disease for distal localization in older patients contribute to the simultaneous occurrence of both disorders in this population<sup>[34]</sup>. Differentiating between IBD and SCAD imposes a challenging task to the clinician as well as the pathologist. Clinical evaluation, laboratory tests, radiological results and endoscopic examinations (especially in diffuse type), in addition to histological studies, may be misleading. Sometimes even intestinal resection cannot provide the clinician with a definitive diagnosis. Luminal mucosal inflammation, although unusual, may occur in diverticular disease due to redundancy and mucosal prolapse<sup>[35]</sup>. When the luminal inflammation appears in what is called crescentic fold disease, a diagnosis of SCAD seems more probable<sup>[36]</sup>. A diagnosis of SCAD becomes more difficult when the inflammation affects a colon segment diffusely. The histology of SCAD may closely mimic ulcerative colitis and the hallmarks of Crohn's disease<sup>[16,37]</sup>. To complicate the issue, many cases of SCAD seem to respond post operatively favorably to treatment with mesalazine preparations similar to that given for IBD<sup>[30]</sup>. That is why a definitive diagnosis may remain obscure for a long time, and only the course of the disease may bring to light the underlying nature of the disorder. Newly emerging instruments in the diagnostic panel such as serological markers<sup>[38-40]</sup>, advanced radiological examinations such as contrast-enhanced magnetic resonance imaging<sup>[41]</sup>, wireless capsule video endoscopy<sup>[42]</sup>, and double-balloon small enteroscopy<sup>[43]</sup> may facilitate an early and correct definite diagnosis. These diagnostic modalities appear to be valuable for patients who have indeterminate colitis or who are failing medical therapy. The multiple harvest of biopsy specimens at each endoscopic session seems to be helpful in differentiating colitis in segmental fashion, especially SCAD that can be cured by limited resection of affected segments, although this conclusion is based on limited data from this retrospective study.

In summary, OAC is not an infrequent problem for the gastroenterologist and should be considered in the evaluation of older patients with clinical features suggestive of IBD. This entity is broader than IBD alone and therefore more challenging. Extra awareness is

required in order to avoid an erroneous diagnosis purely based on histological mimicry of changes seen in SCAD when diagnosing IBD in the presence of diverticulosis coli, and taking multiple biopsies from each part of the colon is recommended.

## COMMENTS

### Background

Inflammatory bowel disease (IBD) is a lifelong uncontrolled inflammation of the intestinal mucosa that mainly affects the young age group but also older individuals.

### Research frontiers

Diagnosis of IBD in older patients may be difficult because it can easily be confused with other forms of colitis commonly occurring in this age group, such as segmental colitis associated with diverticular disease (SCAD).

### Applications

Old-age colitis (OAC) is not an infrequent problem for the gastroenterologist and should be considered in the evaluation of older patients with clinical features suggestive of IBD. Extra awareness is required in order to avoid an erroneous diagnosis.

### Terminology

OAC refers to patients older than 60 years affected by a broad group of colonic diseases, such as infection, carcinoma, drug-induced disease, vasculitis, microscopic colitis, ischemic colitis, and IBD. SCAD has been found to masquerade as IBD on both a clinical and histological basis.

### Peer review

The authors described the characteristics of Dutch patients with chronic IBD with a first diagnosis above 60 years of age, also known as OAC, and highlighted one of the conditions that has the appearance of IBD, namely SCAD. This is an interesting study. The authors concluded that OAC is not an infrequent problem for the gastroenterologist and should be considered in the evaluation of older patients with clinical features suggestive of IBD.

## REFERENCES

- 1 Guindi M, Riddell RH. Indeterminate colitis. *J Clin Pathol* 2004; **57**: 1233-1244
- 2 Satsangi J, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut* 2006; **55**: 749-753
- 3 Hanauer SB. Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities. *Inflamm Bowel Dis* 2006; **12** Suppl 1: S3-S9
- 4 Binder V, Both H, Hansen PK, Hendriksen C, Kreiner S, Torp-Pedersen K. Incidence and prevalence of ulcerative colitis and Crohn's disease in the County of Copenhagen, 1962 to 1978. *Gastroenterology* 1982; **83**: 563-568
- 5 Sinclair TS, Brunt PW, Mowat NA. Nonspecific proctocolitis in northeastern Scotland: a community study. *Gastroenterology* 1983; **85**: 1-11
- 6 Morris T, Rhodes J. Incidence of ulcerative colitis in the Cardiff region 1968-1977. *Gut* 1984; **25**: 846-848
- 7 Andres PG, Friedman LS. Epidemiology and the natural course of inflammatory bowel disease. *Gastroenterol Clin North Am* 1999; **28**: 255-281, vii
- 8 Loftus EV Jr, Silverstein MD, Sandborn WJ, Tremaine WJ, Harmsen WS, Zinsmeister AR. Ulcerative colitis in Olmsted County, Minnesota, 1940-1993: incidence, prevalence, and survival. *Gut* 2000; **46**: 336-343
- 9 Riegler G, Tartaglione MT, Carratu R, D'Inca R, Valpiani D, Russo MI, Papi C, Fiorentini MT, Ingrosso M, Andreoli A, Vecchi M. Age-related clinical severity at diagnosis in 1705 patients with ulcerative colitis: a study by GISC (Italian Colon-Rectum Study Group). *Dig Dis Sci* 2000; **45**: 462-465
- 10 Grimm IS, Friedman LS. Inflammatory bowel disease in the elderly. *Gastroenterol Clin North Am* 1990; **19**: 361-389
- 11 Harpaz N, Sachar DB. Segmental colitis associated with



- diverticular disease and other IBD look-alikes. *J Clin Gastroenterol* 2006; **40**: S132-S135
- 12 **Manousos ON**, Truelove SC, Lumsden K. Prevalence of colonic diverticulosis in general population of Oxford area. *Br Med J* 1967; **3**: 762-763
  - 13 **Hughes LE**. Postmortem survey of diverticular disease of the colon. I. Diverticulosis and diverticulitis. *Gut* 1969; **10**: 336-344
  - 14 **Peppercorn MA**. The overlap of inflammatory bowel disease and diverticular disease. *J Clin Gastroenterol* 2004; **38**: S8-S10
  - 15 **Lennard-Jones JE**. Classification of inflammatory bowel disease. *Scand J Gastroenterol Suppl* 1989; **170**: 2-6; discussion 16-19
  - 16 **Gledhill A**, Dixon MF. Crohn's-like reaction in diverticular disease. *Gut* 1998; **42**: 392-395
  - 17 **Triantafyllidis JK**, Emmanouilidis A, Pomonis E, Cheracakis P, Hereti I, Merikas E, Nicolakis D, Argyros N. Ulcerative colitis in the elderly: clinical patterns and outcome in 51 Greek patients. *J Gastroenterol* 2001; **36**: 312-316
  - 18 **Rutgeerts P**, Van Assche G, Vermeire S. Optimizing anti-TNF treatment in inflammatory bowel disease. *Gastroenterology* 2004; **126**: 1593-1610
  - 19 **Michelassi F**, Block GE. Surgical management of Crohn's disease. *Adv Surg* 1993; **26**: 307-322
  - 20 **Kadish SL**, Brandt LJ. Inflammatory bowel disease in the elderly. In: Kirshner JB, Shorter RG, editors. *Inflammatory Bowel Disease*. 4th ed. Philadelphia: Williams & Wilkins, 1995: 390-406
  - 21 **Polito JM 2nd**, Childs B, Mellits ED, Tokayer AZ, Harris ML, Bayless TM. Crohn's disease: influence of age at diagnosis on site and clinical type of disease. *Gastroenterology* 1996; **111**: 580-586
  - 22 **Robertson DJ**, Grimm IS. Inflammatory bowel disease in the elderly. *Gastroenterol Clin North Am* 2001; **30**: 409-426
  - 23 **Stonnington CM**, Phillips SF, Melton LJ 3rd, Zinsmeister AR. Chronic ulcerative colitis: incidence and prevalence in a community. *Gut* 1987; **28**: 402-409
  - 24 **Softley A**, Myren J, Clamp SE, Bouchier IA, Watkinson G, de Dombal FT. Factors affecting recurrence after surgery for Crohn's disease. *Scand J Gastroenterol Suppl* 1988; **144**: 31-34
  - 25 **Jones HW**, Hoare AM. Does ulcerative colitis behave differently in the elderly? *Age Ageing* 1988; **17**: 410-414
  - 26 **Seiderer J**, Goke B, Ochsenuhn T. Safety aspects of infliximab in inflammatory bowel disease patients. A retrospective cohort study in 100 patients of a German University Hospital. *Digestion* 2004; **70**: 3-9
  - 27 **Ljung T**, Karlson P, Schmidt D, Hellstrom PM, Lapidus A, Janczewska I, Sjoqvist U, Lofberg R. Infliximab in inflammatory bowel disease: clinical outcome in a population based cohort from Stockholm County. *Gut* 2004; **53**: 849-853
  - 28 **Brandt LJ**. Bloody diarrhea in an elderly patient. *Gastroenterology* 2005; **128**: 157-163
  - 29 **Shepherd NA**. Pathological mimics of chronic inflammatory bowel disease. *J Clin Pathol* 1991; **44**: 726-733
  - 30 **Shepherd NA**. Diverticular disease and chronic idiopathic inflammatory bowel disease: associations and masquerades. *Gut* 1996; **38**: 801-802
  - 31 **Makapugay LM**, Dean PJ. Diverticular disease-associated chronic colitis. *Am J Surg Pathol* 1996; **20**: 94-102
  - 32 **Van Rosendaal GM**, Andersen MA. Segmental colitis complicating diverticular disease. *Can J Gastroenterol* 1996; **10**: 361-364
  - 33 **Yantiss RK**, Odze RD. Diagnostic difficulties in inflammatory bowel disease pathology. *Histopathology* 2006; **48**: 116-132
  - 34 **Carr N**, Schofield PF. Inflammatory bowel disease in the older patient. *Br J Surg* 1982; **69**: 223-225
  - 35 **Mathus-Vliegen EM**, Tytgat GN. Polyp-simulating mucosal prolapse syndrome in (pre-) diverticular disease. *Endoscopy* 1986; **18**: 84-86
  - 36 **Gore S**, Shepherd NA, Wilkinson SP. Endoscopic crescentic fold disease of the sigmoid colon: the clinical and histopathological spectrum of a distinctive endoscopic appearance. *Int J Colorectal Dis* 1992; **7**: 76-81
  - 37 **Makapugay LM**, Dean PJ. Diverticular disease-associated chronic colitis. *Am J Surg Pathol* 1996; **20**: 94-102
  - 38 **Joossens S**, Reinisch W, Vermeire S, Sendid B, Poulain D, Peeters M, Geboes K, Bossuyt X, Vandewalle P, Oberhuber G, Vogelsang H, Rutgeerts P, Colombel JF. The value of serologic markers in indeterminate colitis: a prospective follow-up study. *Gastroenterology* 2002; **122**: 1242-1247
  - 39 **Linskens RK**, Mallant-Hent RC, Groothuismink ZM, Bakker-Jonges LE, van de Merwe JP, Hooijkaas H, von Blomberg BM, Meuwissen SG. Evaluation of serological markers to differentiate between ulcerative colitis and Crohn's disease: pANCA, ASCA and agglutinating antibodies to anaerobic coccoid rods. *Eur J Gastroenterol Hepatol* 2002; **14**: 1013-1018
  - 40 **Laghi A**, Borrelli O, Paolantonio P, Dito L, Buena de Mesquita M, Falconieri P, Passariello R, Cucchiara S. Contrast enhanced magnetic resonance imaging of the terminal ileum in children with Crohn's disease. *Gut* 2003; **52**: 393-397
  - 41 **Schreyer AG**, Seitz J, Feuerbach S, Rogler G, Herfarth H. Modern imaging using computer tomography and magnetic resonance imaging for inflammatory bowel disease (IBD) AU1. *Inflamm Bowel Dis* 2004; **10**: 45-54
  - 42 **Legnani P**, Abreu MT. Use of capsule endoscopy for established Crohn's disease. *Gastrointest Endosc Clin N Am* 2006; **16**: 299-306
  - 43 **Oshitani N**, Yukawa T, Yamagami H, Inagawa M, Kamata N, Watanabe K, Jinno Y, Fujiwara Y, Higuchi K, Arakawa T. Evaluation of deep small bowel involvement by double-balloon enteroscopy in Crohn's disease. *Am J Gastroenterol* 2006; **101**: 1484-1489

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RAPID COMMUNICATION

## Effect of probiotic *Lactobacillus rhamnosus* GG intervention on global serum lipidomic profiles in healthy adults

Riina A Kekkonen, Marko Sysi-Aho, Tuulikki Seppänen-Laakso, Ilkka Julkunen, Heikki Vapaatalo, Matej Orešič, Riitta Korpela

Riina A Kekkonen, Heikki Vapaatalo, Riitta Korpela, University of Helsinki, Institute of Biomedicine, Pharmacology, University of Helsinki, Helsinki 00014, Finland

Riina A Kekkonen, Riitta Korpela, Valio Ltd, Research Centre, Helsinki 00370, Finland

Marko Sysi-Aho, Tuulikki Seppänen-Laakso, Matej Orešič, VTT Technical Research Centre of Finland, Espoo 02044, Finland

Ilkka Julkunen, National Public Health Institute, Department of Viral Diseases and Immunology, Helsinki 00300, Finland

**Author contributions:** Kekkonen RA, Korpela R, Vapaatalo H and Julkunen I designed and conceived the clinical intervention; Seppänen-Laakso T performed the lipidomic analysis; Orešič M and Sysi-Aho M analysed the data; Kekkonen RA wrote the paper.

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**Correspondence to:** Riitta Korpela, PhD, Institute of Biomedicine, Pharmacology, PO Box 63, University of Helsinki, Helsinki 00014, Finland. [riitta.korpela@valio.fi](mailto:riitta.korpela@valio.fi)

Telephone: +358-10-3813026 Fax: +358-10-3813019

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### Abstract

**AIM:** To investigate the effect of three weeks' intervention with a probiotic *Lactobacillus rhamnosus* GG (LGG) bacteria on global serum lipidomic profiles and evaluate whether the changes in inflammatory variables (CRP, TNF- $\alpha$  and IL-6) are reflected in the global lipidomic profiles of healthy adults.

**METHODS:** We performed UPLC/MS-based global lipidomic platform analysis of serum samples ( $n = 26$ ) in a substudy of a randomised, double-blind, placebo-controlled 3-wk clinical intervention trial investigating the immunomodulatory effects of probiotics in healthy adults.

**RESULTS:** A total of 407 lipids were identified, corresponding to 13 different lipid classes. Serum samples showed decreases in the levels of lysophosphatidylcholines (LysoGPCho), sphingomyelins (SM) and several glycerophosphatidylcholines (GPCho), while triacylglycerols (TAG) were mainly increased in the probiotic LGG group during the intervention. Among the inflammatory variables, IL-6 was moderately

associated by changes in global lipidomic profiles, with the top-ranked lipid associated with IL-6 being the proinflammatory LysoGPCho (20:4). There was a weak association between the lipidomic profiles and the two other inflammatory markers, TNF- $\alpha$  and CRP.

**CONCLUSION:** This was the first study to investigate the effects of probiotic intervention on global lipidomic profiles in humans. There are indications that probiotic LGG intervention may lead to changes in serum global lipid profiles, as reflected in decreased GPCho, LysoGPCho and SM as well as mainly increased TAG.

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**Key words:** Probiotic; *Lactobacillus rhamnosus* GG; Lipidomic; Inflammatory mediators; Healthy adults

**Peer reviewers:** Laura E Matarese, MS, RD, LDN, FADA, CNSD, Thomas E. Starzl Transplantation Institute, UPMC Montefiore, 7 South, 3459 Fifth Avenue, Pittsburgh, PA 15213, United States; Dr. Raymund Rabe Razonable, Division of Infectious Diseases, Mayo Clinic Institution, 200 First Street SW, Rochester 55905, United States; Dr. Francesco Costa, Dipartimento di Medicina Interna, U.O. di Gastroenterologia, Università di Pisa, Via Roma, Pisa 67-56122, Italy

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### INTRODUCTION

The new global metabolic profiling techniques or 'metabolomics', have made it possible to measure large numbers of different metabolites, and are currently being applied to increase our understanding of the health and disease continuum<sup>[1]</sup>. High-dimensional lipid analysis technologies (lipidomics) provide an opportunity to measure lipids on a broad scale<sup>[2]</sup>. The majority of lipid pathways involved in lipid metabolism are known, but new lipid metabolites are being discovered all the time. It is not fully known how different pathways affect individual

metabolic health and how changes in the regulation of these pathways can influence major metabolic and inflammatory diseases like diabetes, cardiovascular and inflammatory diseases and obesity<sup>[2]</sup>. The new analytical capacity of lipidomics as a branch of metabolomics can increase our understanding of lipid biology, improve the characterisation of global lipid profiles and result in the identification of previously unknown changes in lipid metabolism<sup>[3]</sup>.

One study has evaluated the transgenomic metabolic effects of two probiotic lactobacilli in mice<sup>[4]</sup>, but as far as we know, the effects of probiotics on global lipidomic profiles in humans have not been characterised before. Previously, probiotics have been shown to possess immunomodulatory effects in *in vitro* assays, animal models and clinical trials<sup>[5,6]</sup>, and their effects have been studied mainly in specific conditions such as allergy<sup>[7,8]</sup> and inflammatory diseases<sup>[9]</sup>.

In the present study, we characterised the effect of the probiotic *Lactobacillus rhamnosus* GG (LGG) on global serum lipidomic profiles and investigated whether the changes in inflammatory variables (CRP, TNF- $\alpha$  and IL-6) are reflected in the lipidomics profiles of healthy adults.

## MATERIALS AND METHODS

### Subjects

The subjects were healthy adults ( $n = 26$ , 14 females, 12 males) with a mean age of 42 years (range 23–55) and a mean BMI of 24 kg/m<sup>2</sup> (range 20–30). The subjects were recruited to the study by an advertisement in the Helsinki area. The inclusion criteria were being healthy (no chronic illnesses), taking regular exercise (at least three times per week), and not participating in any other clinical trial. The exclusion criteria were milk allergy (due to the nature of the study products), use of antibiotics during the 2 mo before the study, acute gastrointestinal disorders during the 2 mo before the study, gastrointestinal diseases and related medication, pregnancy, and lactation. Before entering the study, the subjects gave their written informed consent. The study protocol was approved by the Ethics Committee of the Hospital District of Helsinki and Uusimaa.

### Intervention

The present study was a substudy of a randomised, double-blind and placebo-controlled parallel group intervention study investigating the immunomodulatory effects of probiotic bacteria with four treatment groups; placebo, LGG, *Bifidobacterium animalis* ssp. *lactis* Bb12 and *Propionibacterium freudenreichii* ssp. *shermanii* JS<sup>[10]</sup>. Only the placebo ( $n = 15$ ) and LGG ( $n = 11$ ) groups were included in the present pilot study since the LGG exhibited the best anti-inflammatory potential in the original study. Prior to the intervention period, there was a 3-wk run-in period during which no probiotic-containing products were allowed. For 3 wk thereafter, the subjects consumed either a 250 mL milk-based fruit drink containing LGG bacteria (ATCC 53103,  $6.2 \times 10^7$  cfu/mL) or a similar

placebo drink without probiotic bacteria daily. A list of probiotic-containing products was given to the subjects, and they were asked not to consume any other probiotic-containing products at any point during the study. Otherwise they were allowed to eat freely. Venous blood samples from the antecubital vein were taken at baseline and after the 3-wk intervention. The blood samples were stored at -70°C for global lipidomic analyses.

### Inflammatory variables and serum lipids

Serum levels of C-reactive protein (CRP) were measured by a high-sensitivity particle-enhanced immunoturbidimetric CRP assay using a Tina-quant CRP (latex) high-sensitivity reagent and a Roche Hitachi 912 analyser (Roche Diagnostics GmbH, Mannheim, Germany) with a detection limit of 0.04 mg/L. All samples were over the detection limit. Cytokine levels (TNF- $\alpha$ , IL-6) in serum were determined using Quantikine HS, Human TNF- $\alpha$ /TNFSF1A (Catalog Number HSTA00D) and IL-6 (HS600B) immunoassays purchased from R&D Systems (Minneapolis MN, USA) according to the manufacturer's instructions. The detection limit was 0.5 pg/mL for TNF- $\alpha$  and 0.16 pg/mL for IL-6. Serum total cholesterol, high-density lipoprotein (HDL) cholesterol and triglyceride concentrations were measured with their respective enzymatic kits from Roche Diagnostics using an autoanalyser (Roche/Hitachi 912 Automatic Analyzer). Low-density lipoprotein (LDL) cholesterol concentrations were calculated using Friedewald's equation<sup>[11]</sup>.

### Sample preparation for global lipidomic analysis

An aliquot (10  $\mu$ L) of an internal standard mixture containing 11 lipid classes and 0.05 mol/L sodium chloride (10  $\mu$ L) was added to the serum samples (10  $\mu$ L). The lipids were extracted with chloroform/methanol (2:1, 100  $\mu$ L). A standard mixture containing 3 labelled standard lipids was added (10  $\mu$ L) to the extracts. The sample order for LC/MS analysis was determined by randomization.

### Global lipidomics analysis by UPLC/MS

Lipid extracts were analysed on a Waters Q-ToF Premier mass spectrometer combined with an Acquity Ultra Performance LC<sup>TM</sup> (UPLC). The column, which was kept at 50°C, was an Acquity UPLC<sup>TM</sup> BEH C18 10 mm  $\times$  50 mm with 1.7  $\mu$ m particles. The binary solvent system included (A) water (1% 1 mol/L NH<sub>4</sub>Ac, 0.1% HCOOH) and (B) LC/MS grade (Rathburn) acetonitrile/isopropanol (5:2, 1% 1 mol/L NH<sub>4</sub>Ac, 0.1% HCOOH). The gradient started from 65% A/35% B, reached 100% B in 6 min and remained there for the next 7 min. The total run time including a 5 min re-equilibration step was 18 min. The flow rate was 0.200 mL/min and the injected amount 0.75  $\mu$ L. The temperature of the sample organiser was set at 10°C.

The lipid profiling was carried out on Waters Q-ToF Premier mass spectrometer using ESI+ mode. The data were collected at mass range of  $m/z$  300–1200 with a scan duration of 0.2 s. The source temperature was

set at 120°C, and nitrogen was used as desolvation gas (800 L/h) at 250°C. The voltages of the sampling cone and capillary were 39 V and 3.2 kV, respectively. Reserpine (50 µg/L) was used as the lock spray reference compound (5 µL/min; 10 s scan frequency).

Data were processed using MZmine software, version 0.60<sup>[12]</sup>. Lipids were identified using an internal spectral library or by tandem mass spectrometry using UPLC/MS/MS as described previously<sup>[13]</sup>. The normalisation of lipidomic data was performed as follows: All monoacyl lipids except cholesterol esters, such as monoacylglycerols and monoacylglycerol-PL, were normalised with GPCho (17:0/0:0); diacyl lipids except ethanolamine PL were normalised with GPCho (17:0/17:0); ceramides with Cer (d18:1/17:0); the diacyl ethanolamine phospholipids with GPEtn (17:0/17:0); and the TG and cholesterol esters with TG (17:0/17:0/17:0). Other (unidentified) molecular species were calibrated with GPCho (17:0/0:0) for a retention time of < 300 s, GPCho (17:0/17:0) for between 300 s and 410 s, and TG (17:0/17:0/17:0) for higher retention times. Only the identified lipid molecular species were included in further data analyses.

### Lipid nomenclature

Lipids from the global lipidomic analysis were named according to Lipid Maps (<http://www.lipidmaps.org>). For example, lysophosphatidylcholine (LysoGPCho) with 16:0 fatty acid chain was named monoacyl-glycerophosphocholine GPCho (16:0/0:0). In cases where the fatty acid composition could not be determined, the total number of carbons and double bonds was marked. For example, a phosphatidylcholine species PCho (16:0/20:4) is represented as GPCho (36:4). However, GPCho (36:4) could also represent other molecular species, for example, GPCho (20:4/16:0) or GPCho (18:2/18:2).

### Statistical analysis

Principal component analysis (PCA) and partial least squares discriminant analysis (PLS/DA) were utilised as modelling methods for clustering and discrimination<sup>[14]</sup>. PLS/DA is a pattern recognition technique that correlates variation in the dataset with class membership. The resulting projection model gives latent variables (LVs) that focus on maximum separation ("discrimination"). The random subsets cross-validation method<sup>[15]</sup> and Q2 scores were used to develop the models. The VIP (variable importance in the projection) values<sup>[16]</sup> were calculated to identify the most important molecular species for the clustering of specific groups. PLS/DA and PCA analyses were performed using Matlab, version 7.2 (Mathworks, Natick, MA, USA) and PLS Toolbox, version 4.0, of the Matlab package (Eigenvector Research, Wenatchee, WA, USA). All other analyses were performed using R statistical language (<http://www.r-project.org/>).

Comparisons between the levels of selected molecular species were performed using the paired Wilcoxon test. For the PLS/DA analyses as well as paired univariate analyses, the data were first log-transformed for

each lipid so that  $X = \log(z_2/z_1)$ , where  $z_2$  was the lipid concentration at 21 d and  $z_1$  at baseline. With such transformation, the distribution of data was closer to normal and the within-person changes could better be analysed. Chance detection plotting was used to account for multiple hypothesis testing in univariate comparisons. The chance detection plot described how many lipids show more significant differences at random than those actually observed.

In order to assess whether any of the inflammatory variables were explained by global lipidomic profile data, we regressed global lipidomic profile data on selected inflammatory variables using an elastic net method<sup>[17]</sup>. The method selects an optimal subset of lipids, based on predictive performance of the regression model using extensive bootstrap-based cross-validation. The model is selected by minimum cross-validation-error criterion, which balances the bias against the variance of the estimates. For these analyses, the data were first log-transformed for each lipid/clinical variable so that  $X = \log(z_2/z_1)$ .

## RESULTS

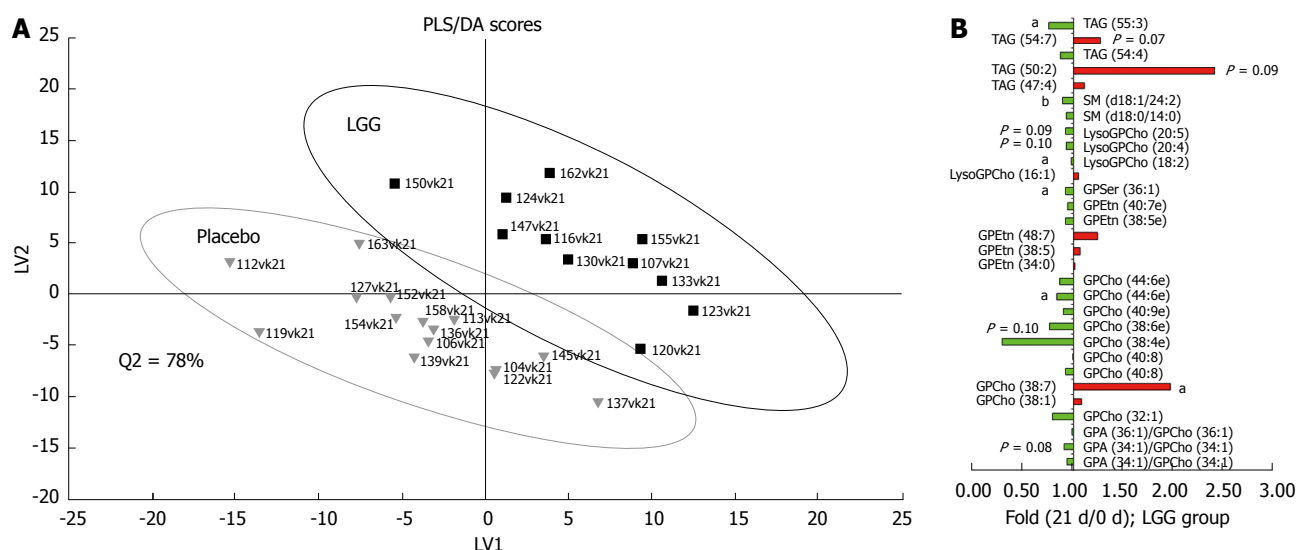
### Serum lipids

The mean (SD) baseline value (mmol/L) for total cholesterol was 5.1 (1.1), for LDL cholesterol 3.1 (1.0), for HDL cholesterol 1.5 (0.4) and for triglycerides 1.0 (0.4) in the placebo group and, in the LGG group, 5.4 (1.2), 3.3 (1.0), 1.5 (0.4) and 1.4 (1.1), respectively. The mean (SD) change (mmol/L) during the 3-wk intervention in total cholesterol was 0.2 (0.5), in LDL cholesterol 0.1 (0.5), in HDL cholesterol 0.1 (0.2) and in triglycerides 0.0 (0.5) in the placebo group and 0.0 (0.4), 0.1 (0.3), 0.0 (0.2) and 0.0 (0.6) in the LGG group, respectively. There were no significant differences in serum lipids during the intervention.

### Global lipidomic analysis

The global lipidomic analysis led to 407 identified lipid species, corresponding to 13 different lipid classes. PCA analysis revealed that no major outliers exist in the data, thus confirming that any changes detected in further analyses would not be due to specific outliers. PLS/DA analysis revealed that the LGG and the placebo groups differed at baseline, and therefore only the within-person changes were utilised in the later statistical analyses. PLS/DA analysis on log-transformed data indicated that the global lipidomic profiles of the groups were separable (Figure 1A). In the LGG group, significant changes ( $P < 0.05$ ) in lipids were observed during the intervention using paired Wilcoxon test, although when accounting for multiple hypothesis testing by using the chance detection plot, no lipid changes were found to be significant within the 95% confidence interval. However, the VIP analysis revealed some common trends in the lipidomic profile data. Decreased LysoGPCho and sphingomyelins (SM), mainly decreased glycerophosphatidylcholines (GPCho) and mainly increased triacylglycerols (TAG) were among the most important





**Figure 1** A: Partial least squares discriminant analysis (PLS/DA) of global serum lipidomic data during the probiotic intervention in healthy adults. The labels in the picture indicate subject ID numbers; B: Fold changes for the top 30 ranking lipids contributing to the PLS/DA model based on VIP analysis (variable important in the projection) (<sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.001$ ).

variables contributing to the separation between the LGG and the placebo groups (Figure 1B).

### Associations between global lipidomics profiles and inflammatory variables

In order to investigate whether the changes in inflammatory variables during the 3-wk intervention were reflected in global lipidomic profiles, we regressed the lipidomic profile data on measured serum TNF- $\alpha$ , IL-6, and CRP values (Figure 2). The results revealed that a reasonably good model based on global lipidomic profiles was found for the proinflammatory cytokine IL-6, while the regression model was poor for CRP and TNF- $\alpha$ . The top-ranking lipid associated with the changes in IL-6 was the proinflammatory LysoGPCCho (20:4) (Figure 2).

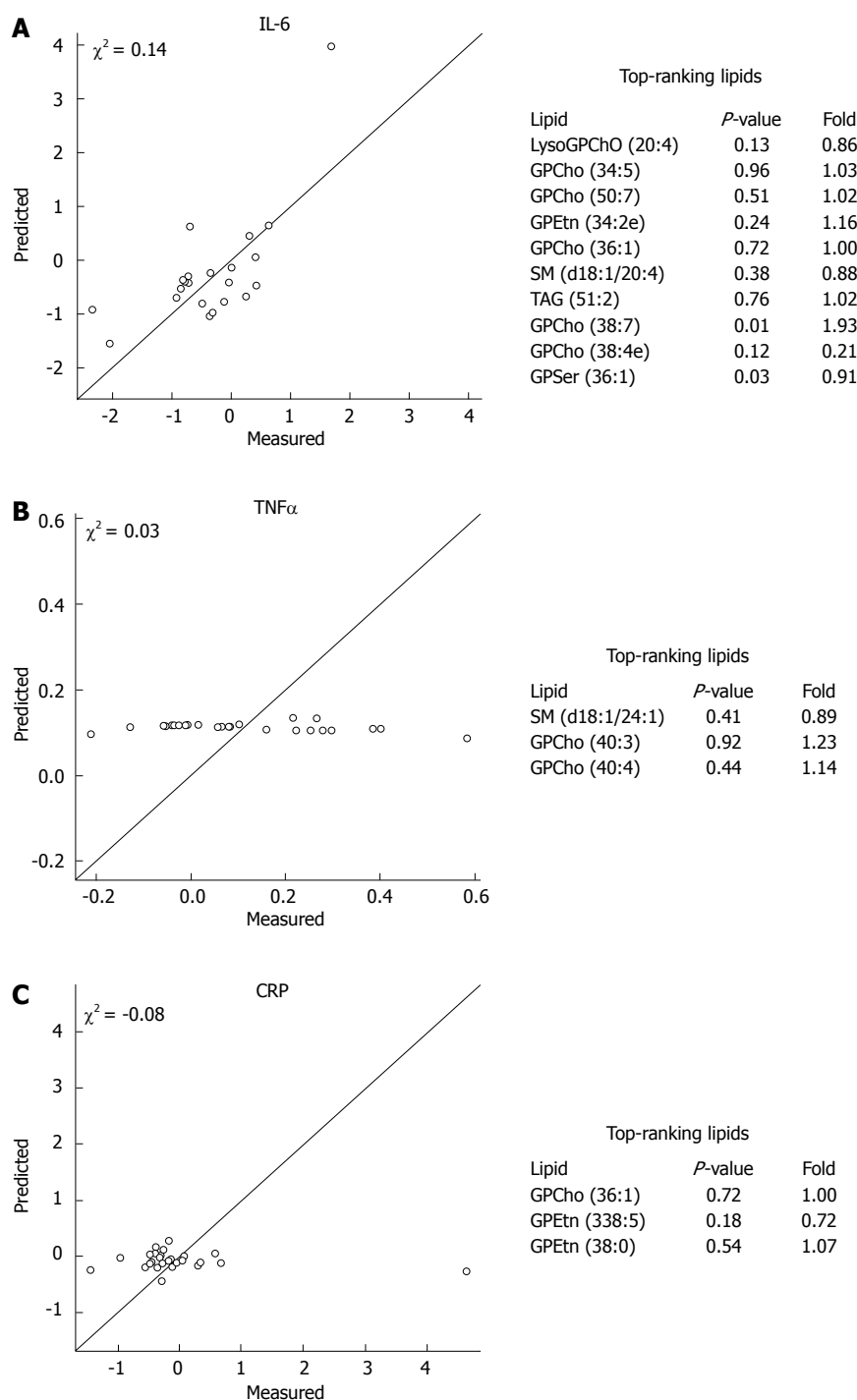
## DISCUSSION

This study is the first to apply lipidomic techniques to analyse the global lipidomic profiles of healthy adults after a probiotic intervention. The lipidomic platform has already been applied in multiple studies investigating the pathophysiology of different diseases<sup>[18–21]</sup>. In the present study, we characterised the effect of probiotic LGG on global serum lipidomic profiles and investigated whether the changes in inflammatory variables (CRP, TNF- $\alpha$  and IL-6) were reflected in global lipidomic profiles in healthy adults. We observed that the probiotic LGG intervention may lead to changes in global lipidomic profiles.

We found a trend towards decreased LysoGPCCho after the probiotic LGG intervention. LysoGPCCho, derived from phosphatidylcholines, are mediators that affect numerous functions in many types of cells, from proliferation and survival to migration and secretion. They are also involved in oxidative metabolism, angiogenesis, and carcinogenesis<sup>[22]</sup>. LysoGPCCho is a

major atherogenic lipid of oxidised LDL<sup>[23]</sup>, and it has been associated with vascular inflammation, endothelial dysfunction and coronary atherosclerosis<sup>[24]</sup>. LysoGPCCho induces an increase in several inflammatory cytokines (IL-1 $\beta$ , IL-6, TNF- $\alpha$ ) in human peripheral mononuclear cells (PBMCs)<sup>[25]</sup>. Therefore, the reduction of LysoGPCCho in the present study could be related to our, and previous, results showing a decreased production of TNF- $\alpha$  in PBMCs in healthy adults<sup>[10,26]</sup>. Interestingly, a high LysoGPCCho level has been connected also to inflammatory bowel disease (IBD)<sup>[27,28]</sup>, impaired mucosal barrier function and increased gut permeability<sup>[29–32]</sup>. LGG has not been effective in treating Crohn's disease<sup>[33,34]</sup>, but it has been shown to maintain remission in patients with ulcerative colitis<sup>[35]</sup>. In addition, LGG normalises gut permeability<sup>[36,37]</sup> and enhances mucosal integrity and epithelial cell survival<sup>[38,39]</sup>. Taken together, the decrease in LysoGPCCho after LGG intervention observed in the present study may be one of the metabolic events behind the beneficial clinical effects of LGG seen in ulcerative colitis and in normalised gut permeability.

In the present study, we also observed a decrease in SM after the LGG intervention. SM is a major membrane sphingolipid and the precursor of important signalling molecules like ceramide and sphingosine<sup>[40]</sup>. Recent studies reveal that metabolites of SM are critically important for the initiation and maintenance of diverse aspects of immune cell activation and also function as regulators of inflammatory responses<sup>[41–43]</sup>. High concentrations of sphingolipids and lipids of the SM/ceramide pathway have been connected to inflammatory processes in the development of atherosclerosis<sup>[44]</sup> and IBD<sup>[45,46]</sup>. The harmful effects of these lipids may be partly mediated via the production of reactive oxygen species in cells<sup>[42,47]</sup>. As in the case of LysoGPCCho, the generation of ceramide by sphingomyelinases from SM and epithelial oxidative stress might contribute to



**Figure 2** Cross-validated regression model prediction for IL-6 (A), TNF- $\alpha$  (B) and CRP (C) based on the global lipidomic profile data with the top-ranking lipids explaining the changes in inflammatory variables during the probiotic intervention.

the disturbed barrier function seen in diseases such as IBD<sup>[45]</sup>. Therefore, the decrease in SM seen after LGG intervention in the present study may also contribute to the beneficial effects on gut barrier function seen in the previous intervention studies with LGG<sup>[36-39]</sup>.

Although we observed some common trends in the global lipidomic profiles after LGG intervention, one should notice that, when accounting for multiple hypothesis testing using the chance detection plot, no lipid changes were found to be statistically significant. This suggests that the study was either underpowered for investigations of global lipidomic profile changes in the described setting, or the observed baseline differences in the global lipidomic profiles dominated over responses

to the intervention, masking potential effects of the LGG intervention. One thus cannot exclude the possibility that some of the significant changes were detected by chance. Furthermore, this pilot study was conducted with healthy individuals alone, whereas the effect of LGG intervention on global lipidomic profiles should also be investigated in subjects suffering from inflammatory conditions or disturbed gut barrier function before further conclusions can be drawn.

In conclusion, there are indications that probiotic LGG intervention may lead to changes in global lipidomic profiles reflected in decreased LysoGPCho and SM, mainly decreased GPCho and mainly elevated TAG. These changes may contribute, for example, to the

metabolic events behind the beneficial effects of LGG on gut barrier function seen in previous studies. IL-6 was moderately associated with the changes in lipidomic profiles. Lipidomics may provide powerful tools for identifying new biomarkers behind the clinical effects of probiotic intervention trials and for establishing relationships between molecular profiles and other known data from the same individual.

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## COMMENTS

### Background

The new global metabolic profiling technique 'metabolomics' has made it possible to measure a large number of metabolites, and is currently being applied to increase the understanding of the health and disease continuum. The new analytical capacity of lipidomics as a branch of metabolomics can increase the understanding of lipid biology, improve the characterisation of global lipid profiles and result in the identification of previously unknown changes in lipid metabolism. Probiotics have been mostly studied in the prevention and treatment of different gastrointestinal diseases and allergy, but the mode of action of probiotics is poorly understood.

### Innovations and breakthroughs

This study is the first to apply lipidomic techniques to analyse the global lipidomic profiles of healthy adults after a probiotic intervention. Lipidomic analysis showed that there were decreases in the levels of lysophosphatidylcholines (LysoGPCCho), sphingomyelins (SM) and several glycerophosphatidylcholines (GPCCho), and increases in triacylglycerols (TAG) in the probiotic LGG group. These changes may contribute, for example, to the metabolic events behind the beneficial effects of LGG on gut barrier function seen in previous studies.

### Applications

Metabolomics and lipidomics may help to understand the action mechanisms of different agents, such as probiotics.

### Terminology

Lipidomics is a branch of metabolomics which enables identification of lipids in a large scale. Probiotic bacteria are defined as living microorganisms that have beneficial effects on human health.

### Peer review

This was an interesting paper and overall it was well written and well-presented. However, this is a small study. One good point is the randomized cohort. The study population was healthy individuals and the results may not be applicable to a population with lipid-related disease states. There were multiple comparisons and one thus cannot exclude the possibility that some of the significant changes were detected by chance.

## REFERENCES

- 1 Schnackenberg LK, Beger RD. Monitoring the health to disease continuum with global metabolic profiling and systems biology. *Pharmacogenomics* 2006; **7**: 1077-1086
- 2 Wiest MM, Watkins SM. Biomarker discovery using high-dimensional lipid analysis. *Curr Opin Lipidol* 2007; **18**: 181-186
- 3 Oresic M, Vidal-Puig A, Hanninen V. Metabolomic approaches to phenotype characterization and applications to complex diseases. *Expert Rev Mol Diagn* 2006; **6**: 575-585
- 4 Martin FP, Wang Y, Sprenger N, Yap IK, Lundstedt T, Lek P, Rezzi S, Ramadan Z, van Bladeren P, Fay LB, Kochhar S, Lindon JC, Holmes E, Nicholson JK. Probiotic modulation of symbiotic gut microbial-host metabolic interactions in a humanized microbiome mouse model. *Mol Syst Biol* 2008; **4**: 157
- 5 Ezendam J, van Loveren H. Probiotics: immunomodulation and evaluation of safety and efficacy. *Nutr Rev* 2006; **64**: 1-14
- 6 Galdeano CM, de Moreno de LeBlanc A, Vinderola G, Bonet ME, Perdigon G. Proposed model: mechanisms of immunomodulation induced by probiotic bacteria. *Clin Vaccine Immunol* 2007; **14**: 485-492
- 7 Boyle RJ, Tang ML. The role of probiotics in the management of allergic disease. *Clin Exp Allergy* 2006; **36**: 568-576
- 8 Osborn DA, Sinn JK. Probiotics in infants for prevention of allergic disease and food hypersensitivity. *Cochrane Database Syst Rev* 2007; CD006475
- 9 Limdi JK, O'Neill C, McLaughlin J. Do probiotics have a therapeutic role in gastroenterology? *World J Gastroenterol* 2006; **12**: 5447-5457
- 10 Kekkonen RA, Lummela N, Karjalainen H, Latvala S, Tynkkynen S, Jarvenpaa S, Kautiainen H, Julkunen I, Vapaatalo H, Korpela R. Probiotic intervention has strain-specific anti-inflammatory effects in healthy adults. *World J Gastroenterol* 2008; **14**: 2029-2036
- 11 Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 1972; **18**: 499-502
- 12 Katajamaa M, Miettinen J, Oresic M. MZmine: toolbox for processing and visualization of mass spectrometry based molecular profile data. *Bioinformatics* 2006; **22**: 634-636
- 13 Yetukuri L, Katajamaa M, Medina-Gomez G, Seppanen-Laakso T, Vidal-Puig A, Oresic M. Bioinformatics strategies for lipidomics analysis: characterization of obesity related hepatic steatosis. *BMC Syst Biol* 2007; **1**: 12
- 14 Barker M, Rayens W. Partial least squares for discrimination. *J Chemometr* 2003; **17**: 166-173
- 15 Wise BM, Gallagher NB, Bro R, Shaver JM, Windig W, Koch JS. PLS Toolbox 3.5 for use with Matlab. Manson: Eigenvector Research Inc, 2005
- 16 Wold S, Esbensen K, Geladi P. Principal component analysis. *In Chemometr Intell Lab Syst* 1987; **2**: 37-52
- 17 Zou H, Hastie T. Regularization and variable selection via the elastic net. *J R Statist Soc* 2005; **B67**: 301-320
- 18 Medina-Gomez G, Gray SL, Yetukuri L, Shimomura K, Virtue S, Campbell M, Curtis RK, Jimenez-Linan M, Blount M, Yeo GS, Lopez M, Seppanen-Laakso T, Ashcroft FM, Oresic M, Vidal-Puig A. PPAR gamma 2 prevents lipotoxicity by controlling adipose tissue expandability and peripheral lipid metabolism. *PLoS Genet* 2007; **3**: e64
- 19 Medina-Gomez G, Virtue S, Lelliott C, Boiani R, Campbell M, Christodoulides C, Perrin C, Jimenez-Linan M, Blount M, Dixon J, Zahn D, Thresher RR, Aparicio S, Carlton M, Colledge WH, Kettunen MI, Seppanen-Laakso T, Sethi JK, O'Rahilly S, Brindle K, Cinti S, Oresic M, Burcelin R, Vidal-Puig A. The link between nutritional status and insulin sensitivity is dependent on the adipocyte-specific peroxisome proliferator-activated receptor-gamma2 isoform. *Diabetes* 2005; **54**: 1706-1716
- 20 Laaksonen R, Katajamaa M, Paiva H, Sysi-Aho M, Saarinen L, Junni P, Latjohann D, Smet J, Van Coster R, Seppanen-Laakso T, Lehtimäki T, Soini J, Oresic M. A systems biology strategy reveals biological pathways and plasma biomarker candidates for potentially toxic statin-induced changes in muscle. *PLoS ONE* 2006; **1**: e97
- 21 Pietiläinen KH, Sysi-Aho M, Rissanen A, Seppanen-Laakso T, Yki-Jarvinen H, Kaprio J, Oresic M. Acquired obesity is associated with changes in the serum lipidomic profile independent of genetic effects - a monozygotic twin study. *PLoS ONE* 2007; **2**: e218
- 22 Goetzl EJ, Rosen H. Regulation of immunity by lysophingolipids and their G protein-coupled receptors. *J Clin Invest* 2004; **114**: 1531-1537
- 23 Inoue N. Vascular C-reactive protein in the pathogenesis of coronary artery disease: role of vascular inflammation and oxidative stress. *Cardiovasc Hematol Disord Drug Targets* 2006; **6**: 227-231
- 24 Kougias P, Chai H, Lin PH, Lumsden AB, Yao Q, Chen C.

- Lysophosphatidylcholine and secretory phospholipase A2 in vascular disease: mediators of endothelial dysfunction and atherosclerosis. *Med Sci Monit* 2006; **12**: RA5-RA16
- 25 **Shi Y**, Zhang P, Zhang L, Osman H, Mohler ER 3rd, Macphee C, Zalewski A, Postle A, Wilensky RL. Role of lipoprotein-associated phospholipase A2 in leukocyte activation and inflammatory responses. *Atherosclerosis* 2007; **191**: 54-62
- 26 **Schultz M**, Linde HJ, Lehn N, Zimmermann K, Grossmann J, Falk W, Scholmerich J. Immunomodulatory consequences of oral administration of *Lactobacillus rhamnosus* strain GG in healthy volunteers. *J Dairy Res* 2003; **70**: 165-173
- 27 **Minami T**, Tojo H, Shinomura Y, Matsuzawa Y, Okamoto M. Increased group II phospholipase A2 in colonic mucosa of patients with Crohn's disease and ulcerative colitis. *Gut* 1994; **35**: 1593-1598
- 28 **Haapamaki MM**, Gronroos JM, Nurmi H, Irjala K, Alanen KA, Nevalainen TJ. Phospholipase A2 in serum and colonic mucosa in ulcerative colitis. *Scand J Clin Lab Invest* 1999; **59**: 279-287
- 29 **Tagesson C**, Franzen L, Dahl G, Westrom B. Lysophosphatidylcholine increases rat ileal permeability to macromolecules. *Gut* 1985; **26**: 369-377
- 30 **Otamiri T**, Sjodahl R, Tagesson C. Lysophosphatidylcholine potentiates the increase in mucosal permeability after small-intestinal ischaemia. *Scand J Gastroenterol* 1986; **21**: 1131-1136
- 31 **Karlqvist PA**, Franzen L, Sjodahl R, Tagesson C. Lysophosphatidylcholine and taurodeoxycholate increase stomach permeability to different-sized molecules. *Scand J Gastroenterol* 1986; **21**: 1039-1045
- 32 **Sawai T**, Lampman R, Hua Y, Segura B, Drongowski RA, Coran AG, Harmon CM. Lysophosphatidylcholine alters enterocyte monolayer permeability via a protein kinase C/Ca<sup>2+</sup> mechanism. *Pediatr Surg Int* 2002; **18**: 591-594
- 33 **Prantera C**, Scribano ML, Falasco G, Andreoli A, Luzi C. Ineffectiveness of probiotics in preventing recurrence after curative resection for Crohn's disease: a randomised controlled trial with *Lactobacillus* GG. *Gut* 2002; **51**: 405-409
- 34 **Bousvaros A**, Guandalini S, Baldassano RN, Botelho C, Evans J, Ferry GD, Goldin B, Hartigan L, Kugathasan S, Levy J, Murray KF, Oliva-Hemker M, Rosh JR, Tolia V, Zhouludev A, Vanderhoof JA, Hibberd PL. A randomized, double-blind trial of *Lactobacillus* GG versus placebo in addition to standard maintenance therapy for children with Crohn's disease. *Inflamm Bowel Dis* 2005; **11**: 833-839
- 35 **Zocco MA**, dal Verme LZ, Cremonini F, Piscaglia AC, Nista EC, Candelli M, Novi M, Rigante D, Cazzato IA, Ojetti V, Armuzzi A, Gasbarrini G, Gasbarrini A. Efficacy of *Lactobacillus* GG in maintaining remission of ulcerative colitis. *Aliment Pharmacol Ther* 2006; **23**: 1567-1574
- 36 **Isolauri E**, Majamaa H, Arvola T, Rantala I, Virtanen E, Arvilommi H. *Lactobacillus casei* strain GG reverses increased intestinal permeability induced by cow milk in suckling rats. *Gastroenterology* 1993; **105**: 1643-1650
- 37 **Gotteland M**, Cruchet S, Verbeke S. Effect of *Lactobacillus* ingestion on the gastrointestinal mucosal barrier alterations induced by indometacin in humans. *Aliment Pharmacol Ther* 2001; **15**: 11-17
- 38 **Lam EK**, Tai EK, Koo MW, Wong HP, Wu WK, Yu L, So WH, Woo PC, Cho CH. Enhancement of gastric mucosal integrity by *Lactobacillus rhamnosus* GG. *Life Sci* 2007; **80**: 2128-2136
- 39 **Yan F**, Cao H, Cover TL, Whitehead R, Washington MK, Polk DB. Soluble proteins produced by probiotic bacteria regulate intestinal epithelial cell survival and growth. *Gastroenterology* 2007; **132**: 562-575
- 40 **Kee TH**, Vit P, Melendez AJ. Sphingosine kinase signalling in immune cells. *Clin Exp Pharmacol Physiol* 2005; **32**: 153-161
- 41 **Olivera A**, Rivera J. Sphingolipids and the balancing of immune cell function: lessons from the mast cell. *J Immunol* 2005; **174**: 1153-1158
- 42 **Won JS**, Singh I. Sphingolipid signaling and redox regulation. *Free Radic Biol Med* 2006; **40**: 1875-1888
- 43 **Melendez AJ**. Sphingosine kinase signalling in immune cells: potential as novel therapeutic targets. *Biochim Biophys Acta* 2008; **1784**: 66-75
- 44 **Bismuth J**, Lin P, Yao Q, Chen C. Ceramide: a common pathway for atherosclerosis? *Atherosclerosis* 2008; **196**: 497-504
- 45 **Homaidan FR**, El-Sabban ME, Chakroun I, El-Sibai M, Dbaiibo GS. IL-1 stimulates ceramide accumulation without inducing apoptosis in intestinal epithelial cells. *Mediators Inflamm* 2002; **11**: 39-45
- 46 **Sakata A**, Yasuda K, Ochiai T, Shimeno H, Hikishima S, Yokomatsu T, Shibuya S, Soeda S. Inhibition of lipopolysaccharide-induced release of interleukin-8 from intestinal epithelial cells by SMA, a novel inhibitor of sphingomyelinase and its therapeutic effect on dextran sulphate sodium-induced colitis in mice. *Cell Immunol* 2007; **245**: 24-31
- 47 **Andrieu-Abadie N**, Gouaze V, Salvayre R, Levade T. Ceramide in apoptosis signaling: relationship with oxidative stress. *Free Radic Biol Med* 2001; **31**: 717-728

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## Factors that influence outcome in non-invasive and invasive treatment in polycystic liver disease patients

Josué Barahona-Garrido, Jesús Camacho-Escobedo, Eduardo Cerda-Contreras, Jorge Hernández-Calleros, Jesús K Yamamoto-Furusho, Aldo Torre, Misael Uribe

Josué Barahona-Garrido, Jesús Camacho-Escobedo, Eduardo Cerda-Contreras, Jorge Hernández-Calleros, Jesús K Yamamoto-Furusho, Aldo Torre, Misael Uribe, Department of Gastroenterology, National Institute of Health Sciences and Nutrition "Salvador Zubirán", Mexico City 14000, Mexico

**Author contributions:** Barahona-Garrido J and Torre A designed research; Barahona-Garrido J, Camacho-Escobedo J, Cerda-Contreras E, and Torre A performed research; Barahona-Garrido J, Hernández-Calleros J, Yamamoto-Furusho JK, and Torre A analyzed data and wrote the paper.

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**Correspondence to:** Aldo Torre, MD, Department of Gastroenterology, National Institute of Health Sciences and Nutrition "Salvador Zubirán". Vasco de Quiroga 15, Colonia Sección XVI, Tlalpan, Mexico City 14000, Mexico. [detoal@yahoo.com](mailto:detoal@yahoo.com)

Telephone: +52-55-55733418 Fax: +52-55-56550942

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second IT in 66.7% (OF 100%). Follow-up mortality rate was 0.

**CONCLUSION:** Presence of symptoms, elevated AP, and CC are associated with IT requirement. HRT is associated with presence of symptoms and IT requirement. Patients with BMI > 25 have a trend to be susceptible to IT complications. The proportions of complications are higher in FHR and second IT groups. RS is more frequent after OF.

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**Key words:** Hepatic cysts; Open fenestration; Laparoscopic fenestration; Hepatic resection; Recurrence of symptoms; Hormonal replacement therapy

**Peer reviewer:** Frank J Burczynski, Professor, Faculty of Pharmacy, University of Manitoba, 50 Sifton Road, Winnipeg, Manitoba, R3T 2N2, Canada

Barahona-Garrido J, Camacho-Escobedo J, Cerda-Contreras E, Hernández-Calleros J, Yamamoto-Furusho JK, Torre A, Uribe M. Factors that influence outcome in non-invasive and invasive treatment in polycystic liver disease patients. *World J Gastroenterol* 2008; 14(20): 3195-3200 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3195.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3195>

### Abstract

**AIM:** To evaluate the factors that influence outcome of both non-invasive and invasive treatment of polycystic liver disease.

**METHODS:** Analysis of clinical files of patients with complete follow-up from July 1986 to June 2006.

**RESULTS:** Forty-one patients (male, 7; female, 34), 47.8 ± 11.9 years age, and 5.7 ± 6.7 years follow-up, were studied. Alkaline phosphatase (AP) elevation (15% of patients) was associated with the requirement of invasive treatment (IT,  $P = 0.005$ ). IT rate was higher in symptomatic than non-symptomatic patients (65.4% vs 14.3%,  $P = 0.002$ ), and in women taking hormonal replacement therapy (HRT) ( $P = 0.001$ ). Cysts complications (CC) were more frequent (22%) in the symptomatic patients group ( $P = 0.023$ ). Patients with body mass index (BMI) > 25 (59%) had a trend to complications after IT ( $P = 0.075$ ). Abdominal pain was the most common symptom (56%) and indication for IT (78%). Nineteen patients (46%) required a first IT: 12 open fenestration (OF), 4 laparoscopic fenestration (LF) and 3 fenestration with hepatic resection (FHR). Three required a second IT, and one required a third procedure. Complications due to first IT were found in 32% (OF 16.7%, LF 25%, FHR 66.7%), and in the

### INTRODUCTION

Polycystic liver disease (PLD) is an autosomic dominant disease related to chromosome 19 alterations in patients with hepatic involvement alone and in chromosomes 4 and 16 in those with renal cysts<sup>[1]</sup>. At autopsy, the prevalence appears to be 0.13%-0.6%, and the association to renal cysts about 30%<sup>[2-4]</sup>. PLD is generally asymptomatic and incidentally diagnosed. Abdominal pain, distension, early satiety, nausea and vomiting are common and hepatic function is rarely affected. Ultrasound (US) and computed tomography (CT) are common diagnostic methods. PLD is considered when more than 5 cysts are observed in the liver that typically appear anechoic, round and smooth-walled with distal echo enhancement in the US<sup>[5]</sup> (Figure 1A); and in CT with homogeneous fluid density and without wall or content enhancement after contrast administration<sup>[6]</sup>

(Figure 1B). Invasive treatment (IT) such as cyst aspiration with sclerotherapy, open fenestration (OF), laparoscopic fenestration (LF), fenestration plus hepatic resection (FHR), and hepatic transplantation in selected cases are preferred<sup>[1,4,7-10]</sup>. Symptoms are controlled with surgical liver volume reduction<sup>[11]</sup>. In a Mexican population, surgery for PLD has shown to modify quality of life<sup>[12]</sup>. We report a descriptive analysis of 41 patients with clinical and imaging diagnosis of PLD that have a complete follow-up during July 1986 to June 2006, making special emphasis on factors that influence outcome of both non-invasive and invasive treatment.

## MATERIALS AND METHODS

### Materials

This is a descriptive study of all patients diagnosed with PLD diagnosis from July 1986 to June 2006 at the National Institute of Health Sciences and Nutrition "Salvador Zubirán". Forty-nine clinical records with PLD diagnosis were reviewed; however, 8 patients were excluded from the analysis because the follow-up was not completed.

### Methods

Variables as gender, age at diagnosis, time of diagnosis delay, BMI, symptoms, diagnosis method, cyst diameter, cyst complications (CC), liver function tests (LFT: bilirubin, transaminases, AP, lactic dehydrogenase, gamma glutamyl transpeptidase, albumin, prothrombin time, glucose and complete blood count at diagnosis, 1 and 6 months, and at 1, 5, 10, 15 and 20 years), comorbidity, extrahepatic cysts, hormonal replacement therapy (HRT) intake, IT requirement, IT complications, recurrence of symptoms (RS), and follow up procedures and outcome were analyzed.

### Statistical analysis

Statistical data are expressed as mean  $\pm$  SD. Numerical variables were analyzed by *t*-test and the categorical with  $\chi^2$  test or Fisher's exact test. A *P* value  $\leq 0.05$  was accepted as being statistically significant. The SPSS 13.0 software (SPSS Inc., Chicago, Illinois, 2004) statistical program was used for the analysis.

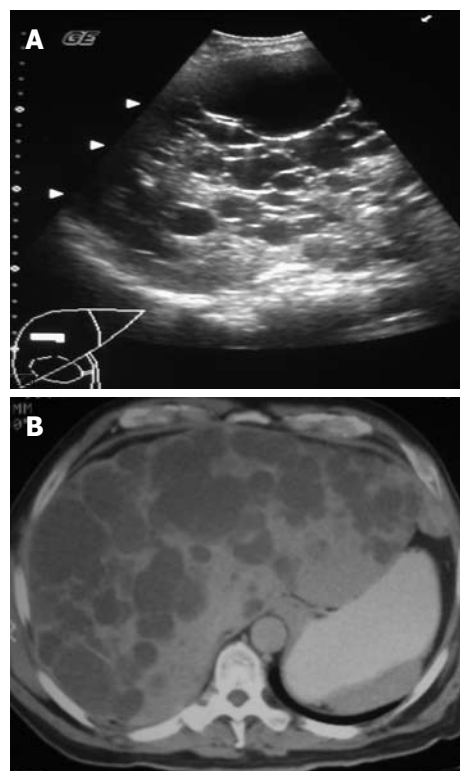
## RESULTS

### Demographical data

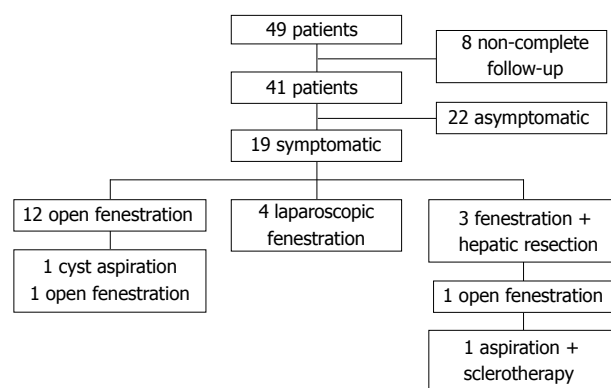
A total of 49 patients with PLD were evaluated in our institution. Eight patients lost follow-up and were not included for the analysis. Forty-one (male 7, female 34) complete patients files were included (Figure 2). The mean age at diagnosis was  $47.8 \pm 11.9$  years (range 27-82) and the mean follow-up time was 5.7 years. Eighteen (44%) patients had familiar history of PLD. Demographical data is shown in Table 1.

### Hormonal replacement therapy is associated with symptoms occurrence

We found that 23.8% of postmenopausal women



**Figure 1** US (A) and CT (B) images showing numerous hepatic cysts in a 47-year-old asymptomatic male patient.



**Figure 2** Clinical course and interventions of 49 PLD patients (July 1986-June 2006).

were taking HRT. In those patients the prevalence of symptoms at diagnosis was significantly higher than those without HRT (100% *vs* 43.8%, *P* = 0.039; OR = 2.286; 95% CI, 1.311-3.984). The requirement of IT also was higher in the HRT group than in non-HRT (80% *vs* 6.3%, *P* = 0.001; OR = 20; 95% CI, 35-115). The size of the major cyst was not associated with HRT (*P* > 0.05). The oral contraceptive intake was not associated with occurrence of symptoms (*P* > 0.05).

### Symptoms, diagnosis, and PLD complications

Based on the initial symptoms the mean time of diagnosis delay was 2.8 (range 0-9) years. We found that 65.4% of the symptomatic *versus* 14.3% of non-symptomatic patients at diagnosis required IT (*P* = 0.002, OR = 11.333, 95% CI, 2.068-62.105). The symptoms described were abdominal pain 56%, early satiety 42%, increase of the abdominal perimeter 34%, and nausea 12%. Two patients

**Table 1** Demographical data, extrahepatic cysts and comorbidity

N (Male/Female)	41 (7/34)
Age at diagnosis (range, yr)	47.8 ± 11.9 (27-82)
Mean diagnosis retard time (range, yr)	2.8 (0-9)
Mean follow up time (yr)	5.7
Familiar history of PLD	44%
Body mass index at diagnosis	
≤ 25	41%
> 25-≤ 30	44%
> 30	15%
Largest cyst diameter (range, cm)	8.2 ± 4.9 (2-25)
Extrahepatic cysts	
Renal cysts	68%
Pancreatic cysts	15%
Spleen cysts	8%
Ovary cysts	2%
Comorbidity	
Arterial hypertension	46%
Chronic renal insufficiency	24%
Dyslipidemia	15%
Hypothyroidism	7%
Type 2 diabetes	5%
Gastroesophageal reflux	5%

(4.9%) had portal hypertension manifested by esophageal varices and splenomegaly, and none of them was found with ascites or encephalopathy.

During the follow up time, symptomatic patients at diagnosis developed more CC than non-symptomatic (29.6% *vs* 0,  $P = 0.023$ ). At this time, nine (22%) patients had CC: cyst infection 5 (55.6%), cyst bleeding 3 (33.3%), and cholangitis 1 (11.1%), at a mean time of 4 months (range 0-9, Table 2). In this group of patients, four (44.4%) had a complication at the time of diagnosis (cyst infection 2, cyst bleeding 2).

Renal cysts were present in 68% of PLD patients (Table 1). Liver CC were seen in 25% of patients with renal cysts, and in 7.7% of patients without them ( $P > 0.05$ ).

At diagnosis, overweight (BMI > 25 but ≤ 30), was found in 44%; and obesity (BMI > 30), in 15%. Patients with BMI > 25 had a trend to develop surgical complications ( $P = 0.075$ ) such as abdominal pain, bleeding or infection (Table 1), and no significant association was found with the presence of symptoms, largest cyst diameter, CC, or IT requirement ( $P > 0.05$ ).

Diagnosis was achieved by US in 78% and by CT in 22%. The largest cyst mean diameter was  $8.2 \pm 4.9$  cm (range 2-25) and no association to symptoms or requirement of IT ( $P > 0.05$ ) were found. The largest cyst mean diameter was  $12.3 \pm 8.8$  cm in postmenopausal women taking HRT *vs*  $8.3 \pm 3.9$  cm in those without HRT ( $P > 0.05$ ).

#### **Elevated alkaline phosphatase at diagnosis is associated with IT requirement**

No significant alterations in bilirubin, transaminases, lactic dehydrogenase, gamma glutamyl transpeptidase, albumin, prothrombin time, glucose, and in complete blood count, were found at diagnosis or during follow up.

**Table 2** Outcome of PLD patients

Symptomatic patients at diagnosis	27 (66%)
Abdominal pain	15
Early satiety	12
Increase of abdominal perimeter	9
Nausea	3
Complications during follow up	9 (22%)
Cyst infection	5
Cyst hemorrhage	3
Cholangitis	1
Invasive treatment patients	19 (46%)
Open fenestration	12
Laparoscopic fenestration	4
Fenestration + hepatic resection	3
Overall symptoms recurrence	4 (17%)
Open fenestration	3
Laparoscopic fenestration	0
Fenestration + hepatic resection	1
Surgery complications	6 (32%)
Bleeding	3
Infection	2
Pain	1

Because we considered IT requirement as an end point in PLD patients, we studied its association with elevated levels of LFT at diagnosis, finding that AP was elevated ( $\geq 132$  IU/mL) in 15.5% of patients. During follow-up, 100% of patients in the elevated AP group required IT *versus* 35.5% in the normal AP group ( $P = 0.005$ , OR = 2.818, 95% CI, 1.753-4.530).

#### **Invasive treatment and complications**

Abdominal pain was the most common indication for surgery (78%). Other indications were satiety (10%), cyst hemorrhage (5%), and cyst infection (5%).

Nineteen patients (46%) required IT (OF 12, LF 4, FHR 3) to control symptoms (Figure 2), at a mean time of 19 months (range 0-85) after PLD diagnosis. Mean age for the first IT was  $47 \pm 10$  years.

Because of RS, 3 patients required a second IT at a mean time of 19 months (range 8-24): 2 (16.7%) in the OF group (cyst aspiration 1, OF 1) and 1 (33.3%) in the FHR group (OF 1). The last patient required a third IT (aspiration + sclerotherapy) 6 months later. None of the patients in the LF group showed RS.

Complications due to the first IT were found in 6 (32%) patients. Three (16.7%) patients had complications in the OF group (bleeding 3), 1 (25%) in the LF group (severe abdominal pain 1), and 2 (66.7%) in the FHR group (bleeding 1, infection 1; Table 2). No significant difference in complications between OF and LF was found.

Two (66.7%) patients after a second IT (OF 2) developed important complications (hemorrhage 1, pleural effusion 1), and the one who required a third IT had severe abdominal pain. Follow up mortality rate was 0.

## **DISCUSSION**

This is one of the largest series published to date and includes a large follow-up time in both symptomatic and non-symptomatic patients with a detailed description

and associations to anthropometric and biochemical data, HRT intake, CC, IT requirement, IT complications, and outcome.

As in other case series<sup>[1,9,10]</sup>, female gender predominated (83%), suggesting a possible role of estrogens in the development of liver cysts.

Data shows selective increase in liver cyst and parenchymal volume in female patients receiving postmenopausal estrogen therapy<sup>[13]</sup>, but it is not clear if it correlates with symptoms and IT requirement. The association found between HRT, the presence of symptoms and IT requirement supports that HRT has an important role in the development of symptoms and so in the requirement of IT in PLD patients. Interestingly HRT was not associated to the size of the major cyst, suggesting that symptoms are not due to the size of cysts but maybe to the number of cysts or the liver volume occupied by them. As in other liver diseases, PLD may contraindicate HRT. Further and prospective studies are recommended to confirm such associations.

As others centers that inform that the diagnosis is more common during the fourth and the fifth decade of life<sup>[9,14]</sup>, in Mexican patients seems to be equal.

No other series report the time evolution of PLD patients. We found a diagnosis delay time near to 3 years. This data may indicate that symptoms at the beginning of the disease are absent or mild and appear or increase as time advances, so patients search for medical attention.

Symptoms predicted IT requirement and were associated to a higher incidence of CC (bleeding, infection, *etc*). Most of the series report that the majority of patients are asymptomatic at diagnosis<sup>[1]</sup>, but we found a high prevalence of symptoms (66%) in Mexican patients. We found a higher prevalence of abdominal pain (56%) than reported in other studies (36.5%)<sup>[9]</sup>.

Hepatic failure in PLD patients is rare and few cases have been reported<sup>[15,16]</sup>. We found 2 (4.9%) cases of portal hypertension and none with ascites or encephalopathy. The cause of portal hypertension in these patients is not clear, but might be due to the mass effect that comprises vessels of the portal circulation. A reported rate of 2.5% for portal hypertension has been described<sup>[9]</sup>. Despite hepatomegaly, portal hypertension and its complications (ascites, variceal bleeding, *etc*) remain quite rare<sup>[1]</sup>, interestingly; we report a higher rate of portal hypertension in our patients, maybe due to a longer follow-up time.

In our knowledge this is one of the largest follow-up in a PLD case series (5.7 years). Bistriz *et al*<sup>[9]</sup> reported that in 40 patients with a follow-up time of 4.69 years, 22.5% had cyst bleeding, 12.5% cyst rupture, 12.5% cyst infection, and 2.5% developed portal hypertension. During follow-up symptomatic patients of our study developed similar incidence of CC. We found a significant association between prevalence of symptoms and the development of CC during follow-up ( $P = 0.023$ ). It suggests that symptomatic patients have increased risk factors that predispose CC. The presence of renal cysts did not significantly increase the incidence of liver CC ( $P > 0.05$ ).

The association between anthropometric data and symptoms in PLD has not been studied. We found a trend to surgical complications in patients with BMI  $> 25$  ( $P = 0.075$ ), and no significant association with symptoms, largest cyst diameter, CC, or IT requirement. The BMI is not a reliable data because it is influenced by the large amount of hepatic weight in PLD patients, so other anthropometric measurement must be achieved in future studies, especially to quantify fat tissue. It is known that fat tissue is a hormonal-active tissue, though may influence the clinical presentation and outcome of PLD patients, as happens with HRT.

By far, US is reported as the most used method for diagnosis<sup>[1]</sup>. Our finding supports that US is a good and reliable method to achieve PLD diagnosis and brings important data such as number of cysts, cyst diameter and cyst complications. It is unclear if the cyst diameter is associated to symptoms or IT requirement. We found that despite a large cyst diameter, symptoms or requirement of IT are not related to it. It suggests that what determines the occurrence of symptoms or indicates surgical therapy is not the cyst diameter, but the number of cysts or the liver volume occupied by them. Further studies are necessary to determine the clinical value of these measures, including measure of liver volume.

The LFT in our patients usually were normal and were not associated to symptoms or outcome. We did not find significant alterations at diagnosis or during follow up. In his review, Arnold<sup>[1]</sup> described that LFT are often normal, but in symptomatic patients, AP levels may be elevated in 30%-47%, GGT in 60%-70%, aspartate aminotransferase in up to 27%, and bilirubin in 17%. We studied those variables, and only the elevation of AP ( $\geq 132$  mg/dL) at diagnosis was significantly associated with requirement of IT. This association suggests that elevated AP may be an important serological marker of disease activity and could be used to indicate IT to control symptoms.

Symptoms and complications are reported indications for IT. According to Chen<sup>[4]</sup> and Que *et al*<sup>[17]</sup> these indications include abdominal distention, abdominal pain, early satiety, fatigue, supine dyspnea, infected cysts, dialysis hypotension, bile duct obstruction, severe ascites and uterine prolapse. We found similar indications in our patients, being pain the most frequent.

RS is frequent with the majority of IT modalities. The reported rate of RS for cyst aspiration is up to 100% and probably does not provide definitive therapy<sup>[18]</sup>. The RS for OF is less common. One of the largest series is the one reported by Koperna *et al*<sup>[19]</sup> who described a RS rate of 21%, but also rates between 11%-33% had been reported<sup>[20-23]</sup>. For LF, a recent case series of 6 PLD patients reported by Garcea *et al*<sup>[24]</sup> showed 16% of RS, but also has been reported in up to 4.5%-71%<sup>[22,25]</sup>. In our patients, the RS after a first IT was low (16.7% for OF, 0% for LF, and 33.3% for FHR). The RS rate for OF and for FHR was as expected, interestingly the non-RS after LF is much lower than reported<sup>[23,24,26]</sup>. We think those findings may be due to patient selection criteria.

For OF the reported morbidity rate is 0%-56%<sup>[21,27]</sup>,



for LF 0%-54%<sup>[21,28]</sup>, and for FHR varies from 20% to 100%<sup>[29,30]</sup>. The higher rate of complications in the LF group compared to the OF group may be due to procedure selection criteria. The rate of complications after FHR was high but as expected. Randomized studies are necessary to know the real rate of complications and RS with each procedure, but as the prevalence of the disease is low, studies are difficult to perform.

In summary, the presence of symptoms at diagnosis, and CC during follow-up time is associated with IT requirement. The HRT is associated to the presence of symptoms and IT requirement. The prevalence of symptoms in PLD patients is high and abdominal pain is the most common. Patients with BMI > 25 have a trend to suffer from complications after IT. Cyst diameter is not associated with the presence of symptoms or need for IT. The AP elevation was associated with IT requirement, suggesting that AP may be a marker of disease severity. In near half of the patients, a first IT is performed and complications are frequent, especially in the FHR group, but the proportion of complications due to the second IT is higher. The RS is more frequent after OF, but this fact may be due to patient selection bias.

## COMMENTS

### Background

Polycystic liver disease (PLD) is generally asymptomatic and incidentally diagnosed. For symptomatic patients invasive treatment (IT) such as cyst aspiration with sclerotherapy, fenestration with or without hepatic resection, and hepatic transplant are options of treatment. There are no known associations that could help clinicians to determine the outcome of invasive or non-invasive treatment.

### Research frontiers

Larger and prospective studies are required in order to find other variables that may affect outcome. It is important to evaluate the physiological basis of the impact of hormonal replacement therapy (HRT) on the outcome of PLD patients.

### Innovations and breakthroughs

Knowledge of factors associated with IT requirement, complications, and recurrence of symptoms.

### Applications

Helpful to determine outcome in both invasive and non-invasive treatment of PLD patients.

### Peer review

This is a retrospective study investigating the association for invasive or noninvasive treatment of polycystic liver disease and biochemical abnormalities. It is a well-written and well-designed paper.

## REFERENCES

- 1 Arnold HL, Harrison SA. New advances in evaluation and management of patients with polycystic liver disease. *Am J Gastroenterol* 2005; **100**: 2569-2582
- 2 Milutinovic J, Fialkow PJ, Rudd TG, Agodoa LY, Phillips LA, Bryant JL. Liver cysts in patients with autosomal dominant polycystic kidney disease. *Am J Med* 1980; **68**: 741-744
- 3 Thomsen HS, Thaysen JH. Frequency of hepatic cysts in adult polycystic kidney disease. *Acta Med Scand* 1988; **224**: 381-384
- 4 Chen MF. Surgery for adult polycystic liver disease. *J Gastroenterol Hepatol* 2000; **15**: 1239-1242
- 5 Spiegel RM, King DL, Green WM. Ultrasonography of primary cysts of the liver. *AJR Am J Roentgenol* 1978; **131**: 235-238
- 6 Mortelet KJ, Ros PR. Cystic focal liver lesions in the adult: differential CT and MR imaging features. *Radiographics* 2001; **21**: 895-910
- 7 Koyama I, Fuchinoue S, Urashima Y, Kato Y, Tsuji K, Kawase T, Murakami T, Tojimbara T, Nakajima I, Teraoka S. Living related liver transplantation for polycystic liver disease. *Transpl Int* 2002; **15**: 578-580
- 8 Tan YM, Ooi LL. Highly symptomatic adult polycystic liver disease: options and results of surgical management. *ANZ J Surg* 2004; **74**: 653-657
- 9 Bistriz L, Tamboli C, Bigam D, Bain VG. Polycystic liver disease: experience at a teaching hospital. *Am J Gastroenterol* 2005; **100**: 2212-2217
- 10 Everson GT, Taylor MR, Doctor RB. Polycystic disease of the liver. *Hepatology* 2004; **40**: 774-782
- 11 van Keimpema L, Ruurda JP, Ernst MF, van Geffen HJ, Drenth JP. Laparoscopic fenestration of liver cysts in polycystic liver disease results in a median volume reduction of 12.5%. *J Gastrointest Surg* 2008; **12**: 477-482
- 12 Orozco H, Mercado MA, Hinojosa CA. [Evaluation of 20 years of experience and quality of life in patients surgically treated for liver cystic disease] *Rev Gastroenterol Mex* 2001; **66**: 179-186
- 13 Sherstha R, McKinley C, Russ P, Scherzinger A, Bronner T, Showalter R, Everson GT. Postmenopausal estrogen therapy selectively stimulates hepatic enlargement in women with autosomal dominant polycystic kidney disease. *Hepatology* 1997; **26**: 1282-1286
- 14 Kornprat P, Cerwenka H, Bacher H, El-Shabrawi A, Tillich M, Langner C, Mischinger HJ. Surgical therapy options in polycystic liver disease. *Wien Klin Wochenschr* 2005; **117**: 215-218
- 15 Elias TJ, Bannister KM, Clarkson AR, Faull RJ. Progressive hepatic failure secondary to adult polycystic kidney disease. *Aust N Z J Med* 1999; **29**: 282-283
- 16 Nakanuma Y, Hosoi M, Hayashi M, Hirai N. Adult polycystic liver presenting with progressive hepatic failure. *J Clin Gastroenterol* 1989; **11**: 592-594
- 17 Que F, Nagorney DM, Gross JB Jr, Torres VE. Liver resection and cyst fenestration in the treatment of severe polycystic liver disease. *Gastroenterology* 1995; **108**: 487-494
- 18 Saini S, Mueller P, Ferrucci J. Percutaneous aspiration of hepatic cysts does not provide definitive therapy. *Am J Roentgenol* 1983; **141**: 559-560
- 19 Koperna T, Vogl S, Satzinger U, Schulz F. Nonparasitic cysts of the liver: results and options of surgical treatment. *World J Surg* 1997; **21**: 850-854; discussion 854-855
- 20 Palanivelu C, Rangarajan M, Senthilkumar R, Madankumar MV. Laparoscopic management of symptomatic multiple hepatic cysts: a combination of deroofting and radical excision. *JLS* 2007; **11**: 466-469
- 21 Gigot JF, Jadoul P, Que F, Van Beers BE, Etienne J, Horsmans Y, Collard A, Geubel A, Pringot J, Kestens PJ. Adult polycystic liver disease: is fenestration the most adequate operation for long-term management? *Ann Surg* 1997; **225**: 286-294
- 22 van Erpecum KJ, Janssens AR, Terpstra JL, Tjon A, Tham RT. Highly symptomatic adult polycystic disease of the liver. A report of fifteen cases. *J Hepatol* 1987; **5**: 109-117
- 23 Martin IJ, McKinley AJ, Currie EJ, Holmes P, Garden OJ. Tailoring the management of nonparasitic liver cysts. *Ann Surg* 1998; **228**: 167-172
- 24 Garcea G, Pattenden CJ, Stephenson J, Dennison AR, Berry DP. Nine-year single-center experience with nonparasitic liver cysts: diagnosis and management. *Dig Dis Sci* 2007; **52**: 185-191
- 25 Bai XL, Liang TB, Yu J, Wang WL, Shen Y, Zhang M, Zheng SS. Long-term results of laparoscopic fenestration for patients with congenital liver cysts. *Hepatobiliary Pancreat Dis Int* 2007; **6**: 600-603
- 26 Katkhouda N, Hurwitz M, Gugenheim J, Mavor E, Mason

- RJ, Waldrep DJ, Rivera RT, Chandra M, Campos GM, Offerman S, Trussler A, Fabiani P, Mouiel J. Laparoscopic management of benign solid and cystic lesions of the liver. *Ann Surg* 1999; **229**: 460-466
- 27 **Hansman MF**, Ryan JA Jr, Holmes JH 4th, Hogan S, Lee FT, Kramer D, Biehl T. Management and long-term follow-up of hepatic cysts. *Am J Surg* 2001; **181**: 404-410
- 28 **Kabbej M**, Sauvanet A, Chauveau D, Farges O, Belghiti J. Laparoscopic fenestration in polycystic liver disease. *Br J Surg* 1996; **83**: 1697-1701
- 29 **Soravia C**, Mentha G, Giostra E, Morel P, Rohner A. Surgery for adult polycystic liver disease. *Surgery* 1995; **117**: 272-275
- 30 **Yang GS**, Li QG, Lu JH, Yang N, Zhang HB, Zhou XP. Combined hepatic resection with fenestration for highly symptomatic polycystic liver disease: A report on seven patients. *World J Gastroenterol* 2004; **10**: 2598-2601

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## Hepatitis B virus prevalence and transmission risk factors in inflammatory bowel disease patients at Clementino Fraga Filho university hospital

Yolanda Faia Manhães Tolentino, Homero Soares Fogaça, Cyrla Zaltman, Lia Laura Lewis Ximenes, Henrique Sérgio Moraes Coelho

Yolanda Faia Manhães Tolentino, Homero Soares Fogaça, Cyrla Zaltman, Henrique Sérgio Moraes Coelho, Internal Medicine Department, Gastroenterology Unit of Clementino Fraga Filho, University Hospital, Federal University of Rio de Janeiro, Rio de Janeiro 21941-913, Brazil

Lia Laura Lewis Ximenes, Virology Department, Oswaldo Cruz Foundation 21045-900, Brazil

**Author contributions:** Tolentino YFM, Fogaça HS and Coelho HSM contributed equally to this work; Tolentino YFM and Fogaça HS performed research and wrote the paper; Fogaça HS and Coelho HSM designed research; Zaltman C interviewed the patients and analyzed data and Ximenes LLL did the laboratories analysis.

**Correspondence to:** Yolanda Faia Manhães Tolentino, Internal Medicine Department, Gastroenterology Unit of Clementino Fraga Filho, University Hospital, Federal University of Rio de Janeiro, Rio de Janeiro 21941-913, Brazil. [yolandafaia@superig.com.br](mailto:yolandafaia@superig.com.br)

Telephone: +55-21-25272566 Fax: +55-21-25272566

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patients that have been submitted to surgery to treat IBD complications received more blood transfusions than patients submitted to other surgical interventions ( $P = 0.015$ ).

**CONCLUSION:** There was a high incidence of positive anti-HBc (17%) and positive HBsAg (2.3%) in IBD patient when compared with the overall population (7.9%).

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**Key words:** Inflammatory bowel disease; Hepatitis B virus; Prevalence; Risk factors

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### Abstract

**AIM:** To evaluate the prevalence of hepatitis B virus (HBV) infection in inflammatory bowel disease (IBD) patients that followed up in our hospital and try to identify the possible risk factors involved in this infection transmission.

**METHODS:** This was a cross-sectional study for which 176 patients were selected according to their arrival for the medical interview. All these patients had already IBD diagnosis. The patient was interviewed and a questionnaire was filled out.

**RESULTS:** In the group of 176 patients whom we examined, we found that 17% (30) were anti-HBc positive. Out of 30 patients with positive anti-HBc, 2.3% (4) had positive HBsAg and negative HBV-DNA. In an attempt to identify the possible HBV infection transmission risk factors in IBD patients, it was observed that 117 patients had been submitted to some kind of surgical procedure, but only 24 patients had positive anti-HBc ( $P = 0.085$ ). It was also observed that surgery to treat IBD complications was not a risk factor for HBV infection transmission, since we did not get a statically significant  $P$  value. However, IBD

### INTRODUCTION

The hepatitis B virus (HBV) infection is a worldwide public health problem. There are two billion people infected by HBV, and among these more than 350 million have chronic infection. Patients with chronic infection have a high death risk for hepatic cirrhosis or liver cancer. These two diseases are responsible for about 1 million people dying every year, in spite of the infection incidence falling recently<sup>[1-4]</sup>.

In developed countries the sexual route is responsible for 30% of infections and is the main route of HBV transmission<sup>[3-5]</sup>.

Health professionals, such as surgeons, pathologists, dialysis and chemotherapy technicians, have a high risk of acquiring HBV infections through small skin lesions or through accident with instruments that cut or perforate<sup>[6]</sup>.

Patients with inflammatory bowel disease (IBD)

have high risk of infection by hepatitis viruses B or C<sup>[7]</sup> because during the course of their disease, they need blood transfusions, and sometimes surgical and endoscopic procedures for diagnosis and treatment<sup>[8-10]</sup>. Biancone *et al* observed that in Crohn's disease (CD), 2/3 of the patients will need an intestinal resection and almost 50% will need multiple surgeries<sup>[11]</sup>. It is important to confirm this data to alert health professionals about prevention and early diagnosis of HBV infection, because the steroids and immunosuppressant drugs used in IBD treatment worsen the HBV liver disease. Few studies exist to verify if these drugs influence HBV infection in IBD patients<sup>[12-15]</sup>.

The Clementino Fraga Filho University Hospital is a reference center for IBD diagnosis and treatment. As it is not known exactly what the HBV infection rate in this group of patients in this institution, we decided to do this study.

The first aim of this study was to evaluate the prevalence of HBV infection in IBD patients that followed up in the hospital. The second aim is to evaluate the possible risk factors involved in HBV infection transmission in this patients group.

## MATERIALS AND METHODS

This study was carried out between May 2002 and November 2004, for which 176 patients were recruited. All these patients had clinical, laboratory, radiological, endoscopic and histopathological IBD diagnosis. Included were patients of both sexes, at least 18 years old, for whom medical records were kept by the hospital and who live in Rio de Janeiro State. Patients with infectious, ischemic, actinic, and uncertain colitis were excluded.

The patients were selected, weekly, according to their order of arrival for the medical interview in the hospital IBD ambulatory. After, if the patient allowed us to include him/her in the study, he/she signed an informed consent term. Next, the patient was interviewed and during this interview, a questionnaire was filled out to obtain identification data such as age, sex and IBD type.

In order to identify possible risk factors for HBV infection transmission in this population, the patients were questioned about blood transfusion histories, surgical and endoscopic interventions, dialysis<sup>[16]</sup>, use of endovenous illicit drugs<sup>[17]</sup>, acupuncture treatment, the presence of tattoos<sup>[7]</sup> or "piercings" and if they engaged in promiscuous sex (defined as more than 3 sexual partners in a year or sexual intercourse with prostitutes)<sup>[18]</sup>.

After the interview, 25 mL of blood were obtained from the patient and the material was submitted to the following analyses: qualitative test for total core antibodies; anti-HBc (Kit Diasorin S.p.A.-Italy); qualitative test for HBV antigen; HBsAg (Kit ELISA-Diasorin S.p.A.-Italy) and qualitative PCR-DNA for HBV (which can detect up to 10 particles/serum milliliter), this last analysis being only for patients with positive anti-HBc, patients with positive HBsAg, and for 14 (8%) patients with negative anti-HBc and HBsAg chosen at random.

Table 1 HBV infection transmission risk factors

Risk factors (n = 176)	n (%)
Blood transfusion	47 (26.7)
Surgery	117 (66.5)
Dialysis	0
Endovenous drug use	8 (4.5)
Tattoo	4 (2.3)
Acupuntura	7 (4.0)
"Piercings"	1 (0.6)
SPL	3 (1.7)
Digestive endoscopes	175 (99.4)

Table 2 Anti-HBc and HBsAg result distribution according to IBD type (n = 176)

	Anti-HBc	HBsAg
Positive	17 UC (56.7%) 13 CD (43.3%)	4 (2.3%)
Negative	146 (83%)	172 (97.7%)

Statistical analysis was processed by the SAS<sup>®</sup> software system. Differences were considered significant for an alpha risk of 5%.

Our objective was to verify if there is a significant association between a positive anti-HBc result and any of the risk factors analyzed. For this purpose the following methods were applied: for proportions comparison (qualitative variables) the chi-square test was used ( $\chi^2$ ) or the exact Fisher test. For numeric variables comparison (quantitative) between two groups, the *t*-test was used for independent samples or the Mann-Whitney test, when the variable did not present normal distribution due to great dispersion or for the ordinal nature of the data.

## RESULTS

In our sample there were 68 (38.6%) men and 108 (61.4%) women. There were 102 (58.0%) CD patients and 74 (42.0%) UC patients.

There were surgical procedure histories in 117 patients (66.5%). Blood transfusion was reported by 47 patients (26.7%). Eight patients (4.5%) confirmed the use of endovenous illicit drugs. None of the patient had undergone dialysis treatment and only 3 patients affirmed having a promiscuous sexual life (Table 1).

Forty-nine patients were without treatment; 7 patients used immunosuppressant drugs; 74 used steroid drugs; while 46 patients used both.

Table 2 shows that among the 176 patients, 30 patients (17%) had positive anti-HBc: 17 (56.7%) with UC and 13 (43.3%) with CD.

Among the 30 patients with positive anti-HBc, 4 had positive HBsAg.

The 30 patients with positive anti-HBc and the 14 patients with negative anti-HBc randomly selected were submitted to the PCR HBV-DNA qualitative test. All of these patients had negative PCR HBV-DNA results. The four patients with positive HBsAg were also



**Table 3** Risk factors according to anti-HBc result-frequency and percentile

Risk factors		Anti-HBc				P
		Positive		Negative		
		n	%	n	%	
Sex	Men	12	40.0	56	38.0	0.86
	Women	18	60.0	90	61.6	
Digestive endoscopy	Yes	16	53.3	91	62.3	0.35
	No	14	46.7	55	37.7	
Retosigmoidoscopy	Yes	13	43.3	50	34.0	0.34
	No	17	56.7	96	65.8	
Blood transfusion	Yes	10	33.3	37	25.3	0.36
	No	20	66.7	109	74.7	
Surgery	Yes	24	80.0	93	63.7	0.085
	No	6	20.0	53	36.3	
Surgery to treat IBD complications	Yes	8	33.3	47	50.5	0.13
	No	16	66.7	46	49.5	

**Table 4** Non-numeric variables risk factors for HBV transmission

Variable	Anti-HBc	n	Mean	SE	Minimum	Maximum	P
Age (yr)	Positive	30	47.7	11.9	26	78	0.001
	Negative	146	39.0	13.9	18	84	
Diagnose time (mo)	Positive	30	114.1	109.3	8	372	0.37
	Negative	146	89.9	90.4	1	600	
Colonoscopy number	Positive	30	2.3	1.9	0	10	0.52
	Negative	146	2.1	1.6	1	8	

submitted to qualitative PCR HBV-DNA tests and they also had negative results. Among these patients, those with positive anti-HBc and HBsAg tests are considered inactive HBV bearers.

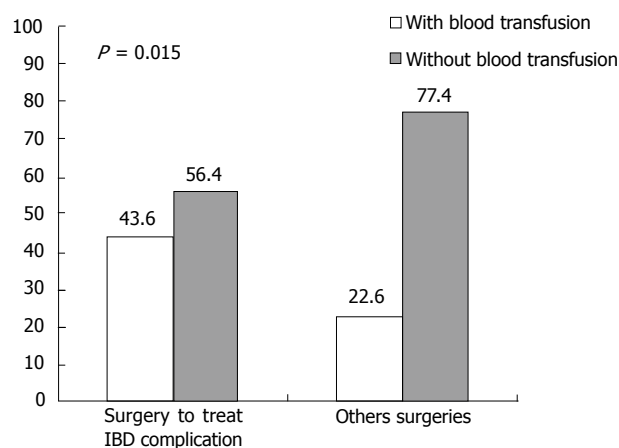
Table 3 supplies the frequency (*n*) and the risk factor percentile (%) according to anti-HBc results and the corresponding *P* value. The statistical analysis was accomplished by the  $\chi^2$  test or by the exact Fisher test.

It was observed that sex, digestive endoscopy, retosigmoidoscopy, and blood transfusions were not considered probable risk factors in HBV infection transmission.

When we calculated surgery only, we observed that 24 patients (80%) with positive anti-HBc were submitted to some type of surgical intervention while the other 20% (6 patients) with positive anti-HBc did not undergo any surgical intervention (*P* = 0.085). We also tried to stratify patients submitted to surgery into two groups: those who underwent surgery to treat IBD complications and those who underwent other surgeries. The *P* value was not significant (*P* = 0.13). Dialysis, endovenous illicit drug use, tattoos, acupuncture, piercing and a sexual promiscuous life style, were not analyzed due to low frequency of observed cases.

Table 4 shows that patients with positive anti-HBc have an average age significantly older (*P* = 0.001) than patients with negative anti-HBc. A significant difference was not observed for disease diagnosis time (*P* = 0.37) neither for number of colonoscopies (*P* = 0.52) among both positive and negative anti-HBc groups.

One hundred and seventeen patients were submitted

**Figure 1** Blood transfusion need according to surgery complexity.

to some form of surgery. We analyzed the relationship between blood transfusion and surgery carried out to treat IBD complications, and it was observed that patients submitted to surgeries to treat IBD complications needed more transfusions (*P* = 0.015) than patients submitted to other types of surgery, as illustrated in Figure 1.

## DISCUSSION

In this study we observed that positive anti-HBc prevalence was 17% (30 patients) in a sample of 176 patients. This data shows that positive anti-HBc prevalence in IBD patient groups is larger when compared with the overall Brazil population (7.9%) and with the Rio de Janeiro state population (2.5%) figures<sup>[18]</sup>.

In the literature, we found only one case-control study that evaluated the HBV prevalence in IBD patients. In that study, the anti-HBc prevalence was larger in CD (10.9%) and ulcerative colitis patients (11.5%) when compared with control group individuals (5.1%)<sup>[11]</sup>. Our study had very similar results for CD and ulcerative colitis for positive anti-HBc prevalence. These results are probably because IBD patients are frequently exposed to surgical interventions and/or endoscopies as well as necessary blood transfusion that can be a means of transmitting HBV<sup>[19,20]</sup>.

Among 30 patients with positive anti-HBc result, 2.3% (4) had positive HBsAg with negative HBV-PCR DNA, patients that are considered HBV inactive bearers. This prevalence is considered high when we compare it with a Brazil Health Ministry study in 2006 in the central west, Northeast and Brasília regions that shows an HBsAg prevalence of 0.5%.

In addition to identifying HBV prevalence, we also tried to identify the possible risk factors for HBV transmission that could increase HBV infection prevalence among IBD patients. Considering 5% to be a significant threshold, we found that such factors as: (1) Sexual activity; (2) Digestive endoscopy; (3) Retosigmoidoscopy and (4) Blood transfusion were ruled out as possible risk factors for HBV infection of IBD patients.

Despite the fact that our sample contained 108 female patients corresponding to 61.4% of the total

sample, when we compared the positive anti-HBc percentile in women and in men, we did not obtain  $P$  value with statistical significance ( $P = 0.86$ ), even when we separated the male and female group according to IBD type, both groups being very similar in this respect. Biancone had a different result in his study. He demonstrated that female status was an important factor to be considered in HBV infection in CD patients<sup>[11]</sup>.

Studies in the last 5 years have verified the possibility of HCV and HBV transmission mainly through endoscopic procedures during therapeutic interventions. Studies demonstrated the presence of HBV-DNA in endoscopic channels that were not submitted to appropriate disinfection processes<sup>[20]</sup>. In our sample procedures, such as digestive endoscopy and retosigmoidoscopy, we did not discover any evidence of HBV transmission risk factors (digestive endoscopy  $P = 0.35$  and retosigmoidoscopy  $P = 0.34$ ).

Considering blood transfusion is an important viral hepatitis transmission route<sup>[7,21]</sup>, it has already been demonstrated by Long *et al* in 2000 and Biancone *et al* in 2001 that blood transfusion was an important risk factor in HCV transmission among IBD patients<sup>[11,22]</sup>. However, we were not able to demonstrate that blood transfusion was a risk factor for HBV infection transmission in our group because in our sample only 10 out of 47 patients with positive anti-HBc received blood transfusions while the other 20 with positive anti-HBc did not have blood transfusion histories ( $P = 0.36$ ).

When we analyzed surgery as a possible risk factor, despite of the fact of not having a  $P$  value smaller than 0.05, we observed that 80% (24) of patients with positive anti-HBc had been submitted to some surgical procedure while the other 20% (6) did not undergo any surgical procedures ( $P = 0.085$ ).

Biancone *et al* showed that surgery, and mainly surgical procedures to treat IBD complications, were an important risk factor in HCV transmission among IBD patients<sup>[11]</sup>. We can try to explain the Biancone *et al* discoveries if we take into consideration that gastrointestinal surgeries to treat IBD complications are high complexity operations<sup>[10]</sup> and probably need blood transfusions during surgical procedure, which could cause a bias in the statistical analysis because the surgery itself was not the cause of transmission but the transfusion. The possible risk factor for HCV infection transmission in these cases was blood transfusion that patients received during these procedures. In our study we separated patients according to surgery type: group 1-patients submitted to surgery to treat IBD complications, and group 2-patients submitted to other surgical interventions; we did not find a significant  $P$  value ( $P = 0.13$ ). However, as can be seen in Figure 1, our hypothesis that patients submitted to surgeries to treat IBD complications received more blood transfusions than patients submitted to other surgical interventions was confirmed ( $P = 0.015$ ).

When we compare our results with Spijkerman *et al*'s study, our data is divergent because according to that study high complexity surgeries (i.e. surgeries with more than one hour of duration, surgeries with a larger

incidence of postoperative complications and those with a higher risk of complication requiring further surgery or more blood transfusions) are associated with a higher risk of HBV infection transmission<sup>[19]</sup>. However, in this study it was demonstrated that the HBV infection was transmitted through an HBV infected surgeon during surgery.

For the other qualitative variables: dialyses, endovenous illicit drug use, tattoos, acupuncture, "piercings" and sexually promiscuous lifestyle, the associations were not analyzed because we had low frequencies of observed cases.

In quantitative-variable analysis (age, disease diagnosis time and number of colonoscopies), the  $P$  value results have statistical significance ( $P = 0.001$ ). The average age of patients with positive anti-HBc was higher (47.7 years) than patients with negative anti-HBc (39.0 years). In the literature, the positive anti-HBc prevalence was associated with ages older than 50 years in CD and in UC<sup>[11]</sup>. These data were found, we believe, because older patients probably have a longer disease duration time and therefore have had more time to develop complications requiring surgical and endoscopic interventions. However, we were not able to prove the veracity of these assumptions.

Biancone *et al* have shown ( $P = 0.37$ ) that disease duration time (number of months since IBD diagnosis) is associated with incidence of positive anti-HBc in UC patients<sup>[1]</sup>.

Steroids, immunosuppressant drugs and the anti-TNF antibodies (anti-necrosis tumor antibodies-Infliximab<sup>®</sup>) in IBD patients<sup>[23,24]</sup>, as some studies have demonstrated, can influence the course of hepatic disease when used in HBV infected patients, mainly patients with positive HBsAg and anti-HBc and negative HBV-DNA (called inactive bearers)<sup>[12-14]</sup>. It is also important to note that in patients with positive anti-HBc and negative HBsAg, the HBV can replicate because the virus stays inside the hepatocytes although there is an apparent serologic cure<sup>[23]</sup>. These studies show that immunological suppression caused by these drugs could cause viral replication and spread infection inside hepatocytes. When these drugs were suspended and the immunological reaction was restored, the infected hepatocytes were destroyed quickly and there was an increase in the transaminases levels ("flare") and an accentuated viremia reduction<sup>[15,25]</sup>. Two cases of fulminant hepatitis were identified after use of Infliximab<sup>®</sup> in rheumatoid arthritis patients infected by HBV<sup>[26]</sup> and one case of hepatic insufficiency and death in a CD patient treated with Infliximab<sup>®</sup><sup>[27,28]</sup>. The reactivation of HBV can happen also to inactive bearers submitted to transplants or in cancer patients who are submitted to chemotherapy. Such patients need higher immunosuppressant drug doses than do IBD patients<sup>[12]</sup>.

Patients with positive HBsAg and anti-HBc and negative HBV-PCR DNA have increased risk of reactivating their HBV infections. Therefore, the use of lamivudine is recommended before immunological suppression therapies<sup>[29]</sup>. Lau and collaborators demon-

strated that patients with lymphoma infected by HBV who were submitted to chemotherapy did not have HBV infection reactivated when they used lamivudine one week before chemotherapy was begun<sup>[30]</sup>.

In conclusion, our study demonstrated that there were high incidences of positive anti-HBc (17%) and positive HBsAg (2.3%) in IBD patients in Clementino Fraga Filho University Hospital when compared with the overall population (7.9%).

These data show that it is important to have an early diagnosis of HBV infection in diagnosed IBD patients before any IBD treatment is initiated using steroids, immunosuppressant drugs, or anti-TNF antibodies, as that IBD treatment may worsen quiescent HBV hepatic disease. We also recommend HBV vaccination in this group of patients.

## COMMENTS

### Background

Hepatitis B virus (HBV) infection is considered a worldwide public health problem. Inflammatory bowel disease (IBD) patients have a high risk of acquiring HBV infection because they sometimes need blood transfusions, invasive surgical and endoscopic procedures. The objective of this study is to verify the seroprevalence of HBV infection and to identify the infection transmission risk factors in IBD patients at Clementino Fraga Filho University Hospital.

### Innovations and breakthroughs

The statistical analysis cannot identify one possible risk factor for HBV transmission but the study found among the IBD patients 4 persons with positive HBsAg who were called inactive bearers. Studies show that immunological suppression caused by steroids, immunosuppressant drugs and the anti-TNF antibodies (anti necrosis antibodies-Infliximab) in IBD patients can influence the course of hepatic disease once used in HBsAg positive patients. These drugs would take a viral replication and infection spread inside hepatocytes. It has already been related to 1 case of hepatic insufficiency and death in a Crohn's disease (CD) patient and 1 case of fulminant hepatitis in rheumatoid arthritis patient, both with positive HBsAg and treated with these drugs. In patients with positive HBsAg lamivudine use would be recommended before immunological suppression.

### Applications

After this study, we recommend HBV vaccination for IBD patients that have never been infected by HBV and also recommend lamivudine for patients with positive anti-HBc and needs to use steroids and immunomodulators.

### Peer review

This article did not identify one risk factor for HBV infection transmission in IBD patients but it shows us that these patients have high risk of acquiring this infection because they need invasive procedures. IBD patients that have been infected already must receive lamivudine before immunological suppression. It is very interesting.

## REFERENCES

- 1 **World Health Organization.** Hepatitis B. World Health Organization, 2000: 204
- 2 **Kim WR, Benson JT, Therau TM, Torgerson HA, Yawn BP, Melton LJ 3rd.** Changing epidemiology of hepatitis B in a U.S. community. *Hepatology* 2004; **39**: 811-816
- 3 **D'Amelio R, Matricardi PM, Biselli R, Stroffolini T, Mele A, Spada E, Chionne P, Rapicetta M, Ferrigno L, Pasquini P.** Changing epidemiology of hepatitis B in Italy: public health implications. *Am J Epidemiol* 1992; **135**: 1012-1018
- 4 **Maddrey WC.** Hepatitis B: an important public health issue. *J Med Virol* 2000; **61**: 362-366
- 5 **Chan HL, Ghany MG, Lok ASF.** Hepatitis B. In: Schiff ER, Sorell MF, Maddrey WC editors. *Diseases of the liver*. 8th ed. Philadelphia: Lippincott Williams & Wilkins, 1999: 758-791
- 6 **Lok ASF, Chan HL.** Viral Hepatitis B and D. In: O'Grady IG, Lake JR, Howdle PD editors. *Comprehensive Clinical Hepatology*. 1st ed. London: Mosby, 2000: 12.1-12.10
- 7 **Nishioka Sde A, Gyorkos TW, MacLean JD.** Tattoos and transfusion-transmitted disease risk: implications for the screening of blood donors in Brazil. *Braz J Infect Dis* 2002; **6**: 172-180
- 8 **Broome U, Glaumann H, Hellers G, Nilsson B, Sorstad J, Hultcrantz R.** Liver disease in ulcerative colitis: an epidemiological and follow up study in the county of Stockholm. *Gut* 1994; **35**: 84-89
- 9 **Gaeta GB, Stroffolini T, Taliani G, Ippolito FM, Giusti G, De Bac C.** Surgical procedures as a major risk factor for chronic hepatitis C virus infection in Italy: evidence from a case-control study. *Int J Infect Dis* 1999; **3**: 207-210
- 10 **Pallone F, Boirivant M, Stazi MA, Cosentino R, Prantera C, Torsoli A.** Analysis of clinical course of postoperative recurrence in Crohn's disease of distal ileum. *Dig Dis Sci* 1992; **37**: 215-219
- 11 **Biancone L, Pavia M, Del Vecchio Blanco G, D'Inca R, Castiglione F, De Nigris F, Doldo P, Cosco F, Vavassori P, Bresci GP, Arrigoni A, Cadau G, Monteleone I, Rispo A, Fries W, Mallardi B, Sturniolo GC, Pallone F.** Hepatitis B and C virus infection in Crohn's disease. *Inflamm Bowel Dis* 2001; **7**: 287-294
- 12 **Biancone L, Del Vecchio Blanco G, Pallone F, Castiglione F, Bresci G, Sturniolo G.** Immunomodulatory drugs in Crohn's disease patients with hepatitis B or C virus infection. *Gastroenterology* 2002; **122**: 593-594
- 13 **Perrillo RP.** Acute flares in chronic hepatitis B: the natural and unnatural history of an immunologically mediated liver disease. *Gastroenterology* 2001; **120**: 1009-1022
- 14 **Marusawa H, Uemoto S, Hijikata M, Ueda Y, Tanaka K, Shimotohno K, Chiba T.** Latent hepatitis B virus infection in healthy individuals with antibodies to hepatitis B core antigen. *Hepatology* 2000; **31**: 488-495
- 15 **Perrillo RP, Schiff ER, Davis GL, Bodenheimer HC Jr, Lindsay K, Payne J, Dienstag JL, O'Brien C, Tamburro C, Jacobson IM.** A randomized, controlled trial of interferon alfa-2b alone and after prednisone withdrawal for the treatment of chronic hepatitis B. The Hepatitis Interventional Therapy Group. *N Engl J Med* 1990; **323**: 295-301
- 16 **Froio N, Nicastrì E, Comandini UV, Cherubini C, Felicioni R, Solmone M, Di Giulio S, Petrosillo N.** Contamination by hepatitis B and C viruses in the dialysis setting. *Am J Kidney Dis* 2003; **42**: 546-550
- 17 **Freeman R.** Barriers to accessing and accepting dental care. *Br Dent J* 1999; **187**: 81-84
- 18 **Secretaria do Estado de Saúde do Rio de Janeiro-Assessoria de Doenças Transmissíveis por Sangue e Hemoderivados.** Hepatites Virais-9º Boletim Informativo, 2005
- 19 **Spijkerman JJ, van Doorn LJ, Janssen MH, Wijkman CJ, Bilkert-Mooiman MA, Coutinho RA, Weers-Pothoff G.** Transmission of hepatitis B virus from a surgeon to his patients during high-risk and low-risk surgical procedures during 4 years. *Infect Control Hosp Epidemiol* 2002; **23**: 306-312
- 20 **Ishino Y, Ido K, Sugano K.** Contamination with hepatitis B virus DNA in gastrointestinal endoscope channels: risk of infection on reuse after on-site cleaning. *Endoscopy* 2005; **37**: 548-551
- 21 **Schreiber GB, Busch MP, Kleinman SH, Korelitz JJ.** The risk of transfusion-transmitted viral infections. The Retrovirus Epidemiology Donor Study. *N Engl J Med* 1996; **334**: 1685-1690
- 22 **Longo F, Hebuterne X, Tran A, Staccini P, Hastier P, Schneider S, Benzaken S, Tirtaine C, Rampal P.** [Prevalence of hepatitis C in patients with chronic inflammatory bowel disease in the region of Nice and evaluation of risk factors] *Gastroenterol Clin Biol* 2000; **24**: 77-81
- 23 **Podolsky DK.** Inflammatory bowel disease. *N Engl J Med* 2002; **347**: 417-429
- 24 **Cottone M, Magliocco A, Trallori G, Brignola C, Vandelli C,**

- Ardizzone S, Meucci G, Zannoni F, Di Maio G, Astegiano M. Clinical course of inflammatory bowel disease during treatment with interferon for associated chronic active hepatitis. *Ital J Gastroenterol* 1995; **27**: 3-4
- 25 **Esteve M**, Saro C, Gonzalez-Huix F, Suarez F, Forne M, Viver JM. Chronic hepatitis B reactivation following infliximab therapy in Crohn's disease patients: need for primary prophylaxis. *Gut* 2004; **53**: 1363-1365
- 26 **Michel M**, Duvoux C, Hezode C, Cherqui D. Fulminant hepatitis after infliximab in a patient with hepatitis B virus treated for an adult onset still's disease. *J Rheumatol* 2003; **30**: 1624-1625
- 27 **Oniankitan O**, Duvoux C, Challine D, Mallat A, Chevalier X, Pawlotsky JM, Claudepierre P. Infliximab therapy for rheumatic diseases in patients with chronic hepatitis B or C. *J Rheumatol* 2004; **31**: 107-109
- 28 **Millonig G**, Kern M, Ludwiczek O, Nachbaur K, Vogel W. Subfulminant hepatitis B after infliximab in Crohn's disease: need for HBV-screening? *World J Gastroenterol* 2006; **12**: 974-976
- 29 **Tillmann HL**, Wedemeyer H, Manns MP. Treatment of hepatitis B in special patient groups: hemodialysis, heart and renal transplant, fulminant hepatitis, hepatitis B virus reactivation. *J Hepatol* 2003; **39** Suppl 1: S206-S211
- 30 **Lau GK**, Yiu HH, Fong DY, Cheng HC, Au WY, Lai LS, Cheung M, Zhang HY, Lie A, Ngan R, Liang R. Early is superior to deferred preemptive lamivudine therapy for hepatitis B patients undergoing chemotherapy. *Gastroenterology* 2003; **125**: 1742-1749

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## Contrast-enhanced intraoperative ultrasonography equipped with late Kupffer-phase image obtained by sonazoid in patients with colorectal liver metastases

Hiroshi Nakano, Yasuo Ishida, Toshiyuki Hatakeyama, Kazuma Sakuraba, Masahiro Hayashi, Osamu Sakurai, Kiyoshi Hataya

Hiroshi Nakano, Yasuo Ishida, Toshiyuki Hatakeyama, Kazuma Sakuraba, Masahiro Hayashi, Osamu Sakurai, Kiyoshi Hataya, Department of Surgery, Yokohama Asahi-Chuo General Hospital, 4-20-1 Wakabadai, Asahi-ku, Yokohama 241-0801, Japan

**Author contributions:** Nakano H designed the study and performed hepatectomies; Ishida Y, Hatakeyama T, Hayashi M, and Sakuraba K helped surgeries; Nakano H, Ishida Y, Hatakeyama T, Sakuraba K, and Hayashi M performed contrast-enhanced intraoperative ultrasonography; Nakano H, Sakurai O, and Hataya K analyzed the data and wrote the paper.

**Correspondence to:** Dr. Hiroshi Nakano, Department of Surgery, Yokohama Asahi-Chuo General Hospital, 4-20-1 Wakabadai, Asahi-ku, Yokohama 241-0801, Japan. [nakahiro@marianna-u.ac.jp](mailto:nakahiro@marianna-u.ac.jp)

Telephone: +81-45-9216111 Fax: +81-45-9214931

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**Peer reviewer:** Kenji Miki, MD, Department of Surgery, Showa General Hospital, 2-450, Tenjin-cho, Kodaira, Tokyo 187-8510, Japan

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### Abstract

**AIM:** To find occult metastases during hepatectomy in patients with colorectal cancer liver metastases (CRCLM), contrast-enhanced intraoperative ultrasonography (CE-IIOUS) was performed using a new microbubble agent, sonazoid, which provides a parenchyma-specific contrast image based on its accumulation in the Kupffer cells.

**METHODS:** Eight patients with CRCLM underwent CE-IIOUS using sonazoid before hepatectomy. The liver was investigated during a late Kupffer-phase imaging, which is a valuable characteristic of sonazoid.

**RESULTS:** CE-IIOUS using sonazoid provided the early vascular- and sinusoidal-phase images for 10 min followed by the late Kupffer-phase image up to 30 min after the injection of sonazoid. IIOUS did not provide new findings of metastatic lesion in the 8 patients. However, during the late Kupffer-phase image of sonazoid, a metastatic lesion was newly found in two of the 8 patients. These newly detected lesions were removed by an additional hepatectomy and histopathologically diagnosed as a metastasis.

**CONCLUSION:** CE-IIOUS using sonazoid can allow surgeons to investigate the whole liver with enough time and to find new metastases intraoperatively.

### INTRODUCTION

Hepatic resection is the only treatment offering a chance of long-term survival to patients with colorectal cancer liver metastases (CRCLM)<sup>[1-4]</sup>. However, a total of 75% of patients with CRCLM who undergo liver resection will develop recurrence and the main site of recurrence is the liver<sup>[5]</sup>. In addition, 65% to 85% of all recurrences appear within the first 2 years<sup>[5]</sup>. Therefore, occult liver metastases may present at the time of hepatectomy and can be undetected preoperatively by computed tomography (CT), magnetic resonance image (MRI), or positron emission tomography (PET)<sup>[6]</sup>.

Intraoperative ultrasound (IOUS) is now considered as a standard method to determine the resection margin or to find preoperatively undetected tumors<sup>[7,8]</sup>. Recently some authors reported that a contrast-enhanced IOUS (CE-IIOUS) was more sensitive than conventional IOUS to identify new lesions and subsequently to influence surgical management<sup>[9,10]</sup>.

Sonazoid (perfluorobutane, GE Healthcare, Oslo, Norway) is a new microbubble agent<sup>[11]</sup> that provides a parenchyma-specific contrast image based on its accumulation in the Kupffer cells in the liver<sup>[12-14]</sup>. Sonazoid was

recently approved for clinical use in Japan, and it presents with a late Kupffer-phase image with a long duration following a vascular- and a sinusoidal-phase images<sup>[14]</sup>. SonoVue (Bracco SpA, Milan, Italy) has been already used as a microbubble agent in CE-IUS<sup>[9,10]</sup>, but it does not have the Kupffer-phase image<sup>[13]</sup>. The present brief clinical report shows our experience of CE-IUS using sonazoid in patients with CRCLM.

## MATERIALS AND METHODS

Examination of IUS and CE-IUS was performed using an Aplio-XV (Toshiba, Tokyo, Japan) and a micro-convex probe (PVT-375BT, 3.5 MHz, Toshiba). CE-IUS was performed under a pulse inversion harmonic (PIH) imaging capability (Toshiba). A bolus intravenous injection of sonazoid [0.015 mL/kg body weight (0.12  $\mu$ L microbubble/kg body weight as perflubutane microbubble)] was performed *via* the peripheral venous line followed by 10 mL of normal saline flush. Immediately after the administration of sonazoid, the portal veins, hepatic veins, and the normal liver parenchyma were uniformly enhanced. Hepatic metastases were identified as a dark contrast free filling defect during an early vascular phase image lasting 3 min after the injection of sonazoid. Approximately 10 min after the injection, the liver was scanned again to observe a late Kupffer-phase image. The hepatic metastases were identified as filling defects clearer than those observed at the vascular phase (Figure 1). The late Kupffer-phase image lasted at least for 30 min.

Eight patients with CRCLM underwent CE-IUS in addition to IUS. The number and size of metastases identified on preoperative CT, MR, and percutaneous contrast-enhanced ultrasonography (CE-US) were compared with those detected by IUS and CE-IUS.

## RESULTS

CE-IUS using sonazoid provided the early vascular- and sinusoidal-phase images for 10 min followed by the late Kupffer-phase image up to 30 min after the injection of sonazoid. Figure 1 shows IUS and CE-IUS images of a metastasis at the Segment 8. The lesion was detected as an unclear slightly hypoechoic mass by IUS (Figure 1A), but the lesion was shown as a clear hypoechoic mass during the late Kupffer-phase (Figure 2B).

Between December 2007 and February 2008, eight patients underwent CE-IUS. Preoperatively detected sites of liver metastases by CT, MRI, and CE-US were listed, and some differences among CT, MRI and CE-US existed as shown in Table 1. Preoperative CT seemed superior to MRI (patient No. 1 and 4). In addition, preoperative CE-US did not seem useful for finding metastases at the Segment 7 (patient No. 1, 2, and 5). Mainly based on the preoperative findings of CT, surgical methods were planned preoperatively in the eight patients (Table 2). IUS did not provide new findings of metastatic lesion in the eight patients. Indeed, IUS could not show some metastatic lesions detected by CT or

MRI (Table 1, patient No. 1 and 2). However, CE-IUS confirmed all hepatic lesions detected by CT or MRI. In addition, metastatic lesions were newly found by CE-IUS in two of the eight patients. These newly detected lesions were removed by an additional hepatectomy and histopathologically diagnosed as a metastasis.

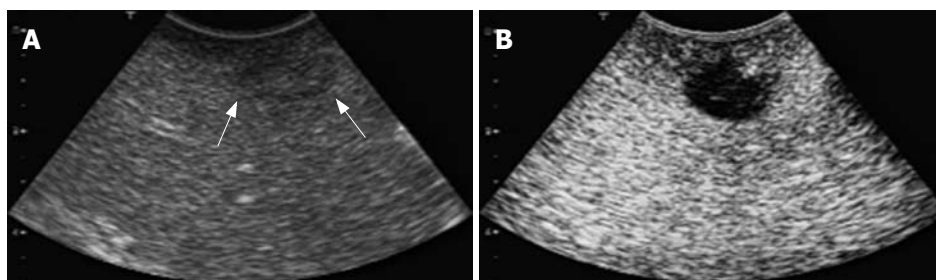
In the patient No. 1 (Table 1), a small hypoechoic lesion with 6 mm in diameter at the Segment 4 was newly detected by the CE-IUS at the late Kupffer-phase view (Figure 2A) although IUS did not show this lesion. This small lesion was resected and histopathologically confirmed as a metastatic nodule (Figure 2C).

In the patient No. 2 who preoperatively presented with liver metastases at the Segment 3 (Table 1), another lesion at the Segment 7 was pointed out as a metastasis with an ill-defined mass by preoperative CT and MRI (Figure 3A and B). Preoperative percutaneous CE-US using sonazoid could not show the lesion at the Segment 7 because of the attenuation of echogenicity. During the surgery, IUS did not show the metastasis at the segment 7, but CE-IUS showed a well-demarcated mass at the Segment 7 (Figure 3C). This lesion was resected by a partial hepatectomy and histopathologically confirmed as a metastasis. In addition, CE-IUS detected a new small lesion at the Segment 6 which was not pointed out by CT or MRI preoperatively (Figure 4). This lesion at the Segment 6 was also removed and histopathologically confirmed as an occult metastasis.

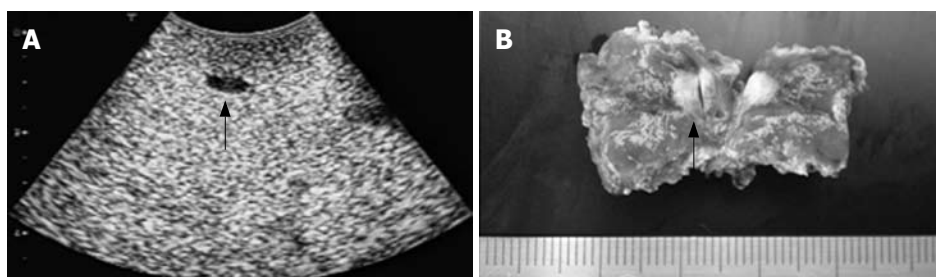
## DISCUSSION

The importance of CE-IUS in patients with CRCLM has been shown by two recent studies<sup>[9,10]</sup>. Indeed, Torzilli *et al* reported that new metastatic lesions, which were not detected by preoperative examinations and IUS, were detected in 5 out of 24 patients (21%) using CE-IUS<sup>[9]</sup>. They also reported that the modification rate of hepatectomy by CE-IUS alone was 21% in the patients with CRCLM. Leen *et al* showed that additional new hepatic metastases were detected in 11 out of 57 patients (19%) and the planned surgical methods were converted in these patients<sup>[10]</sup>. In the present study, an occult metastatic lesion was newly detected in two of the eight patients using CE-IUS and removed by an additional hepatectomy. These metastatic lesions were not detected by preoperative CT, MRI, preoperative percutaneous CE-US, or IUS.

Sonazoid is a novel microbubble-based ultrasound contrast agent, and is classified as a second-generation agent in which the perfluorocarbon gas has enough intravascular stability *in vivo*<sup>[15,16]</sup>. Watanabe *et al* showed that microbubbles of sonazoid were taken up by Kupffer cells immediately after intravenous injection and existed as microbubbles for 30 min within Kupffer cells, and that the hepatic parenchyma-specific contrast by sonazoid was due to the distribution of the microbubbles in Kupffer cells<sup>[14]</sup>. Therefore, sonazoid has a unique "late Kupffer-phase image" in addition to "early-vascular phase image" and "sinusoidal-phase image". This late Kupffer-phase image can provide high echogenic contrast



**Figure 1** IOUS and CE-IOUS views of a metastasis at the Segment 8. **A:** The metastatic lesion was unclearly detected as a slightly hypoechoic mass; **B:** CE-IOUS view of the same lesion. The metastatic lesion was shown as a distinct hypoechoic mass at the late Kupfer-phase.



**Figure 2** An occult metastasis. **A:** An occult metastasis at the segment 4 only detected by CE-IOUS. A clear hypoechoic mass (approximately 6 mm in diameter; black arrow) was newly detected at the delayed Kupfer phase. This metastatic lesion could not be found by CT, MRI, and IOUS. **B:** Macroscopic view of this metastasis (arrow).

**Table 1** Preoperatively diagnosed sites of liver metastases by CT, MRI and CE-US, intraoperatively found lesions by IOUS and CE-IOUS, and intraoperatively newly found metastases by CE-IOUS

Patient No.	Preoperatively diagnosed metastases			Intraoperatively found lesions		
	By CT	By MRI	By CE-US	By IOUS	By CE-IOUS	By CE-IOUS
1	S5, S5-6	S5, S5-6, S7	S5, S5-6	S5, S5-6	S5, S5-6, S7	S4
2	S3, S7	S3, S7	S3	S3	S3, S7	S6
3	S6, S6	S6, S6	S6, S6	S6, S6	S6, S6	(-)
4	S6-7, S1	S6-7	S6-7, S1	S6-7, S1	S6-7, S1	(-)
5	S7	S7	(-)	S7	S7	(-)
6	S7	S7	S7	S7	S7	(-)
7	S7-6, S3	S7-6, S3	S7-6, S3	S7-6, S3	S7-6, S3	(-)
8	S3, S4, S8	S3, S4, S8	S3, S4, S8	S3, S4, S8	S3, S4, S8	(-)

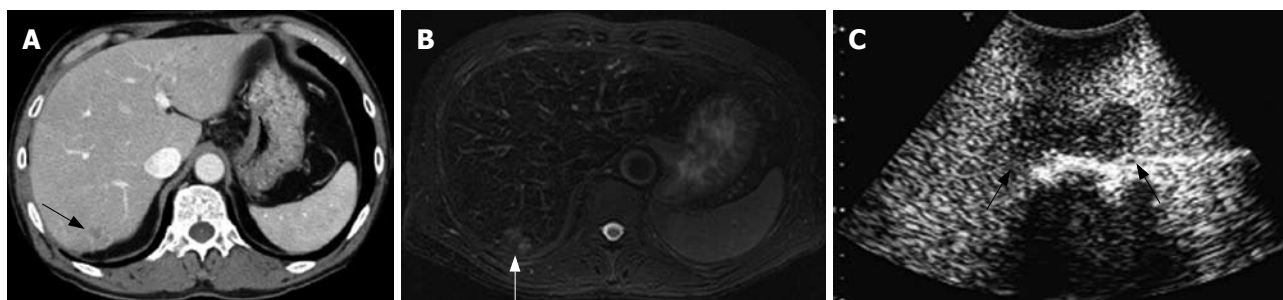
**Table 2** Preoperatively planned surgical methods mainly performed surgical methods, and methods of additional surgery according to the findings of CE-IOUS

Patient No.	Preoperatively planned surgery	Mainly performed operative procedures	Methods of additional surgery based the findings of CE-IOUS
1	Enucleations at S5, S5-S6, and S7	Bisegmentectomy of S5 and S6 Enucleation at S7	Enucleation at S4
2	Left lateral sectionectomy, partial resection of S7	Left lateral sectionectomy, partial resection of S7	Enucleation at S6
3	S6 segmentectomy	S6 segmentectomy	(-)
4	Right posterior sectionectomy, S1 partial resection	Right posterior sectionectomy, S1 partial resection	(-)
5	S7 partial resection	S7 partial resection	(-)
6	S7 segmentectomy	S7 segmentectomy	(-)
7	Posterior sectionectomy, left lateral sectionectomy	Posterior sectionectomy, left lateral sectionectomy	(-)
8	Enucleations at S3, S4, and S8	Enucleations at S3, S4, and S8	(-)

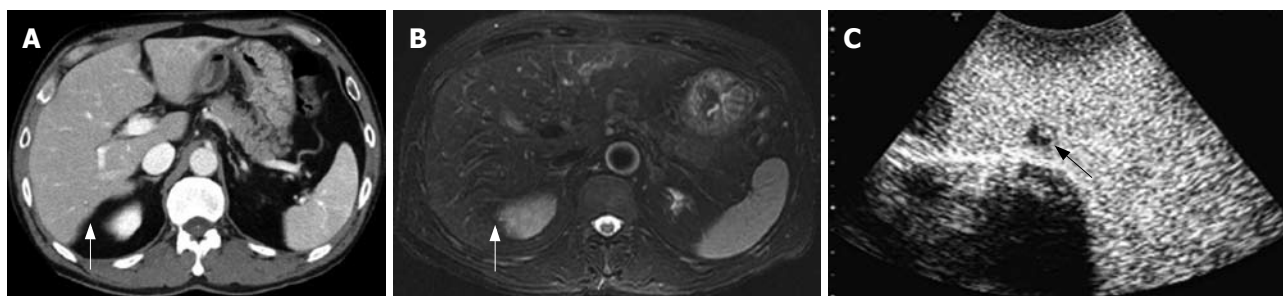
enhancement in the liver parenchyma<sup>[14]</sup>. On the other hand, other microbubble contrast agents such as Imavist and SonoVue provide parenchyma-specific contrast by transient mechanical slowdown of microbubbles within the sinusoid, and these two contrast agents are hardly phagocytosed by the Kupffer cells<sup>[10,13,17]</sup>. Therefore, Imavist and SonoVue cannot provide the late Kupffer-phase image, and the parenchyma-specific contrast image of these two microbubble agents can be seen during only

3 min to 5 min after the injection. Therefore, as previously described, SonoVue needs repeated injections during CE-IOUS to perform a whole liver examination<sup>[9,10]</sup>. Therefore, sonazoid seems a superior microbubble contrast agent for CE-IOUS in patients with CRCLM since a whole precise liver investigation by CE-IOUS, in which determination of surgical margin and examination of occult metastases should be investigated. Therefore, CE-IOUS needs more than 5 min, and the present brief





**Figure 3** Preoperative CT and SPIO-MRI, and CE-IIOUS. **A:** An enhanced-CT view and an ill-defined low density mass was detected at the segment 7 (arrow); **B:** A SPIO MRI view and an ill-defined high intensity mass was detected at the segment 8 (arrow); **C:** CE-IIOUS view at the delayed Kupffer phase and a well-demarcated hypoechoic mass was detected by CE-IIOUS.



**Figure 4** Preoperative CT and SPIO-MRI, and CE-IIOUS. **A:** An enhanced-CT could not detect any lesion at the Segment 6 (arrow); **B:** A SPIO MRI view could not detect any lesion at the Segment 6 (arrow); **C:** CE-IIOUS view at the delayed Kupffer phase and a small hypoechoic mass partially containing an isoechoic lesion was detected by CE-IIOUS.

clinical experience of CE-IIOUS confirmed the usefulness of sonazoid during surgery in patients with CRCLM. In addition, the duration of the approximately 30 min of the late-Kupffer phase image using sonazoid seems useful to perform preoperative percutaneous CE-US compared to SonoVue because the limiting time of SonoVue image (5 min) does not seem convenient to perform preoperative CE-US. Indeed, a routine preoperative CE-US in our institution, in which only the late Kupffer-phase image is performed, can be performed between 10 min and 30 min after the injection of sonazoid. However, based on our experience, small metastases at the Segment 7 seem hardly visualized by percutaneous CE-US using sonazoid because of the attenuation of echogenicity as shown in the present case No. 2.

Sonazoid has been reported as a safe medicine. Indeed, the incidence of adverse effects of sonazoid was shown in 25 out 397 patients (6.3%) in a clinical phase II study performed in Japan. The main side effects were headache (1.0%) and diarrhea (1.0%), but no anaphylactic shock due to sonazoid was reported unlike with contrast-enhanced CT. The image mechanism of CE-IIOUS using sonazoid seems similar to superpara-magnetic iron oxide-enhanced magnetic resonance image (SPIO-MRI) because both images are based on the phagocytosis by Kupffer cells. However, sonazoid is much less expensive compared to SPIO-MRI. Regardless of sensitivity rate of sonazoid for detecting small metastases compared to SPIO-MRI, CE-IIOUS is useful to perform intraoperative liver biopsy of newly detected lesions and to determine an additional hepatectomy.

In conclusion, CE-IIOUS using sonazoid can allow

surgeons to investigate the whole liver with enough time (at least 30 min of the late Kupffer-phase image) and to find new metastases intraoperatively.

## COMMENTS

### Background

Contrast-enhanced intraoperative ultrasonography (CE-IIOUS) seems more sensitive than conventional IIOUS to identify new occult lesions during hepatectomy in patients with colorectal cancer liver metastases (CRCLM). Sonazoid (perfluorobutane, GE Healthcare, Oslo, Norway) is a new microbubble agent that provides late Kupffer-phase image, which cannot be obtained by conventional contrast mediums.

### Research frontiers

No study has investigated the intraoperative efficacy of the late Kupffer-phase image of sonazoid in patients with CRCLM.

### Innovations and breakthroughs

CE-IIOUS using sonazoid enabled whole liver investigation at least for 30 min of the late Kupffer-phase image. Occult metastases, which had not been detected preoperatively, were newly found in some patients and removed by an additional hepatectomy.

### Applications

CE-IIOUS using sonazoid can reduce intrahepatic recurrence after hepatectomy in patients with CRCLM.

### Peer review

This article presented the clinical significance of CE-IIOUS using sonazoid during hepatectomy for colorectal cancer liver metastases. CE-IIOUS for detection of liver metastases requires stable image for enough time to perform repeated whole liver scans. Sonazoid seems to be suitable for this purpose. This article is worthy for publication in *WJG* with minor revision.

## REFERENCES

- 1 Nordlinger B, Jaeck D, Guiguet M, Vaillant JC, Balladur P. Surgical resection of hepatic metastases: Multicentric



- retrospective study by the French Association of Surgery. In: Nordlinger B, Jaeck D. Treatment of hepatic metastases of colorectal cancer. Paris: Springer-Verlag, 1992: 129-146
- 2 **Adam R**, Pascal G, Azoulay D, Tanaka K, Castaing D, Bismuth H. Liver resection for colorectal metastases: the third hepatectomy. *Ann Surg* 2003; **238**: 871-883; discussion 883-884
  - 3 **Jaeck D**, Bachellier P, Nakano H, Oussoultzoglou E, Weber JC, Wolf P, Greget M. One or two-stage hepatectomy combined with portal vein embolization for initially nonresectable colorectal liver metastases. *Am J Surg* 2003; **185**: 221-229
  - 4 **Weber JC**, Nakano H, Bachellier P, Oussoultzoglou E, Inoue K, Shimura H, Wolf P, Chenard-Neu MP, Jaeck D. Is a proliferation index of cancer cells a reliable prognostic factor after hepatectomy in patients with colorectal liver metastases? *Am J Surg* 2001; **182**: 81-88
  - 5 **Fong Y**, Cohen AM, Fortner JG, Enker WE, Turnbull AD, Coit DG, Marrero AM, Prasad M, Blumgart LH, Brennan MF. Liver resection for colorectal metastases. *J Clin Oncol* 1997; **15**: 938-946
  - 6 **Finlay IG**, McArdle CS. The role of occult hepatic metastases in staging colorectal carcinoma. *Scand J Gastroenterol Suppl* 1988; **149**: 150-154
  - 7 **Torzilli G**, Montorsi M, Donadon M, Palmisano A, Del Fabbro D, Gambetti A, Olivari N, Makuuchi M. "Radical but conservative" is the main goal for ultrasonography-guided liver resection: prospective validation of this approach. *J Am Coll Surg* 2005; **201**: 517-528
  - 8 **Jarnagin WR**, Bach AM, Winston CB, Hann LE, Heffernan N, Loumeau T, DeMatteo RP, Fong Y, Blumgart LH. What is the yield of intraoperative ultrasonography during partial hepatectomy for malignant disease? *J Am Coll Surg* 2001; **192**: 577-583
  - 9 **Torzilli G**, Del Fabbro D, Palmisano A, Donadon M, Bianchi P, Roncalli M, Balzarini L, Montorsi M. Contrast-enhanced intraoperative ultrasonography during hepatectomies for colorectal cancer liver metastases. *J Gastrointest Surg* 2005; **9**: 1148-1153; discussion 1153-1154
  - 10 **Leen E**, Ceccotti P, Moug SJ, Glen P, MacQuarrie J, Angerson WJ, Albrecht T, Hohmann J, Oldenburg A, Ritz JP, Horgan PG. Potential value of contrast-enhanced intraoperative ultrasonography during partial hepatectomy for metastases: an essential investigation before resection? *Ann Surg* 2006; **243**: 236-240
  - 11 **Forsberg F**, Piccoli CW, Liu JB, Rawool NM, Merton DA, Mitchell DG, Goldberg BB. Hepatic tumor detection: MR imaging and conventional US versus pulse-inversion harmonic US of NC100100 during its reticuloendothelial system-specific phase. *Radiology* 2002; **222**: 824-829
  - 12 **Kindberg GM**, Tolleshaug H, Roos N, Skotland T. Hepatic clearance of Sonazoid perfluorobutane microbubbles by Kupffer cells does not reduce the ability of liver to phagocytose or degrade albumin microspheres. *Cell Tissue Res* 2003; **312**: 49-54
  - 13 **Yanagisawa K**, Moriyasu F, Miyahara T, Yuki M, Iijima H. Phagocytosis of ultrasound contrast agent microbubbles by Kupffer cells. *Ultrasound Med Biol* 2007; **33**: 318-325
  - 14 **Watanabe R**, Matsumura M, Munemasa T, Fujimaki M, Suematsu M. Mechanism of hepatic parenchyma-specific contrast of microbubble-based contrast agent for ultrasonography: microscopic studies in rat liver. *Invest Radiol* 2007; **42**: 643-651
  - 15 **Hagen EK**, Forsberg F, Aksnes AK, Merton DA, Liu JB, Tornes A, Johnson D, Goldberg BB. Enhanced detection of blood flow in the normal canine prostate using an ultrasound contrast agent. *Invest Radiol* 2000; **35**: 118-124
  - 16 **Yao J**, Teupe C, Takeuchi M, Avelar E, Sheahan M, Connolly R, Ostensen J, Pandian NG. Quantitative 3-dimensional contrast echocardiographic determination of myocardial mass at risk and residual infarct mass after reperfusion: experimental canine studies with intravenous contrast agent NC100100. *J Am Soc Echocardiogr* 2000; **13**: 570-581
  - 17 **Kono Y**, Steinbach GC, Peterson T, Schmid-Schonbein GW, Mattrey RF. Mechanism of parenchymal enhancement of the liver with a microbubble-based US contrast medium: an intravital microscopy study in rats. *Radiology* 2002; **224**: 253-257

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RAPID COMMUNICATION

## Incidence of reflux esophagitis and *Helicobacter pylori* infection in diabetic patients

Ken Ariizumi, Tomoyuki Koike, Shuichi Ohara, Yoshifumi Inomata, Yasuhiko Abe, Katsunori Iijima, Akira Imatani, Yoshitomo Oka, Tooru Shimosegawa

Ken Ariizumi, Tomoyuki Koike, Shuichi Ohara, Yoshifumi Inomata, Yasuhiko Abe, Katsunori Iijima, Akira Imatani, Tooru Shimosegawa, Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Miyagi 9808574, Japan

Yoshitomo Oka, Division of Diabetes and Metabolic Diseases, Tohoku University Graduate School of Medicine, Sendai, Miyagi 9808574, Japan

**Author contributions:** Ariizumi K, Koike T, Ohara S, Oka Y designed the research; Ariizumi K, Koike T, Ohara S, Inomata Y, Abe Y, Iijima K, Imatani A, Oka Y, Shimosegawa T performed the research; Ariizumi K, Koike T, Ohara S wrote the paper.

**Correspondence to:** Ken Ariizumi, Division of Gastroenterology, Tohoku University Graduate School of Medicine, 1-1, Seiryomachi, Aoba-ku, Sendai, Miyagi 9808574, Japan. [kariizumi@int3.med.tohoku.ac.jp](mailto:kariizumi@int3.med.tohoku.ac.jp)

Telephone: +81-22-7177171 Fax: +81-22-7177177

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Malpighi Hospital-Nuove Patologie, Pad. 5-via Massarenti 9, Bologna 40138, Italy

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### INTRODUCTION

At present, the frequency of lifestyle-related illnesses such as diabetes mellitus and obesity is increasing due to the westernization of the Japanese diet. Diabetic patients are now estimated at 7 400 000 in Japan<sup>[1]</sup> and diabetes is showing a world-wide tendency to increase<sup>[2]</sup>.

In gastroesophageal reflux diseases (GERD), frequent gastroesophageal acid reflux causes such symptoms as heartburn, water brash, chest pain, and esophageal discomfort, lowering the quality of life of patients. Also, GERD damages the esophageal mucosa through erosion and the development of ulcers, mainly in the lower esophagus, leading to reflux esophagitis (RE). The incidence of RE has been on the rise in recent years, and today, it is one of the most common chronic diseases for adults in Europe and the United States<sup>[3]</sup>. While the incidence of RE in Japan is considered low as compared with Europe and the United States, the incidence of RE in Japan has increased due to the westernization of the Japanese diet, the rapidly growing elderly population, and lower *H pylori* infection rates<sup>[4]</sup>.

Some investigators have reported that the incidence of RE is high in diabetic patients<sup>[5,6]</sup>, although few reports have examined the incidence of RE in diabetic patient in Japan. There are some reports that hiatal hernia, age, *H pylori* infection and body mass index (BMI) are considered to affect the outbreak of RE<sup>[7,8]</sup>. Recent studies have reported that *H pylori* infection was less prevalent in patients with RE than those without RE, and was considered to suppress the onset of RE by inducing gastric mucosal atrophy and lowering gastric acid secretion<sup>[9,10]</sup>.

Some investigators have reported that the incidence of *H pylori* infection in diabetic patients is higher than controls<sup>[11-14]</sup>, though other investigators have reported

### Abstract

**AIM:** To investigate the incidence of reflux esophagitis (RE) and *H pylori* infection in the diabetic patient.

**METHODS:** The incidence of RE and *H pylori* infection were investigated in 85 patients with diabetes mellitus and the results were compared with controls.

**RESULTS:** The incidence of RE in diabetic patients was 17.6%. Although this tended to be higher in diabetic patients, there were no statistically significant differences between diabetic patients and controls. The incidence of *H pylori* infection in diabetic patients was 53.7% but no statistically significant difference was seen between diabetic patients and controls in the incidence of *H pylori* infection.

**CONCLUSION:** No significant differences could be seen between diabetic patients and controls in the incidence of RE and *H pylori* infection.

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**Key words:** Diabetes mellitus; Reflux esophagitis; *Helicobacter pylori*

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no such significant differences between these groups<sup>[15,16]</sup>.

The objective of the present study is to examine the incidence of RE and *H pylori* infection in diabetic patients.

Patient demographics, incidence of GERD, incidence of columnar lined esophagus (CLE), serum gastrin concentration, and pepsinogen (PG) I / II (an index of gastric mucosal atrophy based on serologic finding) were also evaluated.

## MATERIALS AND METHODS

### Study design

A total of 85 consecutive patients with diabetes mellitus who visited the Department of Diabetes and Metabolic Diseases at Tohoku University Hospital from December 2002 to September 2003 were included in the present study. Patients who had other severe complications, had taken PPI or H<sub>2</sub> receptor antagonists within four wk, or had undergone esophagogastrectomy were excluded from the present study. Nine hundred and forty four patients who had undergone endoscopy at the same period and another 67 age and sex-matched non-diabetic subjects without upper GI tract disorders were also included. Informed consent was obtained from each patient. The study protocol was approved by the ethical committee of the Tohoku University Graduate School of Medicine.

The subjects were divided into two groups, a well glycemic controlled group and a poorly controlled group. Patient demographics, incidence of RE, incidence of GERD, incidence of CLE, incidence of *H pylori* infection, severity grade of RE, serum gastrin concentration and PG I / II were investigated between the good glycemic controlled and poorly controlled group. The incidence of RE, GERD, CLE and the severity grade of RE were compared with 944 patients who had undergone endoscopy at the same study period as controls.

The incidence of RE and GERD were assessed due to HbA1c, disease duration, diabetic complications, and BMI. *H pylori* infection status was investigated between DM patients and 67 age and sex-matched non-diabetic subjects without upper GI tract disorders.

### Patient demographics

The following factors were investigated: gender, age, height, body weight, BMI, type of DM [insulin dependent DM (IDDM) or non insulin dependent DM (NIDDM)], duration of DM, presence of hiatal hernia, with insulin therapy, with calcium antagonists, with complications (retinopathy, nephropathy and neuropathy). All patients were examined by an ophthalmologist for retinopathy. Nephropathy was diagnosed if albuminuria was > 0.3 g/L or there was evidence of chronic renal failure. Neuropathy was diagnosed if the patients had sensory abnormalities, vibration hyposensitivity, orthostatic hypotension, or impotence.

**Good and poorly glycemic controlled groups:** The

good glycemic controlled group had hemoglobin A1c (HbA1c) 6.4% or less, and the poorly controlled group had an HbA1c value of 6.5% or more.

**Assessment of RE:** Subjects were diagnosed as having RE of grade A to D by the Los Angeles Classification<sup>[17]</sup>.

**Diagnosis of hiatal hernia:** In the present study, hiatal hernia was defined as a hernia in which the gastric mucosa could be seen by endoscopy circumferentially from the esophageal hiatus.

**Diagnosis of CLE:** CLE was defined as the replacement of the normal squamous lining of the lower esophagus by columnar epithelium.

**Assessment of *H pylori* infection:** In the present study, patients were diagnosed as having *H pylori* infection if they tested positive to at least one of the following tests: biopsy of the mucosa of the gastric body and gastric antrum along the greater curvature during endoscopy, rapid urease test, and serum *H pylori* antibody test. Patients were diagnosed as being free of *H pylori* infection if they tested negative to all tests.

**PG I / II and gastrin level:** PG I / II<sup>[18]</sup> and gastrin level<sup>[19]</sup> were measured to assess gastric mucosal atrophy. Each blood sample was centrifuged, and the sera were stored frozen at -20°C until testing.

**Diagnosis of GERD, incidence of reflux symptoms:** GERD was diagnosed with a self-administered questionnaire (QUEST). When the sum of the scores was 4 or more, the patient was considered as having GERD<sup>[20,21]</sup>. All patients were interviewed by investigators regarding their symptoms related to RE such as heartburn, burning in the upper abdomen, gastro-esophageal regurgitation, fullness, abdominal distension, anorexia, nausea, abdominal pain, and difficulty in swallowing food.

### Statistical analysis

Of the various patient background factors, gender, type of DM, presence of hiatal hernia, with insulin therapy, with calcium antagonists, with diabetic complications, incidence of RE, reflux symptoms, GERD, CLE and *H pylori* infection status were compared between diabetic patients and controls by a chi-square test. Age, height, body weight, BMI, duration of DM, sum of the QUEST score, PG I / II and gastrin level were expressed as mean  $\pm$  SD, and a one-way ANOVA test was used to compare these parameters between diabetic patients and controls. The Mann-Whitney's *U* test was used to compare RE severity. The significance level was set at < 5%.

## RESULTS

Of the 85 diabetic patients, there were 79 NIDDM and 6IDDM patients, 45 (52.9%) men and 40 (47.1%)

Table 1 Patient characteristics

	Well controlled group (%)	Poorly controlled group (%)	P
Gender (Male/Female)	(20/16)	(25/24)	0.84
Type of DM (IDDM/NIDDM)	(1/35)	(5/43)	0.36
Age (yr)	65.9 ± 10.3	60.0 ± 11.9	0.02
Height (cm)	160.3 ± 8.7	156.9 ± 17.6	0.29
Body weight (kg)	61.4 ± 16.5	61.1 ± 17.9	0.93
BMI	23.5 ± 5.6	23.3 ± 3.1	0.77
Duration of DM (yr)	12.9 ± 10.5	16.7 ± 10.3	0.10
Hiatal hernia	33.3 (12/36)	22.4 (11/49)	0.38
Insulin therapy	33.3 (12/36)	61.2 (30/49)	0.02
Calcium antagonist	13.9 (5/36)	24.5 (12/49)	0.35
Retinopathy	16.7 (6/36)	32.7 (16/49)	0.16
Nephropathy	2.3 (1/36)	8.2 (4/49)	0.56
Neuropathy	13.9 (5/36)	20.4 (10/49)	0.45

Table 2 Comparison between diabetic patients and controls

	Diabetic patients (%)	Controls (%)	P
RE	17.6 (15/85)	10.3 (97/944)	0.056
Los angeles classification			
A	80.0 (12/15)	44.3 (43/97)	0.01
B	20.0 (3/15)	48.5 (47/97)	
C	0	6.2 (6/97)	
D	0	1.0 (1/97)	
Incidence of CLE	32.9 (28/85)	37.7 (356/944)	0.45

CLE: Columnar lined esophagus.

women. The mean age ± SD was 62.5 ± 11.5 years (range 28-85 years, median 64 years). The mean durations of DM was 15.1 ± 10.5 years (range 0-44 years, median 14 years).

Thirty-six patients comprised the well glycemic controlled group and 49 patients comprised the poorly controlled group. In the poorly controlled group, age and the number of patients with insulin therapy were higher than in the well controlled group. Among each group, there were no significant differences in gender, type of DM (IDDM or NIDDM), height, body weight, BMI, duration of DM, presence of hiatal hernia, with calcium antagonists, or diabetic complications (Table 1).

In the diabetic patients, the incidence of RE was 17.6% (15/85), and in the 944 controls who had undergone endoscopy at the same period in our division, the incidence of RE was 10.3% (97/944). Though the incidence of RE tended to be higher in diabetic patients, the difference was not significant between diabetic patients and controls ( $P = 0.056$ ). Under the Los Angeles classification, all diabetic patients were grade A or B, and the severity grade of RE was statistically lower in diabetic patients than controls. The incidence of GERD and reflux symptoms in the diabetic patients were 32.9% (28/85) and 36.6% (31/85), respectively. The incidence of CLE was 32.9% (28/85), and the length all were less than 3 cm long. In the 944 controls, the incidence of CLE was 37.7% (356/944) (more than 3 cm: 11 patients, less than 3 cm: 345 patients) (Table 2).

Table 3 Comparison between good and poorly glycemic controlled groups

	Well controlled group (%)	Poorly controlled group (%)	P
RE	19.4 (7/36)	16.3 (8/49)	0.93
Incidence of GERD	25.0 (9/36)	38.8 (19/49)	0.27
Symptoms	30.1 (11/36)	41.3 (20/49)	0.46
QUEST score	2.06 ± 3.16	3.37 ± 4.22	0.12
Los angeles classification			
A	85.7 (6/7)	75.0 (6/8)	0.62
B	14.3 (1/7)	25.0 (2/8)	
C	0	0	
D	0	0	
Incidence of CLE	27.8 (10/36)	36.7 (18/49)	0.52
PG I / II	4.07 ± 2.61	4.94 ± 2.31	0.19
Gastrin	166.0 ± 126.9	137.6 ± 122.8	0.40

CLE: Columnar lined esophagus.

### Comparison between well and poorly glycemic controlled groups

The incidences of RE in the well glycemic controlled group and poorly controlled group were 17.6% (15/85) and 10.3% (97/944), respectively. The incidences of RE in patients with HbA1c under 5.7, 5.8 to 6.4, 6.5 to 7.9, and higher than 8.0 were 27.3% (3/11), 16% (4/25), 14.6% (6/41), and 25% (2/8), respectively. The incidences of GERD for the same patient groups were 36.4% (4/11), 20% (5/25), 34.1% (14/41), and 62.5% (5/8), respectively. The incidence of RE and GERD did not show any particular tendency.

Among the well and the poorly glycemic controlled groups, there were no significant differences in the frequency of RE, GERD, reflux symptoms, CLE, sum of the QUEST score, severity grade of RE, PG I / II or gastrin level (Table 3).

### Comparison between groups receiving and not receiving calcium antagonists

The incidences of RE in patients with and without calcium antagonists were 23.5% (4/17) and 16.2% (11/68), respectively. The incidences of GERD for the same groups were 29.4% (5/17) and 33.8% (23/68), respectively. There were no significant differences between patients receiving and not receiving calcium antagonists. The incidences of RE in the well and poorly glycemic controlled groups were 16.7% (6/36) and 18.3% (9/49), respectively. The incidences of GERD were 25.0% (9/36) and 38.8% (19/49), respectively. There were no significant differences between the well and the poorly glycemic controlled groups.

### The incidence of RE and GERD in patients according to disease duration

The incidences of RE in patients with disease durations of less than 6 years, 6 to 11 years, 11 to 16 years, and more than 16 years were 11.8% (2/17), 10.5% (2/19), 21.4% (3/14) and 22.9% (8/35), respectively. The incidences of GERD for the same durations were 23.5% (4/17) 42.1% (8/19), 42.9% (6/14) and 28.6% (10/35),



respectively. The incidence of RE tended to rise with increased duration of the disease. The incidence of GERD did not show any particular tendency.

#### **The incidence of RE and GERD with and without complications**

Of the diabetic patients, 22 had retinopathy, 5 had nephropathy and 15 had neuropathy. The incidences of RE in DM patients with and without complications were 20% (6/30) and 16.5% (9/55), respectively. The incidences of GERD were 33.3% (10/30), and 32.7% (18/55), respectively. There were no significant differences between patients with and without diabetic complications.

#### **The incidence of RE and GERD in non-obese and obese patients**

The incidences of RE in non-obese (BMI less than 25) and obese patients (BMI higher than 25) were 17.2% (10/58) and 18.5% (5/27), respectively. The incidences of GERD were 29.3% (17/58) and 40.7% (11/27), respectively. There were no significant differences between non-obese and obese patients. There were also no BMI-related differences between patients with and without RE ( $23.3 \pm 2.8$  vs  $23.4 \pm 4.5$ ) and between those with GERD and without GERD ( $23.8 \pm 6.4$  vs  $23.2 \pm 2.7$ ).

#### ***H. pylori* infection**

Of the 85 diabetic patients, measurement of their *H. pylori* infectious status could be performed in 67 patients, of whom 53.7% (36/67) had *H. pylori* infection. Of the age and sex-matched controls, 68.7% (46/67) had *H. pylori* infection, with no significant differences seen in *H. pylori* infection status between diabetic patients and controls ( $P = 0.11$ ).

The incidences of RE in *H. pylori* (+) and *H. pylori* (-) patients were 19.4% (7/36) and 9.7% (3/31), respectively. The incidences of GERD in *H. pylori* (+) and *H. pylori* (-) patients were 27.8% (10/36) and 35.5% (11/31), respectively. No significant differences in the incidence of RE and GERD could be demonstrated between *H. pylori* (+) and *H. pylori* (-) patients.

## **DISCUSSION**

The incidence of RE has been on the rise in recent years, and today, it is one of the most common chronic diseases for adults in Europe and the United States<sup>[22]</sup>.

Today, the incidence of RE is reported to be 10%-20% in Europe and the United States<sup>[23-25]</sup>, and 14%-16% in Japan<sup>[26-28]</sup>. While the incidence of RE in Japan is considered low as compared to Europe and the United States, the incidence of RE in Japan has increased due to the westernization of the Japanese diet, the rapidly growing elderly population, and lower *H. pylori* infection rates<sup>[26-28]</sup>.

Parkman<sup>[5]</sup> has reported that the incidence of RE in patients was 20% (4/20). Antwi<sup>[6]</sup> reported an incidence of 40.7% (22/54), though in these reports, glycemic control in many of the patients was poor and many had neuropathy. In the present study, the incidence of RE in

diabetic patients was 17.6% (15/85), and the incidence of RE in the 944 controls was 10.3% (97/944). The incidence of RE tended to be higher in diabetic patients, there were no significant differences between diabetic patients and controls.

In Japan, with respect to the severity of RE, most patients are reported to have mild esophagitis (Grade A or B under the Los Angeles classification<sup>[26,27]</sup>). In the present study, all patients had mild esophagitis.

In Japan, Nishida has previously reported that the incidence of GERD diagnosed with the QUEST questionnaire in diabetic patients was 25.3%, and significantly higher than that of controls<sup>[29]</sup>. In this previous study, they used the QUEST questionnaire to investigate GERD, though they did not perform gastrointestinal endoscopy to investigate RE. In the present study, we used the QUEST questionnaire and additionally performed gastrointestinal endoscopy to investigate GERD and RE. To the best of our knowledge, the present study is the first study to investigate both GERD and RE at the same time for diabetic patients in Japan. In the present study, the prevalence of GERD was 32.9%.

HbA1c is an index of DM control over some months beforehand. Complications of DM such as retinopathy, nephropathy and neuropathy occur as a result of poor control of diabetes for several years. So it is conceivable that DM-related complications are more appropriate than HbA1c as an index of the diabetic control. In the present study, there were no significant differences between patients with and without complications in the incidence of RE and GERD.

Some patients with DM, particularly those with NIDDM, are obese, which increases the intra-abdominal pressure, and may worsen RE<sup>[30]</sup>. In the present study, there were no significant differences between obese patients and non-obese patients in the incidence of RE or GERD. There were also no BMI-related differences between patients with and without RE and those with and without GERD. In the present study, obesity was not a risk factor for RE or GERD in the diabetic patient.

DM induces complications such as retinopathy, nephropathy and neuropathy. Diabetic neuropathy occurs in all sensory, motor and autonomic nerves. Some investigators have reported that in diabetic patients, RE can be induced in the presence of lowered LES pressure, abnormal esophageal motility, increased transient lower esophageal sphincter relaxation (TLESR), lowered acid clearance of the esophagus and prolonged gastric emptying time due to the dysfunction of autonomic nerves or the vagal nerve<sup>[31-35]</sup>. Prolonged gastric emptying time sometimes induces an unexpected hyper or hypoglycemic status, especially in the patients using insulin or oral hypoglycemic agents. Disorder of the sensory nerves raises the perception threshold level, and some patients cannot feel reflux symptoms, possibly leading to stricture of the esophagus. In some patients, RE can be discovered for the first time during routine endoscopy. In the present study, the incidence of RE tended to be higher in diabetic patients, although the differences between diabetic patients and controls were not significant.

It would be important to test for the presence of RE in diabetic patients during the daily examination.

The incidence of *H pylori* infection in diabetic patients has attracted some controversy. Some investigators have reported that the incidence of *H pylori* infection in diabetic patients is higher than controls<sup>[9-13]</sup>. Some investigators have reported that in DM patients, due to impaired immune function and impaired gastrointestinal motility, they were prone to *H pylori* infection<sup>[36-38]</sup>. On the other hand, some previous studies show a lower incidence of *H pylori* in diabetic patients than controls<sup>[39]</sup>, whereas in other studies, there was no difference in the prevalence of *H pylori* infection between diabetic patients and controls<sup>[11,14]</sup>. In some studies, *H pylori* infection was assessed by only one or two methods taken from among a biopsy of the mucosa, the rapid urease test, and the presence of serum *H pylori* antibodies. In the present study, *H pylori* infection was investigated by all three methods, so the incidence of *H pylori* infection in DM patients the present study is considered to be accurate, being recorded as 53.7%, but with no significant differences between diabetic patients and controls.

In conclusion, our results indicated that there were no differences in the incidences of either RE or *H pylori* infection between diabetic patients and controls.

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## COMMENTS

### Background

Some investigators had reported that the incidence of reflux esophagitis (RE) and *H pylori* infection is high in diabetic patients. Only a few reports, however, have examined the incidence of RE and *H pylori* infection in diabetic patients in Japan. The present study was designed to investigate the incidence of RE and *H pylori* infection in the diabetic patient.

### Research frontiers

We investigated the incidence of gastroesophageal reflux diseases (GERD), RE and *H pylori* infection in the diabetic patient. The present study is the first study to investigate both GERD and RE at the same time for diabetic patients in Japan.

### Innovations and breakthroughs

We used the questionnaire (QUEST) questionnaire to investigate GERD and performed gastrointestinal endoscopy to investigate RE at the same time for diabetic patients.

### Applications

In the present study, the incidence of RE tended to be higher in diabetic patients. It would be important to test for the presence of RE in diabetic patients during the daily examination.

### Peer review

This manuscript indicated that there were no differences in the incidences of either RE or *H pylori* infection between diabetic patients and controls. The study was well performed and the conclusion was clinically useful.

## REFERENCES

- 1 Health and Welfare Statistics Association: Health services in Japan (Kokumin Eisei no Doko). Indices of Health and Welfare (Kosei no Shihyo), 1998: 102
- 2 King H, Aubert RE, Herman WH. Global burden of diabetes, 1995-2025: prevalence, numerical estimates, and projections. *Diabetes Care* 1998; **21**: 1414-1431
- 3 Locke GR 3rd, Talley NJ, Fett SL, Zinsmeister AR, Melton LJ 3rd. Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Olmsted County, Minnesota. *Gastroenterology* 1997; **112**: 1448-1456
- 4 Furukawa N, Iwakiri R, Koyama T, Okamoto K, Yoshida T, Kashiwagi Y, Ohyama T, Noda T, Sakata H, Fujimoto K. Proportion of reflux esophagitis in 6010 Japanese adults: prospective evaluation by endoscopy. *J Gastroenterol* 1999; **34**: 441-444
- 5 Parkman HP, Schwartz SS. Esophagitis and gastroduodenal disorders associated with diabetic gastroparesis. *Arch Intern Med* 1987; **147**: 1477-1480
- 6 Antwi Ch, Krahulec B, Michalko L, Strbova L, Hlinstakova S, Balazovjeh I. Does diabetic autonomic neuropathy influence the clinical manifestations of reflux esophagitis? *Bratisl Lek Listy* 2003; **104**: 139-142
- 7 Inamori M, Togawa J, Nagase H, Abe Y, Umezawa T, Nakajima A, Saito T, Ueno N, Tanaka K, Sekihara H, Kaifu H, Tsuboi H, Kayama H, Tominaga S, Nagura H. Clinical characteristics of Japanese reflux esophagitis patients as determined by Los Angeles classification. *J Gastroenterol Hepatol* 2003; **18**: 172-176
- 8 El-Serag HB, Graham DY, Satia JA, Rabeneck L. Obesity is an independent risk factor for GERD symptoms and erosive esophagitis. *Am J Gastroenterol* 2005; **100**: 1243-1250
- 9 Koike T, Ohara S, Sekine H, Iijima K, Abe Y, Kato K, Toyota T, Shimosegawa T. *H pylori* infection prevents erosive reflux oesophagitis by decreasing gastric acid secretion. *Gut* 2001; **49**: 330-334
- 10 Shiota T, Kusano M, Kawamura O, Horikoshi T, Mori M, Sekiguchi T. *H pylori* infection correlates with severity of reflux esophagitis: with manometry findings. *J Gastroenterol* 1999; **34**: 553-559
- 11 Quatrini M, Boarino V, Ghidoni A, Baldassarri AR, Bianchi PA, Bardella MT. *H pylori* prevalence in patients with diabetes and its relationship to dyspeptic symptoms. *J Clin Gastroenterol* 2001; **32**: 215-217
- 12 Oldenburg B, Diepersloot RJ, Hoekstra JB. High seroprevalence of *H pylori* in diabetes mellitus patients. *Dig Dis Sci* 1996; **41**: 458-461
- 13 Perdichizzi G, Bottari M, Pallio S, Fera MT, Carbone M, Barresi G. Gastric infection by *H pylori* and antral gastritis in hyperglycemic obese and in diabetic subjects. *New Microbiol* 1996; **19**: 149-154
- 14 Kojecky V, Roubalik J, Bartonikova N. [*H pylori* in patients with diabetes mellitus]. *Vnitř Lek* 1993; **39**: 581-584
- 15 de Luis DA, de la Calle H, Roy G, de Argila CM, Valdezate S, Canton R, Boixeda D. *H pylori* infection and insulin-dependent diabetes mellitus. *Diabetes Res Clin Pract* 1998; **39**: 143-146
- 16 Ko GT, Chan FK, Chan WB, Sung JJ, Tsoi CL, To KF, Lai CW, Cockram CS. *H pylori* infection in Chinese subjects with type 2 diabetes. *Endocr Res* 2001; **27**: 171-177
- 17 Armstrong D, Bennett JR, Blum AL, Dent J, De Dombal FT, Galmiche JP, Lundell L, Margulies M, Richter JE, Spechler SJ, Tytgat GN, Wallin L. The endoscopic assessment of esophagitis: a progress report on observer agreement. *Gastroenterology* 1996; **111**: 85-92
- 18 Ichinose M, Miki K, Furihata C, Kageyama T, Hayashi R, Niwa H, Oka H, Matsushima T, Takahashi K. Radioimmunoassay of serum group I and group II pepsinogens in normal controls and patients with various disorders. *Clin Chim Acta* 1982; **126**: 183-191
- 19 Iinuma K, Ikeda I, Takai M, Yanagawa Y, Kurata K. [Gastrin radioimmunoassay with polyethylene glycol method]. *Radioisotopes* 1982; **31**: 350-356
- 20 Carlsson R, Dent J, Bolling-Sternevald E, Johnsson F, Junghard O, Lauritsen K, Riley S, Lundell L. The usefulness of

- a structured questionnaire in the assessment of symptomatic gastroesophageal reflux disease. *Scand J Gastroenterol* 1998; **33**: 1023-1029
- 21 **Inaba T**, Kawai K, Obara H, Miyatake H, Morimoto T, Hiratuka I, Horiike A, Morita T, Oonishi Y. The usefulness of a structured questionnaire (QUEST) in the assessment of gastro esophageal reflux disease. *J New Remedies Clinics* 1998; **47**: 841-851
- 22 **Locke GR 3rd**, Talley NJ, Fett SL, Zinsmeister AR, Melton LJ 3rd. Prevalence and clinical spectrum of gastroesophageal reflux: a population-based study in Olmsted County, Minnesota. *Gastroenterology* 1997; **112**: 1448-1456
- 23 **Berstad A**, Weberg R, Froyshov Larsen I, Hoel B, Hauer-Jensen M. Relationship of hiatus hernia to reflux oesophagitis. A prospective study of coincidence, using endoscopy. *Scand J Gastroenterol* 1986; **21**: 55-58
- 24 **Cronstedt J**, Carling L, Vestergaard P, Berglund J. Oesophageal disease revealed by endoscopy in 1,000 patients referred primarily for gastroscopy. *Acta Med Scand* 1978; **204**: 413-416
- 25 **Rasmussen CW**. A new endoscopic classification of Chronic Esophagitis. *Am J Gastroenterol* 1976; **65**: 409-415
- 26 **Inamori M**, Togawa J, Nagase H, Abe Y, Umezawa T, Nakajima A, Saito T, Ueno N, Tanaka K, Sekihara H, Kaifu H, Tsuboi H, Kayama H, Tominaga S, Nagura H. Clinical characteristics of Japanese reflux esophagitis patients as determined by Los Angeles classification. *J Gastroenterol Hepatol* 2003; **18**: 172-176
- 27 **Okamoto K**, Iwakiri R, Mori M, Hara M, Oda K, Danjo A, Ootani A, Sakata H, Fujimoto K. Clinical symptoms in endoscopic reflux esophagitis: evaluation in 8031 adult subjects. *Dig Dis Sci* 2003; **48**: 2237-2241
- 28 **Furukawa N**, Iwakiri R, Koyama T, Okamoto K, Yoshida T, Kashiwagi Y, Ohyama T, Noda T, Sakata H, Fujimoto K. Proportion of reflux esophagitis in 6010 Japanese adults: prospective evaluation by endoscopy. *J Gastroenterol* 1999; **34**: 441-444
- 29 **Nishida T**, Tsuji S, Tsujii M, Arimitsu S, Sato T, Haruna Y, Miyamoto T, Kanda T, Kawano S, Hori M. Gastroesophageal reflux disease related to diabetes: Analysis of 241 cases with type 2 diabetes mellitus. *J Gastroenterol Hepatol* 2004; **19**: 258-265
- 30 **Fisher BL**, Pennathur A, Mutnick JL, Little AG. Obesity correlates with gastroesophageal reflux. *Dig Dis Sci* 1999; **44**: 2290-2294
- 31 **Booth DJ**, Kemmerer WT, Skinner DB. Acid clearing from the distal esophagus. *Arch Surg* 1968; **96**: 731-734
- 32 **Mittal RK**, Holloway RH, Penagini R, Blackshaw LA, Dent J. Transient lower esophageal sphincter relaxation. *Gastroenterology* 1995; **109**: 601-610
- 33 **Cadiot G**, Bruhat A, Rigaud D, Coste T, Vuagnat A, Benyedder Y, Vallot T, Le Guludec D, Mignon M. Multivariate analysis of pathophysiological factors in reflux oesophagitis. *Gut* 1997; **40**: 167-174
- 34 **Hüppe D**, Tegenthoff M, Faig J, Brunke F, Depka S, Stuhldreier M, Micklefield G, Gillissen A, May B. Esophageal dysfunction in diabetes mellitus: is there a relation to clinical manifestation of neuropathy? *Clin Investig* 1992; **70**: 740-747
- 35 **Holloway RH**, Hongo M, Berger K, McCallum RW. Gastric distention: a mechanism for postprandial gastroesophageal reflux. *Gastroenterology* 1985; **89**: 779-784
- 36 **Diepersloot RJ**, Bouter KP, Beyer WE, Hoekstra JB, Masurel N. Humoral immune response and delayed type hypersensitivity to influenza vaccine in patients with diabetes mellitus. *Diabetologia* 1987; **30**: 397-401
- 37 **Canturk Z**, Canturk NZ, Cetinarslan B, Ercin C, Dokmetas S, Sencan M. Effects of rhG-CSF on neutrophil functions and bone marrow parameters in diabetic rats. *Endocr Res* 1999; **25**: 381-395
- 38 **Guvener N**, Akcan Y, Paksoy I, Soylu AR, Aydin M, Arslan S, Gedik O. *H pylori* associated gastric pathology in patients with type II diabetes mellitus and its relationship with gastric emptying: the Ankara study. *Exp Clin Endocrinol Diabetes* 1999; **107**: 172-176
- 39 **Mallecki M**, Bien AI, Galicka-Latalla D, Stachura J, Sieradzki J. The prevalence of *H pylori* infection and types of gastritis in diabetic patients. The Krakow Study. *Exp Clin Endocrinol Diabetes* 1996; **104**: 365-369

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RAPID COMMUNICATION

## Reactive oxygen species and chemokines: Are they elevated in the esophageal mucosa of children with gastroesophageal reflux disease?

Engin Tutar, Deniz Ertem, Goksenin Unluguzel, Sevda Tanrikulu, Goncagul Haklar, Cigdem Celikel, Evin Ademoglu, Ender Pehlivanoglu

Engin Tutar, Deniz Ertem, Ender Pehlivanoglu, Division of Pediatric Gastroenterology, Hepatology and Nutrition, Marmara University School of Medicine, Altunizade, İstanbul 81190, Turkey

Goksenin Unluguzel, Goncagul Haklar, Department of Biochemistry, Marmara University School of Medicine, Altunizade, İstanbul 81190, Turkey

Sevda Tanrikulu, Evin Ademoglu, Department of Biochemistry, Istanbul University Istanbul Faculty of Medicine, Çapa, İstanbul 34390, Turkey

Cigdem Celikel, Department of Pathology, Marmara University School of Medicine, Altunizade, İstanbul 81190, Turkey

**Author contributions:** Tutar E, Ertem D designed the study, recruited the study population, performed endoscopies and wrote the paper, Pehlivanoglu E involved in the study design, Tanrikulu S and Ademoglu E performed biochemical parameters, Unluguzel G and Haklar G involved in measurements of reactive oxygen species, Celikel C performed histopathological examinations.

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**Correspondence to:** Deniz Ertem, MD, Associate Professor of Pediatrics, Marmara University School of Medicine, Division of Pediatric Gastroenterology, Hepatology and Nutrition, Tophanelioglu Cd. 13-15, Altunizade, İstanbul 81190, Turkey. [denizertem@marmara.edu.tr](mailto:denizertem@marmara.edu.tr)

Telephone: +90-216-3266639 Fax: +90-216-3269578

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### Abstract

**AIM:** To determine the role of inflammatory cytokines and reactive oxygen species (ROS) in childhood reflux esophagitis.

**METHODS:** A total of 59 subjects who had complaints suggesting GERD underwent esophagogastroduodenoscopy. Endoscopic and histopathologic diagnosis of reflux esophagitis was established by Savary-Miller and Vandenplas grading systems, respectively. Esophageal biopsy specimens were taken from the esophagus 20% proximal above the esophagogastric junction for conventional histopathological examination and the measurements of ROS and cytokine levels. ROS were measured by chemiluminescence, whereas IL-8 and MCP-1 levels were determined with quantitative immunometric ELISA on esophageal tissue. Esophageal

tissue ROS, IL-8 and MCP-1 levels were compared among groups with and without endoscopic/histopathologic esophagitis.

**RESULTS:** Of 59 patients 28 (47.5%) had normal esophagus whereas 31 (52.5%) had endoscopic esophagitis. In histopathological evaluation, almost 73% of the cases had mild and 6.8% had moderate degree of esophagitis. When ROS and chemokine levels were compared among groups with and without endoscopic esophagitis, statistical difference could not be found between patients with and without esophagitis. Although the levels of ROS, IL-8 and MCP-1 were found to be higher in the group with histopathological reflux esophagitis, this difference was not statistically significant.

**CONCLUSION:** These results suggest that the grade of esophagitis is usually mild or moderate during childhood and factors apart from ROS, IL-8 and MCP-1 may be involved in the pathogenesis of reflux esophagitis in children.

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**Key words:** Gastroesophageal reflux disease; Reflux esophagitis; Reactive oxygen species; Interleukine-8; Monocyte chemoattractant protein-1

**Peer reviewers:** Michele Cicala, Professor, Dipartimento di Malattie dell'Apparato Digerente, Università Campus Bio-Medico, Via Longoni, 83-00155 Rome, Italy; William G Paterson, Professor of Medicine, Chair, Division of Gastroenterology, Hotel Dieu Hospital, 166 Brock St., Kingston, Ontario, K7L 5G2, Canada

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### INTRODUCTION

Gastroesophageal reflux disease (GERD) is one of



the most frequently encountered gastrointestinal pathologies in children with a prevalence of 8% during infancy. When the presence of heartburn as a presenting symptom was considered, the prevalence rises up to 40% among adults<sup>[1-4]</sup>. Early diagnosis and treatment is of importance as GERD results in serious problems that have negative influences on the quality of life. Since there is a wide range of symptoms regarding GERD and the results of GER questionnaires (GERQ) are controversial together with the incoherencies among other investigative methods; diagnosis of the disease is difficult<sup>[5]</sup>.

Oxidative stress has been associated with several disease states like atherosclerosis, pulmonary fibrosis, cancer, neurodegenerative diseases and aging<sup>[6-7]</sup>. Furthermore, reactive oxygen species (ROS) are reported to play a role in the pathogenesis of several gastrointestinal diseases such as inflammatory bowel disease and peptic ulcer<sup>[8-11]</sup>. In studies carried out on animal models of esophagitis as well as those on human esophageal tissue, ROS that are generated in the process of reflux esophagitis were found to be responsible for the esophageal tissue damage, and this finding was further supported by the studies showing that tissue damage could be prevented with the use of antioxidants<sup>[12-19]</sup>.

Several chemokines have been shown to increase significantly in inflammatory disease states like pulmonary diseases, viral meningitis, asthma, rhinitis, a topical dermatitis, ulcerative colitis and Crohn's disease<sup>[20-24]</sup>. While IL-8 has chemotactic activity for neutrophils, monocyte chemoattractant protein-1 (MCP-1) is effective in the activation of monocytes, macrophages as well as lymphocytes<sup>[25]</sup>. In studies conducted in adults, chemokine levels were found to be significantly high in the esophageal tissues of the patients with esophagitis as compared to controls; furthermore a significant correlation was reported between the severity of the reflux esophagitis and chemokines levels<sup>[26-30]</sup>. However, there is only limited data on the levels of ROS and tissue cytokines in the pathogenesis of reflux esophagitis in children. In a study including 10 children with reflux symptoms, IL-6 levels of the esophageal tissue was found to be higher than that of the normal resulting in a claim by the researchers that cytokines could be important in the pathogenesis of the reflux disease<sup>[31]</sup>.

The aim of this study is to investigate the roles of chemokines and ROS in reflux esophagitis in children by measuring the levels of ROS, IL-8 and MCP-1 in the esophageal tissues of children having endoscopic and histopathological esophagitis.

## MATERIALS AND METHODS

### *Patient selection, endoscopy and histopathology*

Consecutive children who underwent upper gastrointestinal endoscopy between September 2005 and January 2006 were prospectively considered for the study. Patients who had complaints related to GER such as vomiting, epigastric pain, regurgitation, retrosternal pain, dysphagia and persistent wheezing were included

in this study. Patients with a history of non-steroidal anti-inflammatory drug, proton pump inhibitor, H2 receptor antagonist or antibiotics use or those having severe chronic co-morbidities like diabetes mellitus, renal diseases or neurological diseases were excluded from the study. The study was approved by the local ethics committee of Marmara University School of Medicine, and informed consent forms were obtained from first degree relatives of all the patients.

Same endoscopy team performed the upper gastrointestinal system endoscopy in all patients with a pediatric fiberoptic gastroscope having an inner diameter of 2.8 mm (Fujinon EG 200 HR, Japan). Following an appropriate fasting time based on age of the patients, sedation was established by iv administration of meperidine (2-4 mg/kg) and midazolam (0.2-0.4 mg/kg) and endoscopy was performed. The degree of endoscopic esophagitis was evaluated according to Savary-Miller classification<sup>[32]</sup>. Patients with and without endoscopic esophagitis formed the first group (Group I). During endoscopy, 4 esophageal biopsy samples were obtained from 20% proximal part of esophagogastric junction for conventional histopathological examination and the measurements of ROS and cytokine levels. At the end of the study, histopathological examination of the biopsy samples was carried out at the same time by the same pathologist who was blind for the clinical and laboratory findings of the patient. The diagnosis of histopathological esophagitis was based on the classification by Vandenplas<sup>[33]</sup> and the cases with or without histopathological esophagitis formed the second group (Group II). For simplifying histopathological evaluation; Stage 1a, 1b and 1c were grouped as mild, Stage 2 and 3 as moderate and finally Stage 4 and 5 as severe esophagitis.

The third tissue sample was rinsed with 0.9% physiological saline and placed in eppendorf tubes for measurement of ROS levels in fresh tissues in the biochemistry laboratories. Other tissue samples were stored at -80°C for the measurement of tissue IL-8 and MCP-1 at the end of the study.

For conventional histopathological examination, 3 samples from the antrum, 2 from the corpus and 2 from the duodenum were obtained from all patients, and these were placed in 10% formaldehyde and sent to pathology for examination. The presence of *H. pylori* was confirmed with a positive rapid urease test and histopathological identification of *H. pylori*. Gastric biopsy samples were evaluated with a modified Sydney scoring system that allowed for identifying the presence of gastritis, its severity and the density of *H. pylori*.

### *Biochemical measurements*

For the measurement of free oxygen radicals on esophageal tissue, chemiluminescence method was employed. The measurements were carried out with Mini Lumat LB 9506 Luminometer (EG&G, Berthold, Germany) at room temperature on fresh tissue samples. In the method employed, lucigenin corresponds to superoxide radical, whereas luminol identifies the total

value for hydroxyl radical (OH $\cdot$ ), hydroperoxyl radical (HO $_2\cdot$ ) and peroxy (RO $_2\cdot$ ) radical. The tissues were first placed into 3 mL of PBS solution, on top of the tissues luminol or lucigenin (Sigma Chemicals, USA) were added at a concentration of 4 mmol/L as enhancers and measurements were obtained.

IL-8 and MCP-1 levels were measured by the quantitative immunometric sandwich enzyme linked-immunosorbent assay (ELISA) method. For IL-8 (Diacclone Research, France) and MCP-1 (Biosource, California, USA) measurements, the tissue samples were placed into eppendorf tubes and kept at -80°C. Cytokine measurements were performed on the same day with IL-8 and MCP-1 from the same samples. The tissues obtained were homogenized with phosphate buffered physiological saline to prepare 10% homogenates. After the homogenates were centrifuged at 1000  $\times g$  for 10 min, the supernatant obtained was used for measuring IL-8 and MCP-1 levels. As IL-8 and MCP-1 levels were to be calculated based on total amount of tissue protein, protein measurements were also performed on biopsy samples. The measurement of protein in esophageal homogenates was performed according to the "Bicinchoninic acid" method. For the procedure; bicinchoninic acid solution (Sigma B 9643, Sigma Chemicals) and 4% CuSO $_4$ , protein coloring reagent (0.2 mL 4% CuSO $_4$  on 10 mL bicinchoninic acid solution) were used. Ten  $\mu$ L of homogenate was added onto 200  $\mu$ L of protein coloring reagent. After mixing, the tube was kept at 37°C for 30 min. It was brought back to room temperature and the absorbance of the coloured complex was measured at 562 nm. The values were expressed as pg/mg tissue protein. IL-8 and MCP-1 measurements were carried out with EL  $\times$  800 BIO-TEK Instruments, Inc./USA brand ELISA device in line with the instructions provided in the commercial kits.

### Statistical analysis

In the statistical evaluation of the findings obtained from the study SPSS (Statistical Package for Social Sciences) for Windows 10.0 program was used. The Kolmogorov Smirnov test was used to compare the distributions of luminol, lucigenin, MCP-1 and IL-8 to parameters with normal distribution. As IL-8 and MCP-1 parameters had limit values, logarithmic conversion was used and the values were identified accordingly. In the comparisons of two groups with normal distributions, Student-*t* test was used. In the evaluation of the parameters that do not have a normal distribution, the Kruskal Wallis test was used. Chi-square test, McNemar test, Kappa analysis and diagnostic screening tests (sensitivity, specificity) were used to compare qualitative data. *P* level of < 0.05 was evaluated as being statistically significant.

## RESULTS

A total of 152 children underwent endoscopy during the study period and 59 out of 152 subjects who had complaints suggesting GERD included in the study.

ROS levels were measured in 54 and cytokine levels in 55 out of 59 patients. Mean age of the patients in the study was  $8.9 \pm 4.4$  years (age range 1.5-17 years). Of 59 patients 28 (47.5%) had normal esophagus whereas 31 (52.5%) had endoscopic esophagitis according to Savary Miller classification. In histopathological evaluation, 80% of the cases had mild or moderate degree of esophagitis. Of 31 patients having endoscopic esophagitis, 29 (93.5%) also had histopathological esophagitis whereas of 28 patients not having endoscopic esophagitis 18 (64.3%) had histopathological esophagitis. The sensitivity of endoscopic esophagitis for prediction of histopathological esophagitis was significantly high (93.6%, *P* = 0.0083). However, endoscopy had a low specificity in the diagnosis of histopathological esophagitis in children. Kappa correlation rate between the 2 methods was 30.1%: PPV, 61.7% and NPV, 83.3%.

When esophageal tissue ROS and chemokine levels were compared among groups with and without endoscopic esophagitis, statistical difference could neither be found between the stages of endoscopic esophagitis nor between the patients with and without esophagitis (Table 1).

When a comparison was made in terms of luminol and lucigenin levels of cases with and without histopathological esophagitis, there was a difference of statistical significance between the two groups (*P* = 0.049 and *P* = 0.044, respectively). While luminol levels did not differ among normal and patients with mild esophagitis, luminol levels of patients with moderate esophagitis were significantly higher than the normal patients (Table 2). Since there was not a statistically significant difference in luminol levels between children with mild and moderate esophagitis, two groups were combined, however, groups with and without histopathological esophagitis did not differ for either luminol or lucigenin measurements (*P* > 0.05).

Patients with histopathological esophagitis and normal were compared for MCP-1 and IL-8 levels. The highest cytokine levels were identified in patients with moderate esophagitis while lowest levels were found in normal children. However, this difference did not reach a statistical evaluations (*P* > 0.05, Table 3).

In order to investigate the effect of presence of *H. pylori* gastritis, the patients having esophagitis were classified and patients with and without *H. pylori* gastritis were compared. Patients with *H. pylori* gastritis and those not having gastritis did not show any statistically significant difference between their levels of ROS and chemokines.

## DISCUSSION

Gastroesophageal reflux when untreated in children may be related to some serious complications such as esophagitis, failure to thrive, esophageal strictures, Barrett esophagus and adenocarcinoma<sup>[54]</sup>. Delineation of the pathogenesis of GERD will allow for the development of more effective treatment strategies while making it possible to prevent complications. In

**Table 1** The comparison of luminol, lucigenin, MCP-1 and IL-8 levels between endoscopic esophagitis and normal groups (mean  $\pm$  SD)

	Esophagitis	Normal	P value <sup>1</sup>
Luminol (rlu/mg)	175.2 $\pm$ 98.5	152.7 $\pm$ 71.3	0.349
Lucigenin (rlu/mg)	154.1 $\pm$ 74.9	155.7 $\pm$ 79.9	0.939
MCP-1 (pg/mg)	39.8 $\pm$ 1.7	47.8 $\pm$ 1.7	0.248
IL-8 (pg/mg)	331.1 $\pm$ 2.9	323.6 $\pm$ 2.3	0.898

<sup>1</sup>Student-t test.**Table 2** The comparison of luminol and lucigenin levels according to the severity of histopathological esophagitis (mean  $\pm$  SD)

Vandenplas classification	Normal (n = 12)	Mild esophagitis (n = 43)	Moderate esophagitis (n = 4)	P value <sup>1</sup>
Luminol (rlu/mg)	129.9 $\pm$ 80.9	164.4 $\pm$ 76.3	236.8 $\pm$ 142.8	0.049
Lucigenin (rlu/mg)	156.8 $\pm$ 91.7	145.4 $\pm$ 65.4	244.8 $\pm$ 94.6	0.044

<sup>1</sup>Kruskal Wallis test (normal vs moderate esophagitis).**Table 3** The comparison of MCP-1 and IL-8 levels according to the severity of histopathological esophagitis (mean  $\pm$  SD)

Vandenplas classification	Normal (n = 12)	Mild esophagitis (n = 43)	Moderate esophagitis (n = 4)	P value <sup>1</sup>
MCP-1 (pg/mg)	38.9 $\pm$ 1.7	44.5 $\pm$ 1.7	53.7 $\pm$ 2.7	0.804
IL-8 (pg/mg)	262.4 $\pm$ 2.3	337.3 $\pm$ 2.5	630.9 $\pm$ 5.6	0.614

<sup>1</sup>Kruskal Wallis test.

recent years, the role of ROS and chemokines in the pathogenesis of GERD and reflux esophagitis are being investigated both in experimental esophagitis models and in humans<sup>[12,17,19,27,29,35]</sup>.

The relationship between reflux esophagitis and free oxygen radicals was elaborated in this study, no statistical difference could be identified between normal cases and those having different degrees of endoscopic and histopathologic esophagitis. However, although it was statistically not significant, ROS levels were found to be increased in patient groups compared to the normal group.

Free oxygen radicals in general and superoxide radical in particular were shown to increase in animals with esophagitis and it was claimed that free radical scavengers like SOD and DA-9601 as well as anti-inflammatory agents like ketotifen could prevent the tissue damage<sup>[12,13,15,17,35]</sup>. However, in the study by Soterias *et al*<sup>[36]</sup> free oxygen radicals were found to play a role mostly in severe esophagitis and it was concluded that free oxygen radicals did not increase in the mild histopathological esophagitis model and thus ROS might not be influential on the pathogenesis of mild esophagitis. Studies performed in adults with reflux esophagitis are in support

of the experimental esophagitis models showing that free oxygen radicals do play a role in the pathogenesis of reflux esophagitis<sup>[18,19]</sup>. Olyae *et al*<sup>[37]</sup> have identified a significant correlation between the degree of esophagitis and the levels of free oxygen radicals in the tissue; under the light of this finding they stipulated that in mucosal epithelial cells affected by the injury, the production of ROS was increased. After examining mucosal biopsies of cases with erosive gastritis and Barrett esophagus; another group of researcher reported that the main oxidant product responsible for the development of reflux esophagitis was superoxide anion<sup>[38]</sup>.

Although there are no studies investigating the role of free oxygen radicals in children with reflux esophagitis, our findings are different than the results obtained from adult patients with reflux esophagitis. In our study, neither lucigenin which showed superoxide radical production nor luminol reflecting the productions of other free radicals were found to be increased in children with esophagitis compared to controls. Despite not reaching the level of statistical significance, free oxygen radical levels were found to be higher in children with esophagitis when compared to the controls. The reason behind not identifying a statistically significant difference between the cases of esophagitis and normal may be attributed to the fact that in most of the cases with histopathological reflux esophagitis was of mild degree.

Likewise, the levels of IL-8 and MCP-1 that are chemokines in relation with neutrophil and mononuclear cell migration were found to be higher in the group with histopathological reflux esophagitis compared to normal, however this difference did not reach a level of statistical significance either. In studies performed in adults, tissue chemokine levels are reported to be higher in cases with esophagitis than normal and the increase is reported to be in parallel with the severity of the histopathological esophagitis concerned. Fitzgerald *et al*<sup>[26]</sup> have identified 3-10 fold higher levels of tissue cytokines in the esophageal mucosa of adults with esophagitis when compared to patients with Barrett esophagus and normal controls. Furthermore, adult patients with nonerosive esophagitis were reported to have higher levels of tissue cytokines when compared to normal<sup>[27-30]</sup>. Similarly, in a study comparing 10 children with reflux esophagitis to normal patients, esophageal tissue IL-6 levels were reported to be significantly higher in patients with esophagitis<sup>[31]</sup>. In our study, the lowest levels were measured in normal cases whereas the highest levels were identified in patients with a moderate degree of esophagitis. The difference between these two groups was not statistically significant, this can be explained by the fact that the number of individuals who were normal and who had mild and moderate esophagitis were quite different from each other.

In *H pylori* gastritis, it has been shown that levels of IL-8, MCP-1 and other cytokines increased in gastric mucosa but we do not know the effect of this infection on esophageal tissue which is not normally colonized by the bacteria<sup>[39-41]</sup>. In our study, we could not identify any



effects of *H pylori* gastritis on esophageal tissue ROS and chemokine levels.

One drawback of this study is the lack of a true control group. All the study group was selected from the patients who had GER symptoms. The subjects who had either endoscopic or histopathological reflux esophagitis were compared to their nonreflux counterparts. The subjects who were included in the normal group according to the endoscopic findings might have a nonerosive reflux disease. Similarly, the subjects included in the normal group according to the histopathological examination might have an increased tissue level of proinflammatory substances before the establishment of the well-known histopathological changes. This might be another explanation for the lack of a clear cut statistical significance between the groups. However it is not possible to find a true control group for these kind of studies because it is not ethical and possible to perform endoscopy in totally normal children.

In parallel with the results of the studies on adult GERD or esophagitis, the patients in our study had higher levels of tissue ROS and chemokines; however, this increase did not reach a level of statistical significance. The number of studies aiming at identifying the pathogenesis of reflux esophagitis in children is very limited. Studies to be performed with higher numbers of patients with the aim of identifying the pathogenesis will allow for the development of further diagnostic and therapeutic opportunities for GERD which is a significant cause of morbidity.

## COMMENTS

### Background

Gastroesophageal reflux disease (GERD) is one of the most frequently encountered gastrointestinal pathology not only in adults but also in children. Since it may be related to some serious complications such as esophagitis, failure to thrive, esophageal strictures, Barrett esophagus and adenocarcinoma, early diagnosis and treatment of reflux disease is crucial. In recent years, it has been reported that reactive oxygen species (ROS) and inflammatory chemokines play an important role in the pathogenesis of GERD in adults but there is scarce data regarding children with reflux esophagitis.

### Research frontiers

Inflammatory chemokine levels were found to be significantly high in the esophageal tissues of the patients with esophagitis and a significant correlation was found between the severity of the reflux esophagitis and chemokines levels in adults. The aim was to investigate the role of chemokines and ROS in children with reflux esophagitis. Since the duration of reflux disease might be shorter in children compared to adults, the potential role of chemokines and ROS was interrogated in early or mild cases of reflux esophagitis.

### Innovations and breakthroughs

In this study we showed that the level of ROS and chemokines in esophageal tissue were higher in children with esophagitis compared to the subjects without esophagitis. However the difference was not statistically significant. Hence, factors apart from ROS, IL-8 and MCP-1 might be important in the pathogenesis of reflux esophagitis in children.

### Applications

It was found that ROS and chemokines increased in children with reflux esophagitis. Opposite to the adults, the exposure of the noxious agents to the esophagus is not long enough in children, endoscopic or histopathological esophagitis might be obscure. Hence tissue level of ROS or/and chemokines might be an important finding in early diagnosis of childhood esophagitis. Furthermore, the use of antioxidants or antiinflammatory agents might be alternative treatment modalities to the established treatment regimens.

## Peer review

This manuscript showed that ROS and chemokines are increased in esophageal tissue in children with reflux esophagitis, though not statistically significant. This finding might be important for the delineation of the pathogenesis of reflux esophagitis in children, and therapeutic alternatives targeting these chemokines or ROS could be an option in the future.

## REFERENCES

- 1 Fass R, Ofman JJ. Gastroesophageal reflux disease--should we adopt a new conceptual framework? *Am J Gastroenterol* 2002; **97**: 1901-1909
- 2 Vandenplas Y, Goyvaerts H, Helven R, Sacre L. Gastroesophageal reflux, as measured by 24-hour pH monitoring, in 509 healthy infants screened for risk of sudden infant death syndrome. *Pediatrics* 1991; **88**: 834-840
- 3 Spechler SJ. Epidemiology and natural history of gastro-oesophageal reflux disease. *Digestion* 1992; **51** Suppl 1: 24-29
- 4 Wienbeck M, Barnert J. Epidemiology of reflux disease and reflux esophagitis. *Scand J Gastroenterol Suppl* 1989; **156**: 7-13
- 5 Salvatore S, Hauser B, Vandemaële K, Novario R, Vandenplas Y. Gastroesophageal reflux disease in infants: how much is predictable with questionnaires, pH-metry, endoscopy and histology? *J Pediatr Gastroenterol Nutr* 2005; **40**: 210-215
- 6 Cross CE, Halliwell B, Borish ET, Pryor WA, Ames BN, Saul RL, McCord JM, Harman D. Oxygen radicals and human disease. *Ann Intern Med* 1987; **107**: 526-545
- 7 Halliwell B, Gutteridge JM, Cross CE. Free radicals, antioxidants, and human disease: where are we now? *J Lab Clin Med* 1992; **119**: 598-620
- 8 Keshavarzian A, Sedghi S, Kanofsky J, List T, Robinson C, Ibrahim C, Winship D. Excessive production of reactive oxygen metabolites by inflamed colon: analysis by chemiluminescence probe. *Gastroenterology* 1992; **103**: 177-185
- 9 Simmonds NJ, Allen RE, Stevens TR, Van Someren RN, Blake DR, Rampton DS. Chemiluminescence assay of mucosal reactive oxygen metabolites in inflammatory bowel disease. *Gastroenterology* 1992; **103**: 186-196
- 10 Davies GR, Simmonds NJ, Stevens TR, Grandison A, Blake DR, Rampton DS. Mucosal reactive oxygen metabolite production in duodenal ulcer disease. *Gut* 1992; **33**: 1467-1472
- 11 Wallace JL, Arfors KE, McKnight GW. A monoclonal antibody against the CD18 leukocyte adhesion molecule prevents indomethacin-induced gastric damage in the rabbit. *Gastroenterology* 1991; **100**: 878-883
- 12 Wetscher GJ, Hinder PR, Bagchi D, Perdakis G, Redmond EJ, Glaser K, Adrian TE, Hinder RA. Free radical scavengers prevent reflux esophagitis in rats. *Dig Dis Sci* 1995; **40**: 1292-1296
- 13 Wetscher GJ, Perdakis G, Kretschmar DH, Stinson RG, Bagchi D, Redmond EJ, Adrian TE, Hinder RA. Esophagitis in Sprague-Dawley rats is mediated by free radicals. *Dig Dis Sci* 1995; **40**: 1297-1305
- 14 Lanás A, Royo Y, Ortego J, Molina M, Sainz R. Experimental esophagitis induced by acid and pepsin in rabbits mimicking human reflux esophagitis. *Gastroenterology* 1999; **116**: 97-107
- 15 Oh TY, Lee JS, Ahn BO, Cho H, Kim WB, Kim YB, Surh YJ, Cho SW, Hahm KB. Oxidative damages are critical in pathogenesis of reflux esophagitis: implication of antioxidants in its treatment. *Free Radic Biol Med* 2001; **30**: 905-915
- 16 Liu L, Ergun G, Ertan A, Woods K, Sachs I, Younes M. Detection of oxidative DNA damage in oesophageal biopsies of patients with reflux symptoms and normal pH monitoring. *Aliment Pharmacol Ther* 2003; **18**: 693-698
- 17 Lanás A, Soteras F, Jimenez P, Fiteni I, Piazzuelo E, Royo Y, Ortego J, Inarrea P, Esteva F. Superoxide anion and nitric oxide in high-grade esophagitis induced by acid and pepsin in rabbits. *Dig Dis Sci* 2001; **46**: 2733-2743
- 18 Wetscher GJ, Hinder RA, Bagchi D, Hinder PR, Bagchi M, Perdakis G, McGinn T. Reflux esophagitis in humans is



- mediated by oxygen-derived free radicals. *Am J Surg* 1995; **170**: 552-556; discussion 556-557
- 19 **Wetscher GJ**, Hinder RA, Klingler P, Gadenstatter M, Perdakis G, Hinder PR. Reflux esophagitis in humans is a free radical event. *Dis Esophagus* 1997; **10**: 29-32; discussion 33
  - 20 **Lahrtz F**, Piali L, Nadal D, Pfister HW, Spanaus KS, Baggiolini M, Fontana A. Chemotactic activity on mononuclear cells in the cerebrospinal fluid of patients with viral meningitis is mediated by interferon-gamma inducible protein-10 and monocyte chemotactic protein-1. *Eur J Immunol* 1997; **27**: 2484-2489
  - 21 **Luster AD**, Rothenberg ME. Role of the monocyte chemoattractant protein and eotaxin subfamily of chemokines in allergic inflammation. *J Leukoc Biol* 1997; **62**: 620-633
  - 22 **Garcia-Zepeda EA**, Rothenberg ME, Ownbey RT, Celestin J, Leder P, Luster AD. Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. *Nat Med* 1996; **2**: 449-456
  - 23 **Reinecker HC**, Loh EY, Ringler DJ, Mehta A, Rombeau JL, MacDermott RP. Monocyte-chemoattractant protein 1 gene expression in intestinal epithelial cells and inflammatory bowel disease mucosa. *Gastroenterology* 1995; **108**: 40-50
  - 24 **Grimm MC**, Elsbury SK, Pavli P, Doe WF. Enhanced expression and production of monocyte chemoattractant protein-1 in inflammatory bowel disease mucosa. *J Leukoc Biol* 1996; **59**: 804-812
  - 25 **Mukaida N**, Harada A, Matsushima K. Interleukin-8 (IL-8) and monocyte chemotactic and activating factor (MCAF/MCP-1), chemokines essentially involved in inflammatory and immune reactions. *Cytokine Growth Factor Rev* 1998; **9**: 9-23
  - 26 **Fitzgerald RC**, Onwuegbusi BA, Bajaj-Elliott M, Saeed IT, Burnham WR, Farthing MJ. Diversity in the oesophageal phenotypic response to gastro-oesophageal reflux: immunological determinants. *Gut* 2002; **50**: 451-459
  - 27 **Isomoto H**, Wang A, Mizuta Y, Akazawa Y, Ohba K, Omagari K, Miyazaki M, Murase K, Hayashi T, Inoue K, Murata I, Kohno S. Elevated levels of chemokines in esophageal mucosa of patients with reflux esophagitis. *Am J Gastroenterol* 2003; **98**: 551-556
  - 28 **Isomoto H**, Saenko VA, Kanazawa Y, Nishi Y, Ohtsuru A, Inoue K, Akazawa Y, Takeshima F, Omagari K, Miyazaki M, Mizuta Y, Murata I, Yamashita S, Kohno S. Enhanced expression of interleukin-8 and activation of nuclear factor kappa-B in endoscopy-negative gastroesophageal reflux disease. *Am J Gastroenterol* 2004; **99**: 589-597
  - 29 **Yoshida N**, Uchiyama K, Kuroda M, Sakuma K, Kokura S, Ichikawa H, Naito Y, Takemura T, Yoshikawa T, Okanoue T. Interleukin-8 expression in the esophageal mucosa of patients with gastroesophageal reflux disease. *Scand J Gastroenterol* 2004; **39**: 816-822
  - 30 **Kanazawa Y**, Isomoto H, Wen CY, Wang AP, Saenko VA, Ohtsuru A, Takeshima F, Omagari K, Mizuta Y, Murata I, Yamashita S, Kohno S. Impact of endoscopically minimal involvement on IL-8 mRNA expression in esophageal mucosa of patients with non-erosive reflux disease. *World J Gastroenterol* 2003; **9**: 2801-2804
  - 31 **Corrado G**, Zicari A, Cavaliere M, Rea P, Pacchiarotti C, Cerroni F, Pontieri G, Cardi E. Increased release of interleukin-6 by oesophageal mucosa in children with reflux oesophagitis. *Eur J Gastroenterol Hepatol* 1999; **11**: 839-843
  - 32 **Suys B**, De Wolf D, Hauser B, Blecker U, Vandenplas Y. Bradycardia and gastroesophageal reflux in term and preterm infants: is there any relation? *J Pediatr Gastroenterol Nutr* 1994; **19**: 187-190
  - 33 **Vandenplas Y**. Reflux esophagitis in infants and children: a report from the Working Group on Gastro-Oesophageal Reflux Disease of the European Society of Paediatric Gastroenterology and Nutrition. *J Pediatr Gastroenterol Nutr* 1994; **18**: 413-422
  - 34 **Vandenplas Y**. Gastroesophageal reflux: medical treatment. *J Pediatr Gastroenterol Nutr* 2005; **41** Suppl 1: S41-S42
  - 35 **Naya MJ**, Pereboom D, Ortego J, Alda JO, Lanas A. Superoxide anions produced by inflammatory cells play an important part in the pathogenesis of acid and pepsin induced oesophagitis in rabbits. *Gut* 1997; **40**: 175-181
  - 36 **Soteras F**, Lanas A, Fiteni I, Royo Y, Jimenez P, Inarrea P, Ortego J, Esteve F. Nitric oxide and superoxide anion in low-grade esophagitis induced by acid and pepsin in rabbits. *Dig Dis Sci* 2000; **45**: 1802-1809
  - 37 **Olyae M**, Sontag S, Salman W, Schnell T, Mobarhan S, Eiznhamer D, Keshavarzian A. Mucosal reactive oxygen species production in oesophagitis and Barrett's oesophagus. *Gut* 1995; **37**: 168-173
  - 38 **Jimenez P**, Piazzuelo E, Sanchez MT, Ortego J, Soteras F, Lanas A. Free radicals and antioxidant systems in reflux esophagitis and Barrett's esophagus. *World J Gastroenterol* 2005; **11**: 2697-2703
  - 39 **Isomoto H**, Mizuta Y, Miyazaki M, Takeshima F, Omagari K, Murase K, Nishiyama T, Inoue K, Murata I, Kohno S. Implication of NF-kappaB in Helicobacter pylori-associated gastritis. *Am J Gastroenterol* 2000; **95**: 2768-2776
  - 40 **Isomoto H**, Miyazaki M, Mizuta Y, Takeshima F, Murase K, Inoue K, Yamasaki K, Murata I, Koji T, Kohno S. Expression of nuclear factor-kappaB in Helicobacter pylori-infected gastric mucosa detected with southwestern histochemistry. *Scand J Gastroenterol* 2000; **35**: 247-254
  - 41 **Crabtree JE**, Shallcross TM, Heatley RV, Wyatt JL. Mucosal tumour necrosis factor alpha and interleukin-6 in patients with Helicobacter pylori associated gastritis. *Gut* 1991; **32**: 1473-1477

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RAPID COMMUNICATION

## Prognostic factors in patients with advanced cholangiocarcinoma: Role of surgery, chemotherapy and body mass index

Mirna H Farhat, Ali I Shamseddine, Ayman N Tawil, Ghina Berjawi, Charif Sidani, Wael Shamseddeen, Kassem A Barada

Mirna H Farhat, Ali I Shamseddine, Division of Hematology-Oncology, Department of Internal Medicine, American University of Beirut Medical Center, Beirut, 110 72020, Lebanon

Ayman N Tawil, Department of Pathology, American University of Beirut Medical Center, Beirut, 110 72020, Lebanon

Ghina Berjawi, Charif Sidani, Department of Radiology, American University of Beirut Medical Center, Beirut, 110 72020, Lebanon

Wael Shamseddeen, Department of Public health, American University of Beirut Medical Center, Beirut, 110 72020, Lebanon

Kassem A Barada, Division of Gastroenterology, Department of Internal Medicine, American University of Beirut Medical Center, Beirut, 110 72020, Lebanon

**Author contributions:** Farhat MH, Shamseddine AI, Barada KA designed research; Tawil AN reviewed pathology; Berjawi G and Sidani C reviewed radiology; Shamseddeen W analyzed data; Farhat MH wrote the paper; Shamseddine AI and Barada KA reviewed the paper.

**Correspondence to:** Kassem Barada, MD, Division of Gastroenterology, Department of Internal Medicine, American University of Beirut Medical Center, Beirut, 110 72020, Lebanon. [kb02@aub.edu.lb](mailto:kb02@aub.edu.lb)

Telephone: +961-3-780909 Fax: +961-3-50005112

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only low bilirubin level < 10 mg/dL and chemotherapy administration as independent predictors associated with better survival ( $P < 0.05$ ).

**CONCLUSION:** Our data show that palliative and postoperative chemotherapy as well as a bilirubin level < 10 mg/dL are independent predictors of a significant increase in survival in patients with cholangiocarcinoma.

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**Key words:** Cholangiocarcinoma; Biliary tract cancer; Chemotherapy; Bilirubin; Prognosis

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Farhat MH, Shamseddine AI, Tawil AN, Berjawi G, Sidani C, Shamseddeen W, Barada KA. Prognostic factors in patients with advanced cholangiocarcinoma: Role of surgery, chemotherapy and body mass index. *World J Gastroenterol* 2008; 14(20): 3224-3230 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3224.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3224>

### Abstract

**AIM:** To study the factors that may affect survival of cholangiocarcinoma in Lebanon.

**METHODS:** A retrospective review of the medical records of 55 patients diagnosed with cholangiocarcinoma at the American University of Beirut between 1990 and 2005 was conducted. Univariate and multivariate analyses were performed to determine the impact of surgery, chemotherapy, body mass index, bilirubin level and other factors on survival.

**RESULTS:** The median survival of all patients was 8.57 mo (0.03-105.2). Univariate analysis showed that low bilirubin level (< 10 mg/dL), radical surgery and chemotherapy administration were significantly associated with better survival ( $P = 0.012$ , 0.038 and 0.038, respectively). In subgroup analysis on patients who had no surgery, chemotherapy administration prolonged median survival significantly (17.0 mo vs 3.5 mo,  $P = 0.001$ ). Multivariate analysis identified

### INTRODUCTION

Cholangiocarcinoma is a rare and highly fatal neoplasm that arises from biliary epithelium. It constitutes approximately 2% of all reported cancer<sup>[1]</sup>, and accounts for about 3 percent of all gastrointestinal malignancies<sup>[2]</sup>. Up to date, radical surgery remains the optimal therapy for cholangiocarcinoma offering a potential for cure<sup>[1,3,4]</sup>. In surgical patients with negative margins, five-year survival rates approach 20%-35% as compared to zero in those with positive margins<sup>[5]</sup>. However, most patients present with advanced disease precluding surgery<sup>[6-8]</sup>. Overall prognosis in these patients is poor and survival is limited to a few months<sup>[9]</sup>. Thus, it is crucial to identify factors that would improve survival in such patients.

The role of chemotherapy in cholangiocarcinoma is yet to be determined, with conflicting data regarding its effect on survival. This is due to lack of randomized clinical trials, and absence of a standard chemotherapeutic

regimen<sup>[10]</sup>. While some authors believe that chemotherapy prolongs survival in cholangiocarcinoma<sup>[11,12]</sup>, others deny this survival benefit<sup>[13-15]</sup>.

The impact of excess body weight on survival in patients with different cancers is variable. While it is associated with improved survival in patients with cancers of the gastric cardia<sup>[16]</sup>, less aggressive disease in renal cell cancers<sup>[17]</sup>, and lower malignant potential in ovarian tumors<sup>[18]</sup>, it was found to increase mortality in early stage breast cancers<sup>[19]</sup>, cancer of the esophagus, colon and rectum, liver, gallbladder, pancreas, and kidney<sup>[20]</sup>. However, the relationship of increased body mass index (BMI) and survival in cholangiocarcinoma has not been thoroughly investigated.

Many factors are well known to increase the risk of cholangiocarcinoma; these include age, primary sclerosing cholangitis (PSC), hepatolithiasis, and liver flukes<sup>[6]</sup>.

Several studies have provided evidence that excess body weight and obesity increase the risk of overall cancers. In a study by Lee *et al.*, overweight people had a one and a half times increased risk of cancer compared to those with normal weight in both sexes<sup>[21]</sup>. Similarly, obese people had a 33% increase in overall cancer incidence<sup>[22]</sup>. In cancers of the biliary tract, cancer of the gall bladder was linked previously with increased BMI in women<sup>[23,24]</sup>, whether increased BMI is a risk factor for cholangiocarcinoma is not known yet. In our study, the effect of increased BMI was given special emphasis to investigate its role as a risk factor and as a prognostic indicator.

In this study, we sought to determine the clinicopathologic characteristics of patients with cholangiocarcinoma. We also tried to identify the determinants of prognosis and survival in those patients with special emphasis on the role of surgery, chemotherapy and BMI.

## MATERIALS AND METHODS

Patients diagnosed with cholangiocarcinoma at the American University of Beirut-Medical Center during the 15 year period between 1990 and 2005 were identified. Patients' demographics, clinical data, radiological and histopathologic findings, surgical intervention, chemotherapy administration, and survival data were obtained retrospectively from hospital medical charts and by contacting patients or their family members. All histopathology slides and radiographic studies were reevaluated by a pathologist and a radiologist to obtain data about tumor location, grade, stage, lymphatic spread, vascular invasion and metastasis.

Tumors were classified as intrahepatic if originating from intrahepatic ductules (proximal to the bifurcation of the right and left hepatic ducts), and extrahepatic if perihilar (involving the confluence of the right and left bile ducts) or distal (if originating distal to the confluence of hepatic ducts).

AJCC 2003 criteria were used for TNM staging of the tumor<sup>[25]</sup>.

The impact of high bilirubin level at presentation, tumor location, size, grade, metastasis, presence of

vascular or perineural invasion, positive surgical margins, type of treatment including palliative stenting, surgery and chemotherapy on survival was examined.

Parameters examined as possible risk factors for cholangiocarcinoma were age, gender, diabetes, BMI, history of cholelithiasis, Hepatitis B and C infection, smoking, alcohol consumption, presence of cirrhosis, inflammatory bowel disease (IBD), PSC, and parasitic infestations.

To determine if increasing BMI is a risk factor for cholangiocarcinoma, patients were compared to controls of the same age groups that were selected from a large study about obesity in the Lebanese population<sup>[26]</sup>. According to WHO standards<sup>[27]</sup>, subjects were categorized according to their body mass index (normal: < 25, overweight: 25-30, and obese:  $\geq 30$  kg/m<sup>2</sup>).

All data was coded and entered using SPSS 14.0 computer program. The Kaplan-Meier method was used to estimate survival which was measured from time of presentation to AUB-MC to the date of death or date of last follow up. Differences in survival between subgroups were compared using the log-rank test. Univariate analysis was performed using the chi-squared testing. Multivariate analysis was performed with the Cox proportional hazards model. A *P*-value of less than 0.05 was considered statistically significant.

## RESULTS

### Demographics and clinical data

During the 15-year period, a total of 55 patients diagnosed with cholangiocarcinoma were studied. The demographic and clinical data of all patients are listed in Table 1. There were 34 males (60.7%) and 22 (39.3%) females. The mean age for all patients was  $62.6 \pm 13.0$  years and ranged from 28 years to 91 years. Seventeen patients were older than 70 years (11 females and 8 males). In males, the incidence of the tumor was highest in the age group of 50-59 years, while in females it was in the older than 70 years age group.

The most common presenting symptoms were jaundice (72.7%), dark urine (61.8%), weight loss (43.6%), abdominal pain (43.6%), pruritus (36.4%), and fever (10.9%).

### Risk factors

None of the patients had primary sclerosing cholangitis or any evidence of parasitic infestation on histological examination. One patient had inflammatory bowel disease (1.8%). One patient had a history of hepatitis B infection and two had liver cirrhosis of unknown etiology (3.6%). Ten patients had a history of cholecystectomy (17.8%) and 17 had a history of cholelithiasis (30.9%). Fifteen patients had a history of diabetes mellitus (25.5%) and one third (33%) were obese (BMI  $\geq 30$  kg/m<sup>2</sup>). One patient had a family history of cholangiocarcinoma (1.8%).

### Microscopic and macroscopic appearance

Tissue diagnosis was obtained on 30 patients (54.5%). Histopathologic findings are listed in Table 2. Tumors

**Table 1** Demographic and clinical data of 55 patients with cholangiocarcinoma

	Number of patients (%)	
Age (mean $\pm$ SD)	62.6 $\pm$ 13	
Range (yr)	28-91	61/39
Male/Female	34/22	
Diabetes (Yes/No)	14/41	25/75
BMI (kg/m <sup>2</sup> )		
< 25	18	35
25-30	16	31
$\geq$ 30	18	35
Not available	3	
History of cholelithiasis (Yes /No)	17/38	31/69
Clinical manifestations		
Jaundice	40	72.7
Dark urine	34	61.8
Weight loss	24	43.6
Abdominal pain	24	43.6
Total bilirubin		
< 10 mg/dL	27	49
$\geq$ 10 mg/dL	17	31
Missing	11	20
AJCC staging		
I	1	
II	1	
III	0	
IV	49	89
Missing	4	
Surgery		
Yes	21	38
No	34	62
Adjuvant chemotherapy		
Yes	14	25
No	41	75

larger than 3 cm, as measured in resected specimens and by radiology, comprised 34.5%, compared to 23.6% for those less than 3 cm. The most common morphological type was intraductal growing ( $n = 34$ , 61.8%) followed by mass forming ( $n = 21$ , 38%). Mass forming morphology was present in 63% and 35% of intrahepatic and extrahepatic tumors, respectively, with periductal infiltrating morphology comprising the rest of the patients.

Tumor grade was available on 27 patients. They were mostly moderately (55.6%) and poorly differentiated (29.6%). Vascular involvement on histology was evident in 12 patients (21.8%), while perineural invasion was found in 10 patients (18.2%).

Tumors presented at stage IV in 49 out of 55 patients (89%).

Distribution of tumor could be obtained on 48 patients (Table 2). Thirty-seven tumors (67.2%) were extrahepatic *versus* 11 intrahepatic (20%).

Of the extrahepatic tumors, 19 were distal (34.5%) and 18 were perihilar (32.7%). Nineteen patients had metastatic disease. The most common sites of metastases were the liver (25.4%,  $n = 14/55$ ), followed by the peritoneum (10.9%,  $n = 6/55$ ). Two patients had lymph node metastasis. One patient had brain metastasis and another had bone metastasis.

### Treatment

The resection rate of the tumor was low (21/55, 31.8%).

**Table 2** Tumor location, size, grade, nodal and margin status and relation to survival in patients with cholangiocarcinoma

	<i>n</i>	Median survival (95% CI)	<i>P</i>
Location			
Intrahepatic	10	6.23 (0.76-11.7)	0.68
Perihilar	18	11.47 (6.73-16.2)	
Distal	21	9.17 (0.52-17.8)	
Not available	6		
Size			
< 3 cm	19	3.47 (1.9-5.0)	0.31
$\geq$ 3 cm	13	11 (4.13-17.87)	
Not available	23	13	
Grade			
Poor	4	16.96 (8.9-25.0)	0.31
Moderate	15	11 (2.77-19.2)	
Well	8	13 (0-31)	
Missing	28		
Margin status			
Positive	7	9.9 (4.6-15.19)	0.90
Negative	14	14.3 (5.5-23.1)	
Vascular invasion			
Yes	7	22 (4.9-39.2)	0.78
No	14	10.2 (4.04-16.42)	
Perineural involvement			
Yes	10	6.2 (4.9-7.5)	0.66
No	13	11 (5.8-16.2)	

Rate of radical operation was only 11% (6/55). Extrahepatic tumors were more resectable ( $n = 13$ , 23.6%) as compared to intrahepatic tumors ( $n = 6$ , 10.9%). In 2 surgical patients, location of tumor could not be ascertained.

Of the twenty one patients who underwent surgery: 6 had Whipple procedure, 6 hepatic resection, and 9 en bloc resection of bile ducts and gall bladder. Two patients had positive lymph nodes. Surgical margins were positive in 7 patients ( $n = 7/21$ , 33%). Thirty-four patients had unresectable disease because of gross vascular involvement, locally advanced disease, or peritoneal metastasis discovered by imaging or during surgical exploration.

Twenty-four patients underwent palliative stenting, 9 had endoscopic stenting (37.5%), 14 had percutaneous radiological stenting (58.3%), and only one patient underwent surgical stenting (4.2%).

Fourteen patients received chemotherapy in the form of postoperative chemotherapy ( $n = 6$ ) or as palliative in the setting of non-resectable disease ( $n = 8$ ). Chemotherapy regimens consisted of gemcitabine or 5-Fu. Gemcitabine was given mainly as a single agent. As part of combination therapy, it was co-administered with other drugs such as oxaliplatin, 5-Fu, or CPT-11. 5-Fu was given as part of combination therapy at all times.

### Factors influencing survival in cholangiocarcinoma

The median survival for all patients was 8.57 mo (0.03-105.2), with 1-year, 3-year and 5-year survival rates of 10.8%, 5.4%, and 5.4%, respectively.

The longest survival time among all patients was 103 mo.

Multiple clinical, tumor-related and treatment parameters were evaluated by univariate analysis to determine their impact on survival in cholangiocarcinoma (Table 3).



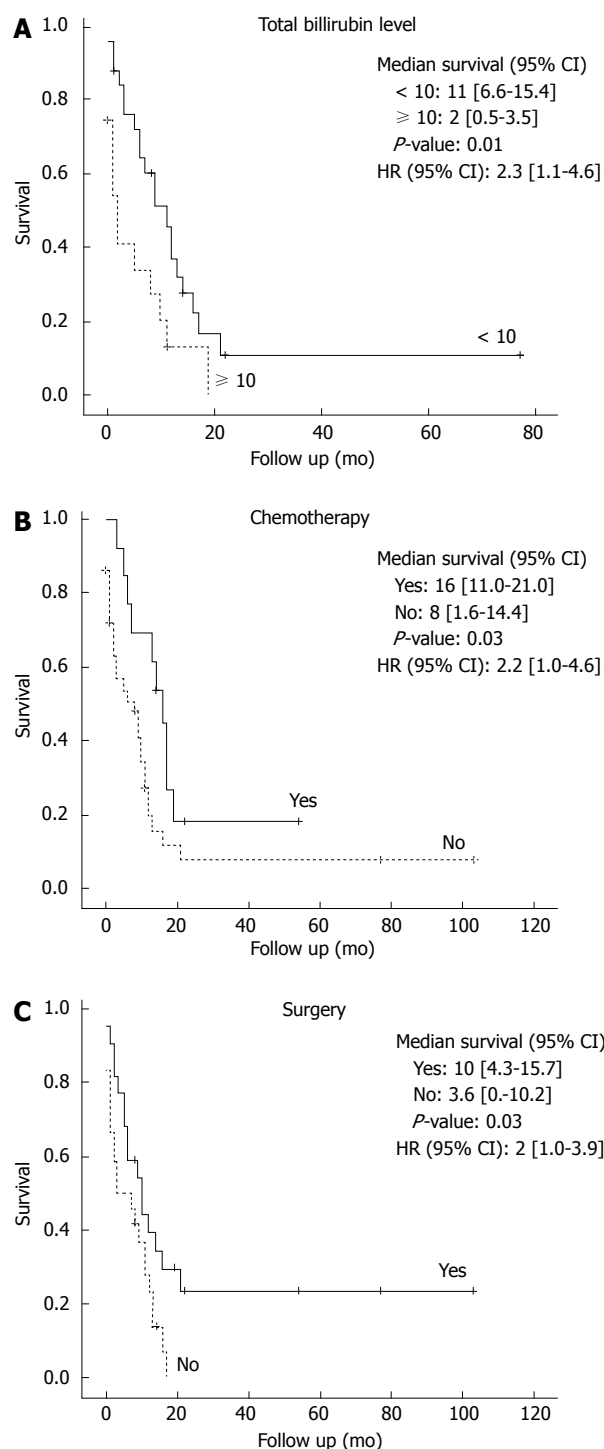
**Table 3** Association between clinical variables and survival in patients with cholangiocarcinoma

Variable (n)	Median survival (mo)	P (Univariate)
Age		
< 50 (11)	10.23 (1.87-18.6)	0.410
≥ 50 (44)	9.17 (3.9-14.4)	
Gender		
Male (33)	9.17 (3.8-14.5)	0.386
Female (22)	9.9 (0.4-19.4)	
Bilirubin		
< 10 (27)	9.9 (3.1-16.7)	0.012
≥ 10 (17)	2.87 (1.2-4.5)	
BMI		
< 25 (18)	13.0 (8.5-17.6)	0.412
25-30 (16)	7.0 (3.2-10.8)	
≥ 30 (18)	4.0 (0.5-7.6)	
Surgery		
Yes (21)	10.23 (4.82-15.64)	0.038
No (34)	8.7 (1.8-15.6)	
Type of surgery		
Whipple (6)	16.6 (0.0-39.4)	0.988
Hepatic lobectomy (6)	10.2 (3.9-16.6)	
Bile duct excision (9)	14.3 (8.5-20.1)	
Metastasis		
Yes (19)	7.07 (0.0-15.83)	0.256
No (36)	9.09 (6.05-13.75)	
Stenting		
Yes (24)	9.9 (0.45-19.35)	0.930
No (32)	9.16 (4.6-13.7)	
Chemotherapy		
Yes (14)	16.96 (11.5-22.4)	0.038
No (41)	6.2 (0-12.9)	
Chemotherapy in unresected patients		
Yes (8)	17 (12.76-21.18)	0.001
No (25)	3.5 (1.12-5.8)	

Parameters that did not influence survival were age, gender, diabetes, history of cholelithiasis, type of operation, resection margin status, presence of metastasis, and stenting. Tumor size, grade, location, vascular and perineural invasion also did not impact survival (Table 2). Increasing BMI was associated with a non-significant decrease in survival.

On univariate analysis, parameters that did influence survival included bilirubin level less than 10 mg/dL at presentation, surgical resection, and chemotherapy administration (Table 3). Since 49 out of 55 patients were stage IV, only these patients were included in Kaplan Meier survival (Figure 1) and multivariate analysis. Using Cox regression, a multivariate analysis was performed and only two were identified as independent predictors of increased survival: bilirubin level less than 10 mg/dL (Figure 1A) and chemotherapy administration (Figure 1B). Compared to patients with bilirubin levels less than 10 mg/dL, patients with higher bilirubin levels had a more than 2-fold increase in death risk from cholangiocarcinoma ( $P < 0.05$ ). Although the risk of dying was less in patients who underwent surgery, results did not attain statistical significance. On the contrary, patients who received chemotherapy had better survival ( $P < 0.05$ , Table 4).

## DISCUSSION



**Figure 1** A: Bilirubin level  $\geq 10$  mg/dL at presentation was associated with decreased survival as compared to lower levels; B: Chemotherapy administration prolonged survival in cholangiocarcinoma patients; C: Surgery added survival benefit in patients with cholangiocarcinoma.

This is the first report of cholangiocarcinoma from Lebanon, a small country in the Middle East, with a population of 3.4 million people. Our study shows the positive impact of surgery, chemotherapy, and low bilirubin level on survival in patients with advanced cholangiocarcinoma.

In previous studies, variables such as low preoperative bilirubin<sup>[4,14,28,29]</sup>, radical resection<sup>[28,29]</sup>, negative resection margin<sup>[11,30]</sup> and well-differentiated tumor histology<sup>[1,31]</sup>

**Table 4** Multivariate analysis of prognostic factors in patients with advanced cholangiocarcinoma

	Hazard's ratio	95% CI	P
Bilirubin			
< 10	1	1.13-0.52	0.023
≥ 10	2.421		
Surgery			
No	1	0.27-1.38	0.238
Yes	0.611		
Chemotherapy			
No	1	0.16-0.92	0.038
Yes	0.383		

were found to be predictors of improved outcome. On the other hand, less-differentiated histology<sup>[32]</sup>, perineural involvement, positive surgical margins, vascular or lymphatic invasion were associated with worse prognosis<sup>[30,32-37]</sup>.

The findings of our study emphasize the importance of increased bilirubin level upon presentation as an independent predictor of decreased survival in cholangiocarcinoma. Due to the small number of patients, we could not confirm the impact of the previously proposed variables on survival.

Radical surgery is considered as the most effective therapy for cholangiocarcinoma<sup>[5]</sup>. Only 20% of patients present with resectable disease<sup>[1]</sup>, yet surgery remains the only potential chance of cure. Without surgery, cholangiocarcinoma is a rapidly fatal disease with 5-year survival rates of less than 5%<sup>[9]</sup>, while in curative resections 5-year survival approaches 20%-35% with negative surgical margins<sup>[5]</sup>. In our findings, we could not document an improved survival after surgical resection which can be explained by the advanced stage at which all patients presented and the small number of the study group.

The significance of chemotherapy in cholangiocarcinoma is still not clear especially with the low response rates<sup>[5]</sup>, disappointing efficacy results and lack of a superior standard chemotherapeutic regimen<sup>[7,38]</sup>. Conflicting data exist regarding the role of chemotherapy in cholangiocarcinoma. Some studies suggest that chemotherapy, whether given in a setting of non-resectable disease or postoperatively, has little or no impact on the course of the disease or on survival outcome<sup>[13-15,29,39-41]</sup> and is therefore considered palliative more than curative<sup>[42]</sup>. Other studies report survival benefit from chemotherapy<sup>[11,12,43]</sup>. Recently, a pooled analysis of all clinical trials from 1985 to 2007 concluded that the combination of gemcitabine with oxaliplatin or cisplatin may improve survival in cholangiocarcinoma<sup>[10]</sup>.

Our study adds further evidence to the previously published reports showing that chemotherapy improves survival in cholangiocarcinoma. We found that chemotherapy markedly improves survival in patients with either resected or unresected cholangiocarcinoma (17.0 mo *vs* 6.0 mo;  $P < 0.01$ ). Additionally, chemotherapy prolonged survival significantly in patients with unresectable tumors (17.0 mo *vs* 3.5 mo;  $P = 0.001$ ). Thus, in patients with advanced cholangiocarcinoma who are not surgical

candidates, Gemcitabine and/or 5-Fu based chemotherapy might offer a survival advantage.

Our results also show a non-significant decrease in survival with increasing BMI. The median survival for patients with BMI < 25 was 17.0 mo (8.5-17.6), 7.0 mo (3.2-10.8) for patients with BMI 25-29.99, and 4 mo (0.5-7.6) for patients with BMI ≥ 30 ( $P = 0.412$ ). The fact that these results did not attain statistical significance may be attributed to the small sample size. Further prospective studies are needed to determine the effect of increased body mass index on prognosis in cholangiocarcinoma.

In line with other reports, cholangiocarcinoma in Lebanon affected older patients<sup>[44,45]</sup> and more males than females<sup>[44-46]</sup>, except in the above 70-year-old group where females were more commonly affected. However, the mean age of our patients was higher than that of patients in the USA<sup>[47]</sup>. The clinical symptoms and signs observed in Lebanese patients with cholangiocarcinoma were mostly of biliary obstruction and abdominal pain, as was previously reported<sup>[28]</sup>.

Most of the tumors in our study were distal extrahepatic lesions, whereas perihilar lesions are the most common type usually reported<sup>[5,6,48]</sup>. A moderate degree of differentiation was noted in the majority of tumors in our patients, while well-differentiated histology is more commonly reported<sup>[5,6]</sup>.

Similar to other reports<sup>[4]</sup>, this series did not identify any risk factors associated with cholangiocarcinoma, which is different from reports of biliary tract cancers elsewhere. In Asian countries, well-established risk factors are hepatolithiasis and liver fluke infestations<sup>[49]</sup>, while in western countries hepatitis B and C infection, HIV, cirrhosis, diabetes, alcohol consumption, and IBD were recently implicated as potential risk factors for cholangiocarcinoma<sup>[44,47,50]</sup>.

Our patients might have had some of the known risk factors for cholangiocarcinoma that might have been missed at the time of patient presentation. Therefore, absence of those risk factors can not be ascertained due to the retrospective design of our study.

The prevalence of diabetes in our patients was 33% with the highest incidence being observed in patients over the age of 65, which was very close to the 29% prevalence rate of diabetes in the general Lebanese population older than 65 years<sup>[51]</sup>. Therefore, diabetes can not be considered a risk factor for cholangiocarcinoma in our population, unlike other populations where diabetes increased the risk 2-3 folds<sup>[44,50,52]</sup>.

Cholelithiasis was present in 30% of our patients. Prevalence of cholelithiasis in cholangiocarcinoma patients was previously reported to fall in the 30% to 48% range<sup>[53]</sup>. It was described as a risk factor for cholangiocarcinoma in a number of studies<sup>[50,53,54]</sup>. However, a definitive cause-effect relationship has not been established yet.

Few reports addressed the association between BMI and bile duct cancer; Samonic *et al* showed that obese black men are at a significant risk of extrahepatic bile duct cancers<sup>[55]</sup>. On the other hand, Welzel *et al* showed

that obesity was not a risk factor for intrahepatic cholangiocarcinoma<sup>[50]</sup>. Others suggested that increased body mass index was associated only with cancer of the extrahepatic duct<sup>[54]</sup>. Furthermore, in a large Korean cohort, a significant positive linear relationship was found between increasing BMI and risk of cholangiocarcinoma<sup>[56]</sup>. The risk of cholangiocarcinoma increased approximately 1.6 folds in patients with BMI > 30 kg/m<sup>2</sup><sup>[56]</sup>. In our study, 68% of all patients with cholangiocarcinoma had excess BMI (median BMI was 26.9 kg/m<sup>2</sup>). In the Lebanese adult population, 53% are overweight (BMI ≥ 25), 17% are obese (BMI ≥ 30) and the mean BMI is estimated to be 25.9 kg/m<sup>2</sup><sup>[26]</sup>, which is comparable to the mean BMI of our study group.

There are several limitations in our study. The first is the small sample size, which is due to the rarity of the disease under investigation. Second, the study represents cases seen at a single tertiary care center, which may limit its utility in patients with cholangiocarcinoma in general. Third, our study is limited by its retrospective design; a key limitation resulting from such a design is the missing data, which may result in fewer patients included in multivariable models, generally increasing the risk for both type one and type two errors. Fourth, our study is non-randomized and lacks a control group. Despite the limitations of retrospective studies, absence of prospective and controlled data in the current literature makes the results of our study of more interest.

In conclusion, bilirubin levels less than 10 mg/dL at presentation and chemotherapy administration both in advanced disease and in postoperative adjuvant settings are associated with better prognosis and prolonged survival in patients with cholangiocarcinoma. None of the well-established or the potential risk factors for cholangiocarcinoma could be identified in the Lebanese population due to the above mentioned limitations. High body mass index was not found to be a risk factor for cholangiocarcinoma; however, increments were associated with a trend towards a decrease in median survival.

## COMMENTARY

### Background

Cholangiocarcinoma is an infrequent malignancy that involves the biliary epithelium. It has a poor prognosis with a survival less than 5% at five years. Radical surgery is the only potentially curative treatment modality, while the impact of chemotherapy on survival remains controversial. Due to small number of patients, determinants of prognosis in cholangiocarcinoma are not well characterized.

### Research frontiers

A retrospective review of the medical records of 55 patients diagnosed with cholangiocarcinoma at the American University of Beirut between 1990 and 2005 was conducted. Univariate and multivariate analysis were performed to determine the impact of surgery, chemotherapy, body mass index, bilirubin level and other factors on survival.

### Innovations and breakthroughs

Bilirubin levels less than 10 mg/dL at presentation and chemotherapy administration both in advanced disease and in postoperative adjuvant settings are associated with better prognosis and prolonged survival in patients with cholangiocarcinoma. High body mass index was not found to be a risk factor for cholangiocarcinoma; however, increments were associated with a trend towards a decrease in median survival.

### Applications

Palliative and postoperative chemotherapy as well as a bilirubin level < 10 mg/dL are independent predictors of a significant increase in survival in patients with cholangiocarcinoma. Large prospective controlled studies are needed to verify these results.

### Peer review

This article reports interesting epidemiological data on cholangiocarcinoma in Lebanon and the effects of surgical resection and chemotherapy on survival. Multivariate analysis identified only a bilirubin level < 10 mg/dL and chemotherapy as independent predictors of better survival.

## REFERENCES

- 1 **Jarnagin WR**, Fong Y, DeMatteo RP, Gonen M, Burke EC, Bodniewicz BS J, Youssef BA M, Klimstra D, Blumgart LH. Staging, resectability, and outcome in 225 patients with hilar cholangiocarcinoma. *Ann Surg* 2001; **234**: 507-517; discussion 517-519
- 2 **Vauthey JN**, Blumgart LH. Recent advances in the management of cholangiocarcinomas. *Semin Liver Dis* 1994; **14**: 109-114
- 3 **Jarnagin WR**, Shoup M. Surgical management of cholangiocarcinoma. *Semin Liver Dis* 2004; **24**: 189-199
- 4 **Su CH**, Tsay SH, Wu CC, Shyr YM, King KL, Lee CH, Lui WY, Liu TJ, P'eng FK. Factors influencing postoperative morbidity, mortality, and survival after resection for hilar cholangiocarcinoma. *Ann Surg* 1996; **223**: 384-394
- 5 **Patel T**, Singh P. Cholangiocarcinoma: emerging approaches to a challenging cancer. *Curr Opin Gastroenterol* 2007; **23**: 317-323
- 6 **Malhi H**, Gores GJ. Review article: the modern diagnosis and therapy of cholangiocarcinoma. *Aliment Pharmacol Ther* 2006; **23**: 1287-1296
- 7 **Lee GW**, Kang JH, Kim HG, Lee JS, Lee JS, Jang JS. Combination chemotherapy with gemcitabine and cisplatin as first-line treatment for immunohistochemically proven cholangiocarcinoma. *Am J Clin Oncol* 2006; **29**: 127-131
- 8 **Khan SA**, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P, Thomas HC. Changing international trends in mortality rates for liver, biliary and pancreatic tumours. *J Hepatol* 2002; **37**: 806-813
- 9 **Farley DR**, Weaver AL, Nagorney DM. "Natural history" of unresected cholangiocarcinoma: patient outcome after noncurative intervention. *Mayo Clin Proc* 1995; **70**: 425-429
- 10 **Eckel F**, Schmid RM. Chemotherapy in advanced biliary tract carcinoma: a pooled analysis of clinical trials. *Br J Cancer* 2007; **96**: 896-902
- 11 **Yoshida T**, Matsumoto T, Sasaki A, Morii Y, Aramaki M, Kitano S. Prognostic factors after pancreaticoduodenectomy with extended lymphadenectomy for distal bile duct cancer. *Arch Surg* 2002; **137**: 69-73
- 12 **Kelley ST**, Bloomston M, Serafini F, Carey LC, Karl RC, Zervos E, Goldin S, Rosemurgy P, Rosemurgy AS. Cholangiocarcinoma: advocate an aggressive operative approach with adjuvant chemotherapy. *Am Surg* 2004; **70**: 743-748; discussion 748-749
- 13 **Takada T**, Amano H, Yasuda H, Nimura Y, Matsushiro T, Kato H, Nagakawa T, Nakayama T. Is postoperative adjuvant chemotherapy useful for gallbladder carcinoma? A phase III multicenter prospective randomized controlled trial in patients with resected pancreaticobiliary carcinoma. *Cancer* 2002; **95**: 1685-1695
- 14 **Yi B**, Zhang BH, Zhang YJ, Jiang XQ, Zhang BH, Yu WL, Chen QB, Wu MC. Surgical procedure and prognosis of hilar cholangiocarcinoma. *Hepatobiliary Pancreat Dis Int* 2004; **3**: 453-457
- 15 **Thongprasert S**. The role of chemotherapy in cholangiocarcinoma. *Ann Oncol* 2005; **16** Suppl 2: ii93-ii96
- 16 **Zhang J**, Su XQ, Wu XJ, Liu YH, Wang H, Zong XN, Wang Y, Ji JF. Effect of body mass index on adenocarcinoma of gastric cardia. *World J Gastroenterol* 2003; **9**: 2658-2661
- 17 **Parker AS**, Lohse CM, Cheville JC, Thiel DD, Leibovich BC,

- Blute ML. Greater body mass index is associated with better pathologic features and improved outcome among patients treated surgically for clear cell renal cell carcinoma. *Urology* 2006; **68**: 741-746
- 18 **Wright JD**, Powell MA, Mutch DG, Rader JS, Gibb RK, Gao F, Herzog TJ. Relationship of ovarian neoplasms and body mass index. *J Reprod Med* 2005; **50**: 595-602
  - 19 **Enger SM**, Greif JM, Polikoff J, Press M. Body weight correlates with mortality in early-stage breast cancer. *Arch Surg* 2004; **139**: 954-958; discussion 958-960
  - 20 **Calle EE**, Rodriguez C, Walker-Thurmond K, Thun MJ. Overweight, obesity, and mortality from cancer in a prospectively studied cohort of U.S. adults. *N Engl J Med* 2003; **348**: 1625-1638
  - 21 **Lee J**, Wang H, Chia KS, Koh D, Hughes K. The effect of being overweight on cancer incidence and all-cause mortality in Asians: a prospective study in Singapore. *Int J Epidemiol* 2002; **31**: 875-876
  - 22 **Wolk A**, Gridley G, Svensson M, Nyren O, McLaughlin JK, Fraumeni JF, Adam HO. A prospective study of obesity and cancer risk (Sweden). *Cancer Causes Control* 2001; **12**: 13-21
  - 23 **Bergstrom A**, Pisani P, Tenet V, Wolk A, Adami HO. Overweight as an avoidable cause of cancer in Europe. *Int J Cancer* 2001; **91**: 421-430
  - 24 **Zatonski WA**, Lowenfels AB, Boyle P, Maisonneuve P, Bueno de Mesquita HB, Ghadirian P, Jain M, Przewozniak K, Baghurst P, Moerman CJ, Simard A, Howe GR, McMichael AJ, Hsieh CC, Walker AM. Epidemiologic aspects of gallbladder cancer: a case-control study of the SEARCH Program of the International Agency for Research on Cancer. *J Natl Cancer Inst* 1997; **89**: 1132-1138
  - 25 **American Joint Committee on Cancer Staging Manual**. 6th ed. Philadelphia: Springer, 2002: 145-150
  - 26 **Sibai AM**, Hwalla N, Adra N, Rahal B. Prevalence and covariates of obesity in Lebanon: findings from the first epidemiological study. *Obes Res* 2003; **11**: 1353-1361
  - 27 **Obesity: preventing and managing the global epidemic**. Report of a WHO consultation. *World Health Organ Tech Rep Ser* 2000; **894**: i-xii, 1-253
  - 28 **Zhang BH**, Cheng QB, Luo XJ, Zhang YJ, Jiang XQ, Zhang BH, Yi B, Yu WL, Wu MC. Surgical therapy for hilar cholangiocarcinoma: analysis of 198 cases. *Hepatobiliary Pancreat Dis Int* 2006; **5**: 278-282
  - 29 **Cheng Q**, Luo X, Zhang B, Jiang X, Yi B, Wu M. Predictive factors for prognosis of hilar cholangiocarcinoma: postresection radiotherapy improves survival. *Eur J Surg Oncol* 2007; **33**: 202-207
  - 30 **Silva MA**, Tekin K, Aytekin F, Bramhall SR, Buckels JA, Mirza DF. Surgery for hilar cholangiocarcinoma: a 10 year experience of a tertiary referral centre in the UK. *Eur J Surg Oncol* 2005; **31**: 533-539
  - 31 **Todoroki T**. Chemotherapy for bile duct carcinoma in the light of adjuvant chemotherapy to surgery. *Hepatogastroenterology* 2000; **47**: 644-649
  - 32 **Kawarada Y**, Yamagiwa K, Das BC. Analysis of the relationships between clinicopathologic factors and survival time in intrahepatic cholangiocarcinoma. *Am J Surg* 2002; **183**: 679-685
  - 33 **Harrison LE**, Fong Y, Klimstra DS, Zee SY, Blumgart LH. Surgical treatment of 32 patients with peripheral intrahepatic cholangiocarcinoma. *Br J Surg* 1998; **85**: 1068-1070
  - 34 **Hanazaki K**, Kajikawa S, Shimozawa N, Shimada K, Hiraguri M, Koide N, Adachi W, Amano J. Prognostic factors of intrahepatic cholangiocarcinoma after hepatic resection: univariate and multivariate analysis. *Hepatogastroenterology* 2002; **49**: 311-316
  - 35 **Havlik R**, Sbisá E, Tullo A, Kelly MD, Mitry RR, Jiao LR, Mansour MR, Honda K, Habib NA. Results of resection for hilar cholangiocarcinoma with analysis of prognostic factors. *Hepatogastroenterology* 2000; **47**: 927-931
  - 36 **Murakami Y**, Uemura K, Hayashidani Y, Sudo T, Hashimoto Y, Ohge H, Sueda T. Prognostic significance of lymph node metastasis and surgical margin status for distal cholangiocarcinoma. *J Surg Oncol* 2007; **95**: 207-212
  - 37 **Ramacciato G**, Di Benedetto F, Cautero N, Masetti M, Mercantini P, Corigliano N, Nigri G, Lauro A, Ercolani G, Del Gaudio M, De Ruvo N, Pinna AD. [Prognostic factors and long term outcome after surgery for hilar cholangiocarcinoma. Univariate and multivariate analysis] *Chir Ital* 2004; **56**: 749-759
  - 38 **Malka D**, Boige V, Dromain C, Debaere T, Pocard M, Ducreux M. Biliary tract neoplasms: update 2003. *Curr Opin Oncol* 2004; **16**: 364-371
  - 39 **Mazhar D**, Stebbing J, Bower M. Chemotherapy for advanced cholangiocarcinoma: what is standard treatment? *Future Oncol* 2006; **2**: 509-514
  - 40 **Takada T**, Nimura Y, Katoh H, Nagakawa T, Nakayama T, Matsushiro T, Amano H, Wada K. Prospective randomized trial of 5-fluorouracil, doxorubicin, and mitomycin C for non-resectable pancreatic and biliary carcinoma: multicenter randomized trial. *Hepatogastroenterology* 1998; **45**: 2020-2026
  - 41 **Nakeeb A**, Pitt HA. Radiation therapy, chemotherapy and chemoradiation in hilar cholangiocarcinoma. *HPB (Oxford)* 2005; **7**: 278-282
  - 42 **Price P**. Cholangiocarcinoma and the role of radiation and chemotherapy. *Hepatogastroenterology* 2001; **48**: 51-52
  - 43 **Glimelius B**, Hoffman K, Sjoden PO, Jacobsson G, Sellstrom H, Enander LK, Linno T, Svensson C. Chemotherapy improves survival and quality of life in advanced pancreatic and biliary cancer. *Ann Oncol* 1996; **7**: 593-600
  - 44 **Shaib YH**, El-Serag HB, Davila JA, Morgan R, McGlynn KA. Risk factors of intrahepatic cholangiocarcinoma in the United States: a case-control study. *Gastroenterology* 2005; **128**: 620-626
  - 45 **Liu XF**, Zhou XT, Zou SQ. An analysis of 680 cases of cholangiocarcinoma from 8 hospitals. *Hepatobiliary Pancreat Dis Int* 2005; **4**: 585-588
  - 46 **Lazaridis KN**, Gores GJ. Cholangiocarcinoma. *Gastroenterology* 2005; **128**: 1655-1667
  - 47 **Nakeeb A**, Pitt HA, Sohn TA, Coleman J, Abrams RA, Piantadosi S, Hruban RH, Lillemoe KD, Yeo CJ, Cameron JL. Cholangiocarcinoma. A spectrum of intrahepatic, perihilar, and distal tumors. *Ann Surg* 1996; **224**: 463-473; discussion 473-475
  - 48 **Watanapa P**, Watanapa WB. Liver fluke-associated cholangiocarcinoma. *Br J Surg* 2002; **89**: 962-970
  - 49 **Shaib YH**, El-Serag HB, Nooka AK, Thomas M, Brown TD, Patt YZ, Hassan MM. Risk factors for intrahepatic and extrahepatic cholangiocarcinoma: a hospital-based case-control study. *Am J Gastroenterol* 2007; **102**: 1016-1021
  - 50 **Welzel TM**, Mellemejaer L, Gloria G, Sakoda LC, Hsing AW, El Ghormli L, Olsen JH, McGlynn KA. Risk factors for intrahepatic cholangiocarcinoma in a low-risk population: a nationwide case-control study. *Int J Cancer* 2007; **120**: 638-641
  - 51 **Salti IS**, Khogali M, Alam S, Nassar N, Abu Haidar N, Masri A. The epidemiology of diabetes mellitus in relation to other cardiovascular risk factors in Lebanon. *Eastern Mediterranean Health Journal* 1997; **3**: 462-471
  - 52 **Wideroff L**, Gridley G, Mellemejaer L, Chow WH, Linet M, Keehn S, Borch-Johnsen K, Olsen JH. Cancer incidence in a population-based cohort of patients hospitalized with diabetes mellitus in Denmark. *J Natl Cancer Inst* 1997; **89**: 1360-1365
  - 53 **Khan ZR**, Neugut AI, Ahsan H, Chabot JA. Risk factors for biliary tract cancers. *Am J Gastroenterol* 1999; **94**: 149-152
  - 54 **Chow WH**, McLaughlin JK, Menck HR, Mack TM. Risk factors for extrahepatic bile duct cancers: Los Angeles County, California (USA). *Cancer Causes Control* 1994; **5**: 267-272
  - 55 **Samancic C**, Chow WH, Gridley G, Jarvholm B, Fraumeni JF Jr. Relation of body mass index to cancer risk in 362,552 Swedish men. *Cancer Causes Control* 2006; **17**: 901-909
  - 56 **Oh SW**, Yoon YS, Shin SA. Effects of excess weight on cancer incidences depending on cancer sites and histologic findings among men: Korea National Health Insurance Corporation Study. *J Clin Oncol* 2005; **23**: 4742-4754





# Prevention and treatment of gastrointestinal dysfunction following severe burns: A summary of recent 30-year clinical experience

Shi-Chu Xiao, Shi-Hui Zhu, Zhao-Fan Xia, Wei Lu, Guang-Qing Wang, Dao-Feng Ben, Guang-Yi Wang, Da-Sheng Cheng

Shi-Chu Xiao, Shi-Hui Zhu, Zhao-Fan Xia, Wei Lu, Guang-Qing Wang, Dao-Feng Ben, Guang-Yi Wang, Da-Sheng Cheng, Department of Burn Surgery, Changhai Hospital, Second Military Medical University, 174 Changhai Road, Shanghai 200433, China

Author contributions: Xiao SC and Zhu SH contributed equally to this work; Xiao SC, Zhu SH and Xia ZF designed the research; Lu W, Wang GQ, Ben DF, Wang GY and Cheng DS performed the research; and Xiao SC and Zhu SH wrote the paper.

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Correspondence to: Zhao-Fan Xia, MD, PhD, Department of Burn Surgery, Changhai Hospital, Second Military Medical University, 174 Changhai Road, Shanghai 200433, China. [xiazhaoan@hotmail.com](mailto:xiazhaoan@hotmail.com)

Telephone: +86-21-25070599 Fax: +86-21-65589829

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## Abstract

**AIM:** To sum up the recent 30-year experience in the prevention and treatment of gastrointestinal dysfunction in severe burn patients, and propose practicable guidelines for the prevention and treatment of gastrointestinal (GI) dysfunction.

**METHODS:** From 1980 to 2007, a total of 219 patients with large area and extraordinarily large area burns (LAB) were admitted, who were classified into three stages according the therapeutic protocols used at the time: Stage 1 from 1980 to 1989, stage 2 from 1990 to 1995, and stage 3 from 1996 to 2007. The occurrence and mortality of GI dysfunction in patients of the three stages were calculated and the main causes were analyzed.

**RESULTS:** The occurrence of stress ulcer in patients with LAB was 8.6% in stage 1, which was significantly lower than that in stage 1 ( $P < 0.05$ ). No massive hemorrhage from severe stress ulcer and enterogenic infections occurred in stages 2 and 3. The occurrence of abdominal distension and stress ulcer and the mortality in stage 3 patients with extraordinarily LAB was 7.1%, 21.4% and 28.5%, respectively, which were significantly lower than those in stage 1 patients

( $P < 0.05$  or  $P < 0.01$ ), and the occurrence of stress ulcer was also significantly lower than that in stage 2 patients ( $P < 0.05$ ).

**CONCLUSION:** Comprehensive fluid resuscitation, early excision of necrotic tissue, staged food ingestion, and administration of specific nutrients are essential strategies for preventing gastrointestinal complications and lowering mortality in severely burned patients.

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**Key words:** Severe burn; Gastrointestinal function; Fluid resuscitation; Staged food ingestion

**Peer reviewer:** Henrike Maria Hamer, MD, Internal Medicine Department, Maastricht University, Division of Gastroenterology (Box 46), Maastricht 6200 MD, The Netherlands

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## INTRODUCTION

Gastrointestinal dysfunction is a common complication of severe burns. Injury to GI function, especially to GI barrier function, is an important initiator as well as a stimulator for occurrence of systemic inflammatory response syndrome (SIRS), sepsis and multiple organ dysfunction syndrome (MODS) following severe burns<sup>[1]</sup>. With the deeper understanding of GI function and changes in the stereotype of clinical treatment in recent 30 years, a series of new therapies including fluid resuscitation, early escharectomy, continuous renal replacement therapy, and use of glutamine and growth factor has been adopted in the treatment of severe burns<sup>[2,3]</sup>. Although animal experiments have shown that these new therapies do play a positive role in the prevention and treatment of GI dysfunction following severe burns, there has been a lack of convincing clinical

data to confirm the outcome<sup>[4-6]</sup>. The present study reviewed the clinical data of 219 patients with large area burns (LAB) in recent more than 20 years, who were classified into different stages according to the therapeutic protocols used at the time. Based on the review, the outcomes of GI function protection and treatment were compared, analyzed and summarized in an attempt to propose some practicable guidelines for the effective prevention and treatment of GI dysfunction.

## MATERIALS AND METHODS

### *Clinical data*

This study included 219 patients with severe burns who were admitted to this burn center from January 1980 to August 2007. They were classified as LAB patients (50%-79% TBSA, or degree III burn area > 20%) and extraordinarily large area burn (ELAB) patients (80%-100% TBSA, or degree III burn area > 50%). According to the therapeutic protocols used at the time, they were assigned to three stages: stage 1 from 1980 to 1989, stage 2 from 1990 to 1995, and stage 3 from 1996 to 2007. The occurrence of GI dysfunction and mortality were analyzed statistically.

**Stage 1 (1980-1989):** Limited fluid resuscitation was advocated during the shock phase of burn patients. In other words, the total fluid input was minimized as long as the vital signs were stably maintained, and the urine output was controlled at a level of 0.5 mL/h per kg body weight. The first escharectomy was usually done 4-7 d after burn injury, and the operation area was 20%-30% TBSA in most cases. Patients were mostly starved in the early stage of burn and relied on intravenous nutrition. To prevent stress ulcer, gastric mucosal protection agents and anti-acid drugs were administered routinely.

**Stage 2 (1990-1995):** The major therapeutic changes were advancing the first escharectomy to 3-4 d after burn injury, expanding the operation area as much as possible, and excising the necrotic tissue as early as possible. When bowel sounds recovered 2-4 d after burn injury, food intake was started gradually through the gastric tube and patients were encouraged to take food orally, with administration of appropriate amounts of gastrokinetic drugs such as domperidone to promote gastrointestinal peristalsis. Oral norfloxacin and nystatin were administered routinely within 2 wk after burn. Selective decontamination of the digestive tract (SDD) was also recommended.

**Stage 3 (1996-2007):** The comprehensive resuscitation strategy was advocated for shock burn, which includes sufficient resuscitation and maintenance of urine output at 1-1.5 mL/h per kg BW; routine intravenous instillation of vasoactive drugs such as small doses of dopamine; adjustment of the gastrointestinal tract and renal blood perfusion; and use of antioxidants such as large doses of Vitamin C and E to eliminate free oxygen radicals. In addition, antibiotics were used prudently,

including shortening the duration of antibiotic administration and reducing the variety of antibiotics. So far as gastrointestinal nutrition is concerned, staged food ingestion was advocated, where small amounts of light fluids (20-40 mL/h) were instilled through the gastric tube 2 h after burn to stimulate gastrointestinal peristalsis. Once bowel sounds recovered, the amount of food was increased gradually. Usually the amount of enteral nutrition fed through the gastric tube was increased to 2000-2500 Kcal/d 3-6 d after burn. Specific nutrients were used such as oral glutamine, L-arginine, dietary fiber and subcutaneous growth factor.

Since 2003, early administration of continuous renal replacement therapy (CRRT) for 5-8 consecutive days has been advocated in patients with GI failure accompanied with sepsis. The content of endotoxin, IL-1 $\beta$ , IL-6 and IL-8 in plasma were analyzed before and after CRRT treatment. TNF- $\alpha$  content was measured by radioimmunoassay. The activity of diamine oxidase (DAO) in plasma was tested according to the previous report<sup>[7]</sup>.

### *Indexes for assessing GI function*

There was no uniformed criterion for assessing GI dysfunction<sup>[8]</sup>. Based on the diagnostic criteria for MODS and GI symptoms commonly seen in burned patients, GI dysfunction is summarized as follows: (1) abdominal distension: bowel sound was reduced and food intolerance exceeded more than 5 d; (2) stress ulcer: gastric fluid aspirated from the gastric tube appeared bloody macroscopically and gastric mucosa was erosive and ulcerative gastroendoscopically; (3) severe stress ulcer: blood loss exceeded 800 mL within 24 h; (4) alteration of intestinal microbiota: Gram-negative *E.coli* was amplified, and the bacillus/coccus ratio was greater than 10:1; and (5) enterogenic infection: highly suspected systemic infection occurred after ruling out wound surface, pulmonary and indwelling catheter infections<sup>[9,10]</sup>.

### *Statistical analysis*

Data were testified by Pearson's Chi-square test, and Fisher's two-tail exact test.

## RESULTS

Of the 219 severe burn cases analyzed (Table 1), 89 cases were LAB and 130 cases were ELAB. There was no significant difference in age distribution and burn area between the three stages of patients.

Table 2 shows that the occurrence of stress ulcer in LAB patients of stage 3 was 8.6%, which was significantly lower than 30.3% of stage 1 patients ( $P < 0.05$ ). No hemorrhage from severe stress ulcer and enterogenic infection occurred in the patients of stage 2 and 3.

Compared with LAB patients, the occurrence of gastrointestinal complications and mortality in ELAB patients were significantly higher, indicating that occurrence of gastrointestinal complications was closely

Table 1 General clinical data of 219 burned patients

Stage	<i>n</i>	LAB		<i>n</i>	ELAB	
		Age (yr)	TBSA (%)		Age (yr)	TBSA (%)
1	33	26.5 ± 18.7	65.1 ± 12.4	45	28.5 ± 13.7	91.4 ± 10.2
2	21	27.7 ± 13.8	74.6 ± 10.3	29	26.9 ± 14.6	89.3 ± 8.2
3	35	25.9 ± 16.3	69.5 ± 12.2	56	26.4 ± 13.3	93.1 ± 9.4

TBSA: Total body surface area.

Table 2 GI complications and mortality in LAB patients (%)

Stage	<i>n</i>	AE	SU	SSU	FA	EI	Mortality
1	33	12.1	30.3	3.0	15.1	3.0	12.1
2	21	4.8	19.0	-	4.8	-	4.8
3	35	2.9	8.6 <sup>a</sup>	-	2.9	-	2.9

AE: Abdominal extension; SU: Stress ulcer; SSU: Severe stress ulcer; FA: Flora alteration; EI: Enterogenic infection. Compared with the stage 1, <sup>a</sup>*P* < 0.05.

associated with the severity of burn. Table 3 shows that the occurrence of abdominal extension and stress ulcer and mortality in the stage 3 ELAB patients were 7.1%, 21.4% and 28.5%, respectively, which were significantly lower than those of stage 1 (*P* < 0.05 or *P* < 0.01), and the occurrence of stress ulcer in the stage 3 ELAB patients was also significantly lower than that of stage 2 patients (*P* < 0.05).

In the 5 patients with GI failure accompanied with severe sepsis, endotoxin, IL-1β, IL-6, IL-8 and TNF-α levels and plasma DAO activity were decreased significantly after CRRT (Table 4) (*P* < 0.01).

## DISCUSSION

Timely and effective fluid resuscitation is the basis and guarantee of curing severely burned patients<sup>[11]</sup>. Before the 1990s, the therapeutic concepts were limited to such that excessive fluid infusion would aggravate edema so that limited resuscitation was addressed. Under the bunker of stable vital signs lies the problems of GI hypoxia and ischemia, or occult GI shock<sup>[12,13]</sup>. Since the mid and late 1990s, comprehensive resuscitation strategies for maintaining the stability of vital signs and splanchnic resuscitation to restore GI blood supply as early as possible and reduce hypoxic and ischemic injuries as much as possible have been recommended<sup>[14]</sup>. It is suggested that small doses of dopamine should be administered to dilate the renal and GI vessels<sup>[15]</sup>, and free oxygen radical clearing agents to attenuate ischemia/reperfusion injury in the process of resuscitation<sup>[16,17]</sup>. These comprehensive resuscitation measures played an important role in protecting GI function, helping resume bowel sound earlier and digestive function<sup>[18]</sup>. No stress ulcer occurred during the shock phase, which laid a sound foundation for future treatment.

It has been generally accepted that early enteric nourishment plays an essential role in preventing GI dysfunction following severe burns<sup>[19,20]</sup>. But as LAB often causes serious edema of GI mucosa, there is a

Table 3 GI complications and mortality in ELAB patients (%)

Stage	<i>n</i>	AE	SU	SSU	FA	EI	Mortality
1	45	24.4	60.0	11.1	20.0	15.5	55.6
2	29	13.8	48.3	6.9	13.8	10.3	41.3
3	56	7.1 <sup>a</sup>	21.4 <sup>bc</sup>	3.6	8.9	7.1	28.5 <sup>b</sup>

Compared with stage 1, <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01; compared with stage 2, <sup>c</sup>*P* < 0.05.

Table 4 Endotoxin, IL-1β, IL-6, IL-8 and TNF-α levels and plasma DAO activity before and after CRRT

	Endotoxin (Eu/mL)	TNF-α (pg/mL)	IL-6 (pg/mL)	IL-8 (pg/mL)	DAO (U/mL)
Before CRRT	0.76 ± 0.13	272 ± 28	518 ± 64	583 ± 51	2.98 ± 0.94
After CRRT	0.045 ± 0.017 <sup>b</sup>	57 ± 15 <sup>b</sup>	98 ± 25 <sup>b</sup>	105 ± 31 <sup>b</sup>	1.27 ± 0.54 <sup>b</sup>

Compared with before CRRT, <sup>b</sup>*P* < 0.01.

concern that early food intake would give additional burden to the GI tract or even cause acute gastric dilation, resulting in vomiting and aspiration. For this reason, there is controversy over when and how to take food. Before the 1990s, food intake was usually started when patients resumed bowel sound. After the mid and late 1990s, the idea of staged food intake was advanced: a small amount of light fluid is started several hours after burn so as to not only supplement nutrition but stimulate GI peristalsis and improve GI blood supply. Once bowel sound resumes, the amount of food can be increased. Using immunoregulatory nutrients such as oral glutamine, L-arginine and dietary fiber promoted post-burn repair of GI mucosa, maintained GI barrier function, and reduced translocation of enterogenic bacteria and endotoxins<sup>[21,22]</sup>.

The microenvironment formed by GI resident bacteria forms an ecologic barrier in the intestinal lumen, preventing intestinal pathologic bacteria from colonization and substantial proliferation<sup>[23]</sup>. To maintain normal intestinal microbiota, we paid special attention to the followings: prudent use of antibiotics and routine use of SDD. In earlier treatment of severe burns, a variety of broad-spectrum antibiotics were often used concomitantly. But our clinical experiences showed that mere use of antibiotics failed to control infections effectively in severely burned patients; instead it often caused alteration of bacterial flora, resulting in superinfection. Since the mid and late 1990s, the principle of “bold use of antibiotics and bold discontinuation of them” has been advocated. In other words, the duration and variety of antibiotics should be minimized, and the use of antibiotics should be enhanced properly during the edema reabsorption phase and the perioperative period. By doing so, the incidence of systemic infection was lowered, and furthermore it avoided alteration of intestinal microbiota effectively. The use of SDD within two weeks of burn injury inhibited the growth of Gram bacteria and fungi and maintained the stability of intestinal microbiota, which may also be beneficial to reducing superinfection

of intestinal bacteria<sup>[24]</sup>.

Early excision of necrotic tissue and closure of the wound surface are essential in the treatment of severe burns<sup>[25]</sup>. Positive surgical treatment has become a generally accepted idea. Our practice is that surgery is started 3-4 d after burn injury and the area of escharectomy at a time is much larger than before, usually reaching 60%-75% TBSA. The wound surface is covered with heterogeneous skin, which plays an important role in preventing systemic inflammatory reaction and protecting organ functions<sup>[26]</sup>.

In some patients in whom fluid resuscitation was not implemented effectively for various reasons, wound surface infection often caused severe injury to the GI function, or even toxic paralytic ileus palsy, greatly increasing toxin absorption and bacterial superinfection. Toxins absorbed in the blood act on the GI tract, which in turn lowers the GI kinetics, resulting in a vicious cycle<sup>[27]</sup>. Treatment of this kind of critically severely burned patients is a real challenge, in whom the mortality rate is usually high. Apart from the above mentioned routine treatments, we also used CRRT to filtrate inflammatory mediators and toxins in the body, which significantly lowered the content of endotoxins and inflammatory factors and DAO activity. As the vicious cycle was broken off, the therapeutic outcome was usually good<sup>[28]</sup>.

In summary, post-burn GI dysfunction is caused by multiple factors, and therefore maintaining GI function is a systematic engineering project. The therapeutic strategy should not rely on a single treatment or a single drug<sup>[29,30]</sup>. Furthermore, as severe burn itself may cause serious injury to various functions of the body, prevention of multi-organ functions should be addressed. Clinical experiences in recent 30 years have demonstrated that comprehensive fluid resuscitation, early excision of necrotic tissue, staged food ingestion, and administration of specific nutrients are essential strategies for preventing gastrointestinal complications in severely burned patients. Once severe GI dysfunction and sepsis occur, individualized comprehensive treatment should be implemented without delay. CRRT developed in recent years appears to be a promising strategy in the treatment of severe burns<sup>[31]</sup>.

## COMMENTS

### Background

Gastrointestinal dysfunction is a common complication of severe burns. Injury to GI function, especially to GI barrier function, is an important initiator as well as a stimulator for occurrence of systemic inflammatory response syndrome (SIRS), sepsis and multiple organ dysfunction syndrome (MODS) following severe burns.

### Research frontiers

The study analyzed and summarized the authors' clinical experiences in recent 30 years in the prevention and treatment of gastrointestinal dysfunction in severely burned patients in an attempt to propose some practicable guidelines for the effective prevention and treatment of GI dysfunction following severe burns.

### Innovations and breakthroughs

Comprehensive fluid resuscitation, early excision of necrotic tissue, staged food ingestion, and administration of specific nutrients are essential strategies for

preventing gastrointestinal complications and lowering the mortality in severely burned patients.

### Applications

The study provided some practicable guidelines for the effective prevention and treatment of GI dysfunction following severe burns.

### Peer review

The paper analyzed and summarized some practicable experience for the effective prevention of GI dysfunction. It is valuable to see the actual results from the therapies by the authors over the last 30 years.

## REFERENCES

- 1 Wang ZT, Yao YM, Xiao GX, Sheng ZY. Risk factors of development of gut-derived bacterial translocation in thermally injured rats. *World J Gastroenterol* 2004; **10**: 1619-1624
- 2 Prelack K, Dylewski M, Sheridan RL. Practical guidelines for nutritional management of burn injury and recovery. *Burns* 2007; **33**: 14-24
- 3 Kim K, Kwok I, Chang H, Han T. Comparison of cardiac outputs of major burn patients undergoing extensive early escharectomy: esophageal Doppler monitor versus thermodilution pulmonary artery catheter. *J Trauma* 2004; **57**: 1013-1017
- 4 Wang Z, Xiao G, Yao Y, Guo S, Lu K, Sheng Z. The role of bifidobacteria in gut barrier function after thermal injury in rats. *J Trauma* 2006; **61**: 650-657
- 5 Sallam HS, Oliveira HM, Gan HT, Herndon DN, Chen JD. Ghrelin improves burn-induced delayed gastrointestinal transit in rats. *Am J Physiol Regul Integr Comp Physiol* 2007; **292**: R253-R257
- 6 Jeschke MG, Herndon DN, Finnerty CC, Bolder U, Thompson JC, Mueller U, Wolf SE, Przgora R. The effect of growth hormone on gut mucosal homeostasis and cellular mediators after severe trauma. *J Surg Res* 2005; **127**: 183-189
- 7 Li JY, Lu Y, Hu S, Sun D, Yao YM. Preventive effect of glutamine on intestinal barrier dysfunction induced by severe trauma. *World J Gastroenterol* 2002; **8**: 168-171
- 8 Mesejo A, Juan M, Garcia-Simon M. [Enteral access and intestinal function assessment in the critically ill patient] *Nutr Hosp* 2007; **22** Suppl 2: 37-49
- 9 Cumming J, Purdue GF, Hunt JL, O'Keefe GE. Objective estimates of the incidence and consequences of multiple organ dysfunction and sepsis after burn trauma. *J Trauma* 2001; **50**: 510-515
- 10 Olguin F, Araya M, Hirsch S, Brunser O, Ayala V, Rivera R, Gotteland M. Prebiotic ingestion does not improve gastrointestinal barrier function in burn patients. *Burns* 2005; **31**: 482-488
- 11 Cochran A, Morris SE, Edelman LS, Saffle JR. Burn patient characteristics and outcomes following resuscitation with albumin. *Burns* 2007; **33**: 25-30
- 12 Secchi A, Ortanderl JM, Schmidt W, Gebhard MM, Martin E, Schmidt H. Effect of endotoxemia on hepatic portal and sinusoidal blood flow in rats. *J Surg Res* 2000; **89**: 26-30
- 13 Cancio LC, Kramer GC, Hoskins SL. Gastrointestinal fluid resuscitation of thermally injured patients. *J Burn Care Res* 2006; **27**: 561-569
- 14 Knotzer H, Pajk W, Maier S, Dunser MW, Ulmer H, Schwarz B, Salak N, Hasibeder WR. Comparison of lactated Ringer's, gelatine and blood resuscitation on intestinal oxygen supply and mucosal tissue oxygen tension in haemorrhagic shock. *Br J Anaesth* 2006; **97**: 509-516
- 15 Biber B, Martner J, Nilsson H, Redfors S, Sjowall H, Winso O. Intestinal vascular responses to dopamine during fentanyl-nitrous oxide anaesthesia, supplemented with dixyrazin. *Acta Anaesthesiol Scand* 1983; **27**: 255-261
- 16 Dokmeci D. Testicular torsion, oxidative stress and the role of antioxidant therapy. *Folia Med (Plovdiv)* 2006; **48**: 16-21
- 17 Mandal R, Kutala VK, Khan M, Mohan IK, Varadharaj S,



- Sridhar A, Carnes CA, Kalai T, Hideg K, Kuppusamy P. N-hydroxy-pyrroline modification of verapamil exhibits antioxidant protection of the heart against ischemia/reperfusion-induced cardiac dysfunction without compromising its calcium antagonistic activity. *J Pharmacol Exp Ther* 2007; **323**: 119-127
- 18 **Biesalski HK**, McGregor GP. Antioxidant therapy in critical care--is the microcirculation the primary target? *Crit Care Med* 2007; **35**: S577-S583
- 19 **Rivas S**, Hernandez F, Martinez L, Lopez Gutierrez JC, Ros Z. [Decrease in bacterial translocation in burned children treated with controlled nutritional support] *Cir Pediatr* 2002; **15**: 79-81
- 20 **Inoue S**, Epstein MD, Alexander JW, Trocki O, Jacobs P, Gura P. Prevention of yeast translocation across the gut by a single enteral feeding after burn injury. *JPEN J Parenter Enteral Nutr* 1989; **13**: 565-571
- 21 **Yan H**, Peng X, Huang Y, Zhao M, Li F, Wang P. Effects of early enteral arginine supplementation on resuscitation of severe burn patients. *Burns* 2007; **33**: 179-184
- 22 **Namiki M**. Nutraceutical functions of sesame: a review. *Crit Rev Food Sci Nutr* 2007; **47**: 651-673
- 23 **Deloris Alexander A**, Orcutt RP, Henry JC, Baker J Jr, Bissahoyo AC, Threadgill DW. Quantitative PCR assays for mouse enteric flora reveal strain-dependent differences in composition that are influenced by the microenvironment. *Mamm Genome* 2006; **17**: 1093-1104
- 24 **Silvestri L**, van Saene HK, Milanese M, Gregori D, Gullo A. Selective decontamination of the digestive tract reduces bacterial bloodstream infection and mortality in critically ill patients. Systematic review of randomized, controlled trials. *J Hosp Infect* 2007; **65**: 187-203
- 25 **Wong P**, Burd A. Meta or better: analysis of early excision. *Burns* 2006; **32**: 662
- 26 **Ong YS**, Samuel M, Song C. Meta-analysis of early excision of burns. *Burns* 2006; **32**: 145-150
- 27 **Garcia-Rodenas CL**, Bergonzelli GE, Nutton S, Schumann A, Cherbut C, Turini M, Ornstein K, Rochat F, Cortesey-Theulaz I. Nutritional approach to restore impaired intestinal barrier function and growth after neonatal stress in rats. *J Pediatr Gastroenterol Nutr* 2006; **43**: 16-24
- 28 **Peng Y**, Yuan Z, Li H. Removal of inflammatory cytokines and endotoxin by veno-venous continuous renal replacement therapy for burned patients with sepsis. *Burns* 2005; **31**: 623-628
- 29 **Chung DH**, Herndon DN. Multiple converging mechanisms for postburn intestinal barrier dysfunction. *Crit Care Med* 2004; **32**: 1803-1804
- 30 **Chen LW**, Hwang B, Wang JS, Chen JS, Hsu CM. Hypertonic saline-enhanced postburn gut barrier failure is reversed by inducible nitric oxide synthase inhibition. *Crit Care Med* 2004; **32**: 2476-2484
- 31 **Sun IF**, Lee SS, Lin SD, Lai CS. Continuous arteriovenous hemodialysis and continuous venovenous hemofiltration in burn patients with acute renal failure. *Kaohsiung J Med Sci* 2007; **23**: 344-351

S- Editor Li DL L- Editor Ma JY E- Editor Ma WH



RAPID COMMUNICATION

## Venous diethylene glycol poisoning in patients with preexisting severe liver disease in China

Bing-Liang Lin, Zhi-Xin Zhao, Yu-Tian Chong, Jian-Guo Li, Xing Zuo, Yu Tao, Tan-Qi Lou, Zhi-Liang Gao

Bing-Liang Lin, Zhi-Xin Zhao, Yu-Tian Chong, Jian-Guo Li, Xing Zuo, Zhi-Liang Gao, Department of Infectious Diseases, Third Affiliated Hospital of Sun Yet-Sen University, Guangzhou 510060, Guangdong Province, China

Yu Tao, Department of Pathology, First Affiliated Hospital of Sun Yet-Sen University, Guangzhou 510060, Guangdong Province, China

Tan-Qi Lou, Department of Internal Medicines, Division of Renal Diseases, Third Affiliated Hospital of Sun Yet-Sen University, Guangzhou 510060, Guangdong Province, China

**Author contributions:** Lin BL and Gao ZL contributed equally to this work; Lin BL, Zhao ZX designed the research; Chong YT, Li JG, Zuo X, Tao Y, Lou TQ performed the research; Lin BL analyzed the data; Lin BL and Gao ZL wrote the paper.

**Correspondence to:** Zhi-Liang Gao, Professor, Department of Infectious Diseases, Third Affiliated Hospital of Sun Yet-Sen University, Tianhe Area, 600 Tianhe Road, Guangzhou 510060, Guangdong Province, China. [lamikin@126.com](mailto:lamikin@126.com)

Telephone: +86-20-85253165 Fax: +86-20-87583501

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revealed acute tubular necrosis and interstitial nephritis. Significant differences in preexisting severe hepatitis, ascites, renal disease, and diuretic therapy were found between groups. Prior to diethylene glycol injections, the mean values for neutral granular cells, BUN, Cr, calcium and phosphorous ions differed significantly between groups.

**CONCLUSION:** Venous diethylene glycol poisoning is characterized by oliguric acute renal failure, metabolic acidosis, digestive symptoms, nervous system impairment, and a high probability of anemia and WBC proliferation. Mortality is high. Correlative factors include preexisting severe liver disease, renal disease, and infection.

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**Key words:** Diethylene glycol; Poisoning; Liver disease; Clinical feature

**Peer reviewer:** Dr. Cynthia Levy, Division of Gastroenterology, Hepatology and Nutrition, University of Florida, MSB-Rm M 440, 1600 SW Archer Road, Gainesville, FL 32608, United States

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### Abstract

**AIM:** To analyze the clinical presentation of venous diethylene glycol (DEG) poisoning in patients with preexisting severe liver disease and factors that correlate with DEG poisoning.

**METHODS:** Retrospective chart review was performed to analyze the epidemiology, clinical presentation, hepatorenal functions, hemodynamics and pathological characteristics of 64 patients with severe liver disease who received intravenous armillarisin-A, the solvent of which was DEG. Comparative analyses of correlating factors and causes for poisoning were based on the presence or absence of poisoning.

**RESULTS:** Of the 64 patients who received armillarisin-A, 15 were found to have DEG poisoning. Twelve poisoned patients died. After a mean of 5 d, the poisoned patients displayed acute renal failure. Metabolic acidosis occurred in 13 cases. BUN, Cr, and CO<sub>2</sub> values were significantly elevated and exacerbation of digestive tract symptoms and/or symptom was noted in 11 cases. Neurological system impairment was observed in 10 cases after 2 wk. Compared to the 49 non-poisoned patients, the poisoned patients exhibited significantly lower RBC and Hb values and higher WBC count. Renal biopsy from the poisoned patients

### INTRODUCTION

Diethylene glycol (DEG) is a chemical substance used primarily for industrial purposes. Tested in animals, DEG induces liver impairment and kidney toxicity presenting as acute renal failure (ARF)<sup>[1,2]</sup>. In 1937, 358 human cases of ARF resulting in 107 deaths were described following ingestion of sulfanilamide dissolved in DEG in America<sup>[3]</sup>. Similar reports of DEG poisoning appeared subsequently in the other countries<sup>[4-10]</sup>, with most cases involving pediatric poisoning through oral ingestion and with fundamentally milder complications.

On April 22th 2006 and April 24th 2006, two patients in the Department of Infectious Diseases, Third Affiliated Hospital of Sun Yat-Sen University, with severe liver disease developed ARF. On April 29th

2006 and April 30th 2006, another six patients in this department with severe liver disease also developed ARF. Upon further investigation, armillarisin-A<sup>[11]</sup>, a drug produced by the Qiqihar No. 2 Pharmaceutical Co. Ltd, for treatment of gall-bladder disease, was found to have been administered to all patients who developed ARF. Administration of the drug was immediately suspended. Subsequently, the situation was reported to the relevant pharmacy. All preparations of armillarisin-A were sealed and forwarded to the Guangdong Drug Examination Center for investigation. Findings revealed that DEG was present in these preparations at a concentration of 30%. Subsequent judiciary investigation disclosed that the Qiqihar No. 2 Pharmaceutical Co. Ltd, selected DEG to serve as an economic substitute for trimethylene glycol in armillarisin-A preparations.

Review of 64 patients who received armillarisin-A in the hospital during the relevant time period was therefore undertaken, and findings were described in the present report. Of the 15 patients subsequently died, 12 were diagnosed with DEG poisoning. No other patients with similar complications have been reported since May 2nd 2006. The investigation described in the present report has the following features: (1) all subjects were adults who received armillarisin-A with DEG intravenously; (2) clinical presentation was recorded before and after DEG poisoning, and the exact injection volumes and DEG concentrations in the preparations were recorded; (3) the majority of patients presented with concurrent severe liver disease. In the present report, the clinical presentation of venous diethylene glycol poisoning and the pathological characteristics of renal tissue of poisoned patients were described and factors that correlate with this form of poisoning were identified.

## MATERIALS AND METHODS

### Subjects

The 64 patients enrolled in the present study were treated with armillarisin-A in the Third Affiliated Hospital of Sun Yat-Sen University in Guangzhou between April 19th 2006 and May 1st 2006. All the patients, including 49 (76.6%) males, were diagnosed with severe liver diseases. Of these 64 patients, 14 had severe hepatitis, 16 had liver cirrhosis caused by hepatitis B virus, 21 had chronic active hepatitis, 6 had primary hepatocellular carcinoma, 2 underwent liver transplantation, 2 had biliary cirrhosis, and 1 had hepatolenticular degeneration and liver impairment due to malignant lymphoma and cholangiocarcinoma.

### Diagnostic methods

Based on the published findings long before and the consensus of experts, the Department of Health of Guangdong Province established the criteria for clinical diagnosis of DEG poisoning. The following three criteria are considered essential: (1) a history of DEG prescription (oral/venous injection), (2) acute renal impairment or renal failure characterized by oliguria or anuria occurring within 2 wk of the last ingestion/injection, and (3) elimination of all other causes of

acute renal impairment or renal failure.

Diagnosis of viral hepatitis was based on the standardized "viral hepatitis prevention study" performed in 2000 by the Society of Infections Diseases and Society of Liver Diseases of the Chinese Medical Association<sup>[12]</sup>.

### Research methods

Retrospective chart review was applied to all 64 patients who received DEG intravenously. These patients were assigned to either the poisoned group or the non-poisoned group. For each poisoned patient, analyses of epidemiology, clinical symptoms, prognosis, hepatorenal functions, hemodynamics and pathology of renal tissue were performed before and after poisoning for comparison purposes. Analyses were also performed for the poisoned group as compared to the non-poisoned group prior to receipt of DEG to identify factors predisposing to DEG poisoning.

Renal tissues from poisoned patients were examined with several methods. Ten or more renal corpuscles were extracted and subjected to HE and PASH staining followed by microscopic observation. The nature and degree of corpuscular and tubular-interstitial pathologies were evaluated. Immunofluorescence staining of frozen sections was performed to observe the deposition sites and degree of deposition of immune-complex compounds. Electron microscopy was performed to identify the ultrastructural changes in renal tissue.

Liver function and biochemical parameters were detected using an automatic chemistry analyzer. The concentration of DEG in armillarisin-A was determined by spectrophotometry.

### Statistical analysis

Normality distribution was analyzed for the continuous variables. The *t*-test was performed to detect significant differences between groups with normality. The data are presented as mean  $\pm$  SD. Group comparison for data without a normal distribution involved evaluation by independent nonparametric testing. Findings were presented as the medians. The Chi-square test was performed to examine numerical data.  $P < 0.05$  was considered statistically significant. SPSS13.0 for windows was used for all statistical analyses.

## RESULTS

### Basic information concerning patients who received DEG intravenously

Sixty-four patients who received intravenous injections of armillarisin-A were observed. On June 30th 2006, DEG poisoning was present in 15 patients and absent in 49 patients. Comparative statistics was performed based on the presence or absence of DEG poisoning, and findings are listed in Table 1. The DEG concentration in the patients ranged from 1.2% to 6%, with a cumulative dosage volume of 2.4-114 mL, but no statistical differences in these values were observed between the poisoned and non-poisoned groups. Liver impairment was more severe in the DEG-poisoned group than in

**Table 1 Basic information concerning patients receiving venous diethylene glycol injections (*n* = 64)**

Item	DEG-poisoned group ( <i>n</i> = 15)	Non-DEG-poisoned group ( <i>n</i> = 49)	Statistical value	<i>P</i> value
Male sex (%)	14 (93.3)	35 (71.4)	3.071	0.080
Age (yr)				
Median	50	48	1.11	0.267
Range	33-76	5-72		
DEG intake-ml				
Median	24	36	0.27	0.787
Range	9-72	2.4-114		
DEG concentration (%)				
Median	6	6	0.713	0.476
Range	3-6	1.2-6		
Alcoholics (%)	7 (46.7)	19 (38.8)	0.296	0.586
Diagnosis			11.691	0.039
TLD (%)	12 (80.0)	21 (42.9)	6.344	0.012
CH (%)	2 (13.3)	19 (38.8)		0.112 <sup>2</sup>
Other (%)	1 (6.7)	9 (18.4)		0.258 <sup>2</sup>
Diuretics (%)	12 (80.0)	16 (32.7)		0.020 <sup>2</sup>
Complication				
Ascites (%)	10 (66.7)	9 (18.4)		0.000
Renal disease <sup>2</sup> (%)	5 (33.3)	3 (6.1)		0.014 <sup>1</sup>
Serum checking				
ALT (U/L)	180.9 ± 269.9	201.4 ± 284.3	0.251	0.804
TB (μmol/L)	359.2 ± 245	239.3 ± 221.5	1.767	0.082
BUN (mmol/L)	7.9 ± 3.8	4.3 ± 2.9	3.372	0.003
Creatinine (μmol/L)	94.2 ± 24.6	58.7 ± 22.6	5.141	0.000
Ca <sup>2+</sup> (mmol/L)	2.37 ± 0.17	2.25 ± 0.21	2.157	0.035
Phosphonium (mmol/L)	0.72 ± 0.43	1.00 ± 0.33	2.574	0.013
WBC (10 <sup>9</sup> /L)	6.47 ± 2.08	6.00 ± 5.34	0.326	0.746
NEUT	0.716 ± 0.114	0.587 ± 0.153	3.003	0.004
RBC (10 <sup>12</sup> /L)	3.03 ± 0.92	3.33 ± 0.79	1.208	0.232
Hemoglobin (g/L)	99.9 ± 25.6	106.6 ± 18.8	1.035	0.305
Platelet count (10 <sup>9</sup> /L)	106.9 ± 50.6	125.9 ± 73.3	0.293	0.354

TLD: Terminal liver disease, including severe hepatitis, liver cirrhosis, recurrence of post liver transplantation; ALT: alanine aminotransferase; CH: Chronic hepatitis; TB: total bilirubin; WBC: White blood cells; RBC: Red blood cells; NEUT: Ratio of neutral leucocyte; BUN: Blood urea nitrogen. <sup>1</sup>Fisher's exact test; <sup>2</sup>Pre-existing renal diseases, including kidney stones, proliferative renal cysts and urinary tract infections. One case of renal carcinoma was observed in the non-poisoned group.

the non-DEG-poisoned group. Of the 15 poisoned patients, 12 had terminal liver disease. Data analyses revealed significant differences between the poisoned and non-poisoned groups with respect to the severity of pre-injection liver conditions, presence of ascites and renal diseases, use of diuretics, pre-injection neutral granular cell count, serum BUN, serum Cr, calcium and phosphate ion (IP) concentrations. Death occurred in 12 patients of the poisoned group and 8 patients of the non-poisoned group. Hepatic failure and multiple organ dysfunction syndromes (MODS) were identified as the main causes of death.

#### **Clinical presentation of patients with DEG poisoning**

Oliguric ARF was present for a mean of 5 d in 15 patients with intravenous DEG poisoning. The clinical characteristics of these 15 patients are presented in Table 2. The urine volume decreased rapidly. The majority of poisoned patients developed digestive tract symptoms,

**Table 2 Clinical characteristics of 15 DEG-poisoned patients**

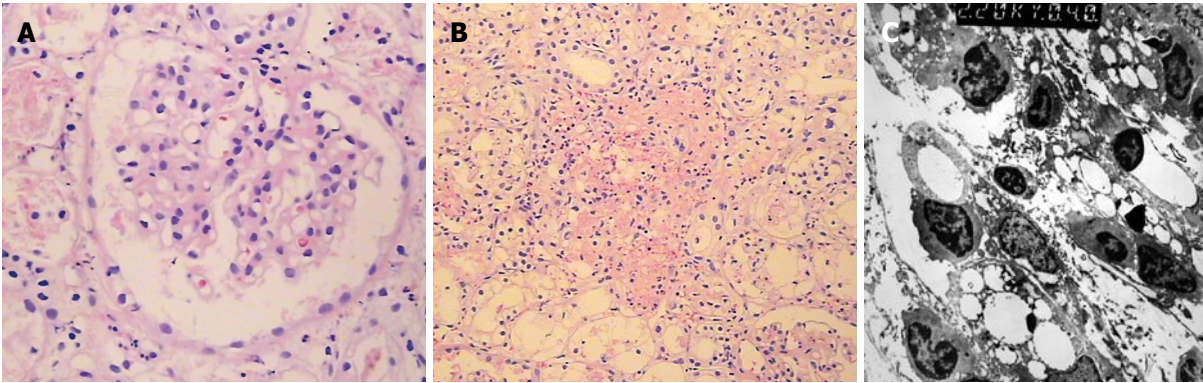
Characteristics	Data
Age (yr)	50 (33-76)
Male sex (%)	14 (93.6)
Injected DEG volume (mL)	24 (9-72)
ARF (%)	15 (100)
Incubation period of ARF (d)	5 (2-12)
Incubation periods of anuria (d)	6 (3-13)
Fever (%)	7 (46.7)
Incubation periods (d)	6 (1-13)
Dig. tract symptoms (%)	11 (73.3)
Incubation period (d)	9 (3-19)
Nerv. syst. impair (%)	10 (66.7)
Incubation periods (d)	14 (7-24)
Cranial nerves (%)	10 (64.7)
Peripheral nerves (%)	5 (33.3)
Central nerv. syst. (%)	6 (40.0)
Metab. acidosis ( <i>n</i> = 13) (%)	13 (100)
Incubation periods of abnormal Cr and/or BUN (d)	5 (2-12)
Time of peak Cr (d)	11 (6-19)
Time of peak BUN (d)	14 (6-23)
Incubation periods of abnormal CO <sub>2</sub> (d)	9 (2-14)
Time of peak CO <sub>2</sub> (d)	10 (5-16)
Death (%)	12 (80.0)
Death time after injection (d)	12.5 (8-65)
Causes of death ( <i>n</i> = 12)	
MODS (%)	7 (58.3)
Infection (%)	4 (33.3)
Dig. tract bleed (%)	1 (8.3)

such as nausea, vomiting and bloating, or exhibited an increase in the severity of these symptoms. Half of the patients exhibited concomitant mild pyrexia. Ten patients displayed nervous system impairment involving the cranial nerves, including the facial, optic, oculomotor and glossopharyngeal nerves, at an average of 14 d after the initial injection. A few patients exhibited peripheral nerve involvement presenting as limb tremor and paralysis. Respiratory muscle paralysis might have been present in some patients, leading inevitably to respiratory failure. DEG poisoning was also associated with an increase in the severity of hepatic encephalopathy among patients previously exhibiting this complication. Retrospective analyses of 13 patients before and after DEG poisoning revealed that all patients experienced metabolic acidosis at an average of 9 d after injection and 4 d following development of ARF. The most severe manifestations of metabolic acidosis occurred on d 10 after initial ingestion of DEG. Twelve of the 15 patients diagnosed with DEG poisoning died. Death generally occurred 1 wk following the initial signs of renal failure. Among the 3 patients who survived the poisoning, however, urine volume was observed to recover 3 wk after poisoning and urine volume was normal 1 mo after poisoning. One of the three patients who survived underwent combined liver-kidney transplantation 16 d after exhibiting DEG poisoning.

#### **Hepatorenal functions and peripheral blood cell count before and after DEG poisoning**

When the liver function, renal function and peripheral blood cell counts before DEG poisoning were compared





**Figure 1** Pathological changes in renal tissue of patients with DEG poisoning. **A:** Glomerulus of a patient poisoned by intravenously administered DEG revealing no remarkable changes (HE, × 200); **B:** Tubular necrosis and interstitial inflammatory response in renal tissue following poisoning by intravenously administered DEG (HE, × 100); **C:** Microscopic observation of tubular vacuolation and interstitial inflammatory response in renal tissue following poisoning by intravenously administered DEG (× 6000).

**Table 3** Liver-renal function measurements and peripheral blood cell counts before and after DEG poisoning

Item	n	BP	AP	t-value	P	CI
TB (μmol/L)	15	376.7 ± 244.6	354.7 ± 257.1	0.945	0.362	-28.36-72.44
PT (s)	15	24.4 ± 13.1	22.4 ± 8.8	1.33	0.210	-1.34-5.41
GGT (U/L)	14	163.2 ± 225.5	109.4 ± 115.8	1.451	0.170	-26.3-133.8
ALP (U/L)	14	217.0 ± 265.4	146.7 ± 148.8	1.888	0.082	10.2-150.7
BUN (mmol/L)	15	7.4 ± 3.9	31.2 ± 9.68	8.373	0.000	17.61-30.00
Cr (μmol/L)	15	94.2 ± 24.1	691.6 ± 197.8	10.659	0.000	475.28-719.51
CO <sub>2</sub> (mmol/L)	13	24.4 ± 3.9	13.1 ± 2.6	11.75	0.000	9.20-13.39
Ca <sup>2+</sup> (mmol/L)	14	2.38 ± 0.18	2.41 ± 0.22	-0.737	0.474	-0.13-0.07
Phosphonium (mmol/L)	14	0.73 ± 0.45	1.31 ± 0.50	-4.088	0.001	-0.90-(-0.28)
WBC (10 <sup>9</sup> /L)	15	6.59 ± 2.33	9.78 ± 3.75	3.325	0.008	1.05-5.33
RBC (10 <sup>12</sup> /L)	15	2.99 ± 0.94	2.32 ± 0.76	2.968	0.014	0.16-1.17
Hb (g/L)	15	99.6 ± 25.1	79.5 ± 23.6	2.823	0.018	4.25-36.11
PLT (10 <sup>9</sup> /L)	15	119.6 ± 50.1	94.6 ± 72.6	1.336	0.211	-16.75-66.93

BP: The last values before DEG poisoning; AP: The peak values after DEG poisoning; GGT: Gamma-glutamyl transpeptidase; ALP: Alkaline phosphatase.

with the peak value after DEG poisoning: (1) the patients' blood urea nitrogen (BUN), creatinine (Cr), and phosphate (P) concentrations increased significantly after DEG poisoning, while serum CO<sub>2</sub> concentration dropped significantly, but serum calcium had no remarkable change; (2) DEG did not cause aggravation of liver function, while serum total bilirubin level, GGT, ALP and prothrombin time did not change significantly; (3) the peripheral blood cell counts increased significantly after DEG poisoning, while the red blood cell counts and hemoglobin value dropped significantly, but platelet counts did not change obviously (Table 3).

**Pathological changes in renal tissue from patients with DEG poisoning**

Renal tissues were taken from two patients with DEG poisoning on the third and forth days after ARF, respectively. Biopsies of renal tissue indicated significant tubular pathological changes, partial dissolution and necrosis of epithelial cells, and interstitial inflammatory cell infiltration (Figure 1). No pathological changes in the glomerular basement membrane of these patients were observed.

**DISCUSSION**

In 1937, the Massengill Company (USA) developed an “elixir of sulfanilamide”, a preparation of 9-10 g of sulfanilamide dissolved in 100 mL of DEG. Other cases of DEG poisoning have been largely associated with foul play or deliberate consumption of alcoholic mixtures containing DEG<sup>[3-10,13]</sup>. In this study, injection of armillarisin-A produced by the Qiqihar No. 2 Pharmaceutical Co. Ltd. resulted in events similar to those described previously in response to DEG poisoning. Sixty-four patients with severe liver disease received venous armillarisin-A injections containing high concentrations of DEG (325.9 mg/mL and 30% concentration as reported by the Heilongjiang Province Drug Inspection Center and the Guangdong Province Drug Inspection Center, respectively). Fifteen patients were diagnosed with DEG poisoning. The rate of poisoning was 23.4%.

Liver impairment was more severe in the DEG-poisoned group than in the non-DEG-poisoned group. Metabolism of DEG involves the actions of alcohol dehydrogenase and aldehyde dehydrogenase<sup>[1]</sup>. Alcohol dehydrogenase ordinarily converts DEG to an aldehyde and aldehyde dehydrogenase ordinarily converts this

aldehyde to certain acids. As alcohol dehydrogenase and aldehyde dehydrogenase are mainly restricted to the liver, loss of these enzymes as a consequence of severe liver disease may significantly impair DEG metabolism. Furthermore, secondary infection is a common complication in patients with terminal liver disease. Peritonitis caused by Gram-negative bacilli and hepatobiliary infection are the most prevalent complications. Data also indicate that the pre-injection rate of neutral granular cells in the poisoned group was significantly higher than that in the non-poisoned group. Infection-induced endotoxemia increases alcohol dehydrogenase activity<sup>[14]</sup>, and accumulation of the aldehyde intermediate can provoke DEG poisoning. Concurrently, serious liver diseases often produce massive ascites requiring diuretic therapy. Resultant renal hemodynamic changes occurring in response to such therapy may inevitably lead to exacerbation of renal damage. Therefore, lower dosages and concentrations of DEG can provoke poisoning *via* the intravenous route in patients with severe liver disease as compared to the oral route in patients without severe liver disease.

In the present study, the poisoned patients had a significantly higher incidence of renal disease and significantly higher serum BUN and Cr concentrations than the non-poisoned patients, suggesting that patients with renal disease are more susceptible to DEG poisoning than those without renal disease.

Currently available information about DEG indicates that this glycol induces acute poisoning, but no chronic poisoning. This apparent discrepancy can be explained by the short half-life of DEG (approximately 3 h)<sup>[15]</sup>. DEG poisoning was previously considered similar to ethylene glycol poisoning, which is associated with renal impairment attributable to renal accumulation of calcium ions and to the final product, oxalic acid, with resultant accumulation of calcium oxalates. Recent findings show that the final product of DEG metabolism is a 2-hydroxy-ethoxyacetic acid rather than an oxalic acid. DEG-induced pathological changes and necrosis of tubular epithelial cells are attributable to a metabolic intermediate that poisons tubular epithelial cells rather than to deposition of calcium oxalates<sup>[16-19]</sup>. Renal impairment is observed at early stages of poisoning and is prominent in all cases of poisoning, as was observed in the present study.

The clinical characteristics of patients poisoned by intravenous DEG were similar to those of patients poisoned following oral ingestion of DEG in the present study. It was reported that renal impairment occurs at early times following ingestion, with metabolic acidosis and delayed neurological impairment mainly involving the cranial and peripheral nerves commonly observed<sup>[20-24]</sup>. Poisoning *via* the intravenous route differs notably from poisoning *via* the oral route in that exhibition of mild fever and an increase in severity of digestive tract symptoms before occurrence of renal failure, along with a later occurrence of organ impairment, is specific for intravenous poisoning. This finding may be attributable to the age of patients in the present study and to their preexisting severe liver disease which could have limited

the actions of alcohol and aldehyde dehydrogenases. Prospective research is warranted for further clarification. Due to the scarcity of DEG poisoning survivors, it is difficult to evaluate the process of systematic recovery. While previous reports indicate that recovery of the nervous system after oral DEG poisoning requires 4-6 mo<sup>[25]</sup>, the present findings disclose that nervous system recovery occurs 1 mo following intravenous poisoning.

In the present study, 80% mortality was observed in the poisoned patients. Seven died of MODS, 4 died of severe infection, and 1 died of severe digestive tract bleeding. The lethal dose of DEG varies with species<sup>[26]</sup>. It was reported that DEG at a cumulative dosage of 0.22-4 mL/kg with a concentration of 17.5%-72% in humans can lead to death<sup>[6,7]</sup>. The DEG concentration in the present study ranged from 3% to 6%, with a cumulative dosage volume of 9-72 mL, but no statistical differences in these values were observed between the poisoned and non-poisoned groups, indicating that the severity of preexisting liver disease leading to loss of alcohol and aldehyde dehydrogenase activities constitutes a primary important predisposing factor for poisoning.

In the present study, the patients with DEG poisoning had higher serum calcium values and lower serum IP values than the non-poisoned patients, the serum IP concentrations were significantly increased after intravenous DEG poisoning. The importance of calcium and phosphates in DEG poisoning remains to be determined.

Although the DEG-poisoned patients described in the present study presented with concomitant severe liver diseases, no exacerbating degenerative features of general liver function (no changes in bilirubin, aldehyde dehydrogenase, albumin, and hemostatic function) were found. Furthermore, no significant increase in gamma-glutamyl transpeptidase or alkaline phosphatase was noted, indicating that venous injections of DEG do not directly affect the hepatobiliary system and drug-induced liver damage is absent. These observations may be attributable to the fact that, in contrast to oral ingestion of DEG<sup>[27]</sup>, venous injection of this glycol does not participate in liver metabolism.

Additional analyses indicated that DEG-poisoned patients might present with anemia characterized by decreased red blood cells and hemoglobin. A similar form of anemia was observed in ethylene glycol poisoning. The DEG-poisoned patients described in the present study also presented with an increase in white blood cell count, with a significant increase in neutral granular cells but no remarkable changes in eosinophils. This phenomenon can be attributed to an increase in the severity of infection in patients with severe liver disease and is indicative of acute renal failure as a result of DEG poisoning rather than allergen induction.

Renal biopsy findings revealed that DEG induced tubular epithelial cell dissolution and necrosis and renal interstitial inflammatory cell infiltration, but no pathological changes in the glomerulus. These alterations differ from those associated with the hepatorenal complications and glomerulonephritis induced by severe

liver disease and are therefore important in differential diagnosis.

In conclusion, venous diethylene glycol poisoning is characterized by oliguric acute renal failure, metabolic acidosis, digestive symptoms, nervous system impairment, and a high probability of anemia and WBC proliferation. Mortality is high, and correlative factors include preexisting severe liver disease, renal disease, and infection.

## COMMENTS

### Background

Diethylene glycol (DEG) is a chemical substance used primarily for industrial purposes. DEG induces kidney toxicity presenting as acute renal failure (ARF) and has been used as a chemical substance for industrial purposes in many countries since 1937. In 2006, 64 patients with severe liver disease received venous armillarisin-A injections containing high concentrations of DEG, and 15 were diagnosed with DEG poisoning in Guangzhou, China. In the present report, the clinical presentation of venous diethylene glycol poisoning and the pathological characteristics of renal tissue from poisoned patients were described and factors that correlate with this form of poisoning were identified.

### Research frontiers

All the clinical researches about DEG poisoning based on events of herbal toxicity, have been limited in oral DEG intake and normal persons.

### Innovations and breakthroughs

The investigation described in the present report was characterized by the following features: (1) all subjects were adults who received armillarisin-A with DEG intravenously, (2) clinical presentation was recorded before and after DEG poisoning and the exact injection volumes and DEG concentrations in the preparations were recorded, (3) the majority of patients presented with concurrent severe liver diseases.

### Applications

This work may help to know the clinical presentation of venous diethylene glycol (DEG) poisoning in patients with preexisting severe liver disease and factors that correlate with this form of poisoning.

### Peer review

This is a nice report on an outbreak of IV DEG poisoning. Authors analyzed the features of venous DEG poisoning and serious consequences to remind government of paying attentions to drug safety and supervising.

## REFERENCES

- Mathews JM, Parker MK, Matthews HB. Metabolism and disposition of diethylene glycol in rat and dog. *Drug Metab Dispos* 1991; **19**: 1066-1070
- Kraul H, Jahn F, Braunlich H. Nephrotoxic effects of diethylene glycol (DEG) in rats. *Exp Pathol* 1991; **42**: 27-32
- Geiling EMK, Cannon PR. Pathologic effects of elixir of sulfanilamide (diethylene glycol) poisoning. *JAMA* 1938; **111**: 919-926
- Okuonghae HO, Ighogboja IS, Lawson JO, Nwana EJ. Diethylene glycol poisoning in Nigerian children. *Ann Trop Paediatr* 1992; **12**: 235-238
- Hanif M, Mobarak MR, Ronan A, Rahman D, Donovan JJ Jr, Bennis ML. Fatal renal failure caused by diethylene glycol in paracetamol elixir: the Bangladesh epidemic. *BMJ* 1995; **311**: 88-91
- Ferrari LA, Giannuzzi L. Clinical parameters, postmortem analysis and estimation of lethal dose in victims of a massive intoxication with diethylene glycol. *Forensic Sci Int* 2005; **153**: 45-51
- Fatalities associated with ingestion of diethylene glycol-contaminated glycerin used to manufacture acetaminophen syrup—Haiti, November 1995-June 1996. *MMWR Morb Mortal Wkly Rep* 1996; **45**: 649-650
- O'Brien KL, Selanikio JD, Hechdivert C, Placide MF, Louis M, Barr DB, Barr JR, Hospedales CJ, Lewis MJ, Schwartz B, Philen RM, St Victor S, Espindola J, Needham LL, Denerville K. Epidemic of pediatric deaths from acute renal failure caused by diethylene glycol poisoning. Acute Renal Failure Investigation Team. *JAMA* 1998; **279**: 1175-1180
- Singh J, Dutta AK, Khare S, Dubey NK, Harit AK, Jain NK, Wadhwa TC, Gupta SR, Dhariwal AC, Jain DC, Bhatia R, Sokhey J. Diethylene glycol poisoning in Gurgaon, India, 1998. *Bull World Health Organ* 2001; **79**: 88-95
- Bowie MD, McKenzie D. Diethylene glycol poisoning in children. *S Afr Med J* 1972; **46**: 931-934
- Sun F, Su JD, Zheng H. Studies on the pharmacological activities and toxicities of armillarisin-A, a new choleric drug. *Yaoxue Xuebao* 1981; **16**: 401-406
- Society of Infectious Diseases and Society of Liver Diseases of the Chinese Medical Association. Viral hepatitis prevention and treatment methods. *Zhonghua Ganzhangbing Zazhi* 2000; **8**: 324-329
- Junod SW. Diethylene glycol deaths in Haiti. *Public Health Rep* 2000; **115**: 78-86
- Potter JJ, Rennie-Tankersley L, Mezey E. Endotoxin enhances liver alcohol dehydrogenase by action through upstream stimulatory factor but not by nuclear factor-kappa B. *J Biol Chem* 2003; **278**: 4353-4357
- Lenk W, Lohr D, Sonnenbichler J. Pharmacokinetics and biotransformation of diethylene glycol and ethylene glycol in the rat. *Xenobiotica* 1989; **19**: 961-979
- Winek CL, Shingleton DP, Shanor SP. Ethylene and diethylene glycol toxicity. *Clin Toxicol* 1978; **13**: 297-324
- Deisinger PJ, Guest D. Metabolic studies with diethylene glycol monobutyl ether acetate (DGBA) in the rat. *Xenobiotica* 1989; **19**: 981-989
- Heilmair R, Lenk W, Lohr D. Toxicokinetics of diethylene glycol (DEG) in the rat. *Arch Toxicol* 1993; **67**: 655-666
- Johnson KA, Baker PC, Kan HL, Maurissen JP, Spencer PJ, Marty MS. Diethylene glycol monobutyl ether (DGBE): two- and thirteen-week oral toxicity studies in Fischer 344 rats. *Food Chem Toxicol* 2005; **43**: 467-481
- Hebert JL, Auzepy P, Durand A. [Acute human and experimental poisoning with diethylene glycol]. *Sem Hop* 1983; **59**: 344-349
- Vale JA, Buckley BM. Metabolic acidosis in diethylene glycol poisoning. *Lancet* 1985; **2**: 394
- Rollins YD, Filley CM, McNutt JT, Chahal S, Kleinschmidt-DeMasters BK. Fulminant ascending paralysis as a delayed sequela of diethylene glycol (Sterno) ingestion. *Neurology* 2002; **59**: 1460-1463
- Alfred S, Coleman P, Harris D, Wigmore T, Stachowski E, Graudins A. Delayed neurologic sequelae resulting from epidemic diethylene glycol poisoning. *Clin Toxicol (Phila)* 2005; **43**: 155-159
- Hari P, Jain Y, Kabra SK. Fatal encephalopathy and renal failure caused by diethylene glycol poisoning. *J Trop Pediatr* 2006; **52**: 442-444
- Hasbani MJ, Sansing LH, Perrone J, Asbury AK, Bird SJ. Encephalopathy and peripheral neuropathy following diethylene glycol ingestion. *Neurology* 2005; **64**: 1273-1275
- Schwetz BA, Price CJ, George JD, Kimmel CA, Morrissey RE, Marr MC. The developmental toxicity of diethylene and triethylene glycol dimethyl ethers in rabbits. *Fundam Appl Toxicol* 1992; **19**: 238-245
- Kawamoto T, Matsuno K, Kayama F, Arashidani K, Yoshikawa M, Kodama Y. The effect of ethylene glycol monomethyl ether and diethylene glycol monomethyl ether on hepatic gamma-glutamyl transpeptidase. *Toxicology* 1992; **76**: 49-57

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RAPID COMMUNICATION

## Effects of quercetin on hyper-proliferation of gastric mucosal cells in rats treated with chronic oral ethanol through the reactive oxygen species-nitric oxide pathway

Jing-Li Liu, Jun Du, Ling-Ling Fan, Xiao-Yan Liu, Luo Gu, Ying-Bin Ge

Jing-Li Liu, Jun Du, Xiao-Yan Liu, Luo Gu, Ying-Bin Ge, Department of Physiology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China

Ling-Ling Fan, Department of Physiology, Henan University of Science and Technology, Luoyang 471003, Henan Province, China

**Author contributions:** Liu JL and Fan LL developed the chronic drinking rat model and performed the assay of lipid peroxidation, protein oxidation, nitric oxide, and nitrotyrosine (NT); Du J assayed the cell proliferation by Western blot; Liu XY did the immunohistochemistry; Gu L carried out the statistical analysis and assisted in the experiment design; Ge YB designed the experiment and wrote the paper.

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**Correspondence to:** Ying-Bin Ge, MD, PhD, Department of Physiology, Nanjing Medical University, Nanjing 210029, Jiangsu Province, China. [ybge@njmu.edu.cn](mailto:ybge@njmu.edu.cn)

Telephone: +86-25-86862016 Fax: +86-25-86862016

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in the gastric mucosa of animals subjected to ethanol treatment for 7 days was significant increased (increased to 290% for PCNA density  $P < 0.05$ , increased to 150 for Cyclin D1 density  $P < 0.05$  and  $21.6 \pm 0.8$  vs  $42.3 \pm 0.7$  for PCNA positive cells per view field), accompanied by an increase in ROS generation ( $1.298 \pm 0.135 \mu\text{mol}$  vs  $1.772 \pm 0.078 \mu\text{mol}$  for TBARS  $P < 0.05$ ;  $4.36 \pm 0.39 \text{ mmol}$  vs  $7.48 \pm 0.40 \text{ mmol}$  for carbonyl contents  $P < 0.05$ ) and decrease in NO generation ( $11.334 \pm 0.467 \mu\text{mol}$  vs  $7.978 \pm 0.334 \mu\text{mol}$   $P < 0.01$  for NOx;  $8.986 \pm 1.351 \mu\text{mol}$  vs  $6.854 \pm 0.460 \mu\text{mol}$  for nitrotyrosine  $P < 0.01$ ) and nNOS activity (decreased to 43%  $P < 0.05$ ). This function was abolished by the co-administration of quercetin.

**CONCLUSION:** The antioxidant action of quercetin relies, in part, on its ability to stimulate nNOS and enhance production of NO that would interact with endogenously produced reactive oxygen to inhibit hyper-proliferation of gastric mucosal cells in rats treated with chronic oral ethanol.

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**Key words:** Quercetin; Cell proliferation; Reactive oxygen species; Nitric oxide; Gastric mucosa; Ethanol

**Peer reviewer:** Serhan Karvar, MD, Assistant Professor of Medicine, University of Southern California, Keck School of Medicine, Division of Gastrointestinal & Liver Diseases, 2011 Zonal Avenue, HMR 101, Los Angeles, CA 90089, United States

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### Abstract

**AIM:** To investigate the effect of quercetin (3,3',4',5,7-pentahydroxy flavone), a major flavonoid in human diet, on hyper-proliferation of gastric mucosal cells in rats treated with chronic oral ethanol.

**METHODS:** Forty male Sprague-Dawley rats, weighing 200-250 g, were randomly divided into control group (tap water *ad libitum*), ethanol treatment group (6 mL/L ethanol), quercetin treatment group (intragastric gavage with 100 mg/kg of quercetin per day), and ethanol plus quercetin treatment group (quercetin and 6 mL/L ethanol). Expression levels of proliferating cell nuclear antigen (PCNA) and Cyclin D1 were detected by Western blot to assay gastric mucosal cell proliferation in rats. To demonstrate the influence of quercetin on the production of extra-cellular reactive oxygen species/nitrogen species (ROS/RNS) in rats, changes in levels of thiobarbituric acid reactive substance (TBARS), protein carbonyl, nitrite and nitrate (NOx) and nitrotyrosine (NT) were determined. The activity of inducible nitric oxide synthase (NOS) including iNOS and nNOS was also detected by Western blot.

**RESULTS:** Compared to control animals, cell proliferation

### INTRODUCTION

Chronic ethanol consumption is a major risk factor for oropharyngeal, esophageal, and rectal cancer<sup>[1]</sup>. Chronic ethanol consumption resulting in gastric mucosal lesions might thus be expected to influence the kinetic balance between cell proliferation and cell death. Because hyper-



regenerative gastrointestinal mucosa has an increased susceptibility to chemical carcinogens and thus influences carcinogenesis. Various studies have been performed to evaluate the effect of chronic ethanol consumption on gastric mucosal cell turnover<sup>[2,3]</sup>. However, the role of ethanol in the altered cell proliferation in rat stomach remains poorly understood. There is evidence that alcohol is involved in gastric mucosa oxidant injury as studies showed that ethanol-induced damage can be prevented if antioxidant treatment or therapy is given concurrently or prior to alcohol exposure<sup>[4-6]</sup>. Previous studies in our laboratory found that cell proliferation is enhanced in gastric mucosa of rats treated with ethanol in a dose- and time-dependent manner<sup>[7]</sup>. These findings indicate that ethanol-associated gastric cell proliferation may involve oxidative stress<sup>[8]</sup>.

Oxidative stress occurs when there is a significant imbalance between generation of reactive oxygen species (ROS) and nitrogen species (RNS) and its clearance by antioxidant defenses<sup>[9]</sup>. 3, 3', 4', 5, 7-pentahydroxy flavone (quercetin) is a potent bioflavonoid widely distributed throughout vegetables and fruits. It was reported that quercetin has many beneficial effects on human health, including cardiovascular protection, anticancer activity, anti-ulcer effects, anti-allergy activity, cataract prevention, antiviral activity and anti-inflammatory effects<sup>[10,11]</sup>. These effects of quercetin due to its antioxidant properties of potent anti-oxidant, scavenge free radicals directly<sup>[12]</sup>, inhibit xanthine oxidase and lipid peroxidation<sup>[13,14]</sup>, and alter the anti-oxidant defense pathway *in vivo* and *in vitro*<sup>[15]</sup>. It was recently reported that quercetin inhibits oxidative damage in ethanol-induced gastric lesions of rats<sup>[16]</sup>.

In light of these findings, we hypothesized that quercetin has an effect on gastric mucosa cell proliferation in rats that chronically administer ethanol involving inhibition of the ROS-nitric oxide (ROS-NO) pathway. To establish the potential antiproliferative mechanism of quercetin, we detected the expression levels of proliferating cell nuclear antigen (PCNA) and Cyclin D1, which are significantly associated with gastric mucosal cell proliferation in rats. To demonstrate the influence of quercetin on the production of extra-cellular ROS/RNS, changes in thiobarbituric acid reactive substance levels (TBARS) as an index of lipid peroxidation, protein carbonyl content as a marker of free radical-mediated modification of proteins, nitrite and nitrate (NOx) and nitrotyrosine (NT) levels as the marker of NO production, were also determined.

## MATERIALS AND METHODS

### Animals and treatment protocol

Male Sprague-Dawley rats, weighing 200-250 g, were used in this study. Twenty-four rats were housed in plastic cages in an air-conditioned and light controlled room at  $24 \pm 2^\circ\text{C}$  and  $60\% \pm 5\%$  humidity. The study protocol was approved by the Nanjing Medical University Animal Care and Use Committee. After a 3 d adaptation period, the rats were randomly divided into

four groups (6 in each group). Group 1 had free access to tap water, group 2 had drinking water containing 6 mL/L ethanol as previously described<sup>[7]</sup>, group 3 was given 50 mg/kg quercetin (Sigma, St Louis, MO, USA) by intragastric gavage twice a day, group 4 was given 50 mg/kg quercetin by intragastric gavage twice a day and 6 mL/L ethanol. Quercetin was dissolved using DMSO as the vehicle and diluted in PBS to 2 mL, with the maximum concentration of DMSO being 0.1%. As controls, animals in groups 1 and 2 were also treated with 2 mL 0.1% DMSO, twice a day. The time and doses of ethanol and quercetin treatment were determined on the basis of results from our preliminary experiment. The mean ethanol consumption was 6.52 g/kg body weight per day, the mean plasma ethanol concentration at the time of stomach excision was 18.47 mmol/L in animals of groups 3 and 4. The rats were anesthetized with urethane and sacrificed after 7 d. Their stomachs were dissected and used for this study.

### Cell proliferation assay

Nuclear extracts from gastric mucosa were prepared using a nuclear extract kit (Active Motif Japan, Japan) following the instructions of its manufacturer. PCNA and Cyclin D1 detected by Western blot were applied to determination of gastric mucosal cell proliferation in rats.

### Lipid peroxidation

To evaluate the extent of lipid peroxidation, the amount of thiobarbituric acid reactive substances (TBARS) in gastric tissue, a measurement of the extent of lipid peroxidation, was detected with the modified thiobarbituric acid (TBA) method<sup>[17,18]</sup>. Each sample was homogenized in a 1.15% KCl solution containing 10 mmol/L deferoxamine, 0.04% butylated hydroxytoluene (BHT), and 2% ethanol. Each homogenate was incubated for 60 min at  $95^\circ\text{C}$  in an oil bath with a stock TCA-TBA-HCl reagent consisting of 15% (w/v) trichloroacetic acid, 0.375% (w/v) thiobarbituric acid, 0.25 mol/L hydrochloric acid and 2% BHT. After cooling, the precipitate was removed by centrifugation, and the extinction coefficient of the supernatant at 535 nm was determined spectrophotometrically and compared with a known TBARS standard.

### Protein oxidation

Protein carbonyls in gastric tissues were determined by spectrometric DNPH assay according to Fagan *et al* with minor modifications<sup>[19]</sup>. Briefly, gastric tissues were homogenized by sonication in a lysis buffer containing PBS (pH 7.2), 1% Triton X-100, 1 mmol/L EDTA and 1X protease inhibitor cocktail and removal of insoluble cellular debris was performed by centrifugation. Aliquots in protein samples were precipitated with 10 volumes of HCl-acetone (3:100) and washed with 5 mL of 10% TCA solution. Pellets were re-suspended in 500  $\mu\text{L}$  buffer solution and reacted with 500  $\mu\text{L}$  of 10 mmol/L DNPH (in 2 mol/L HCl) by vortexing for 15 min. To remove the un-reacted DNPH, the centrifuged pellets were washed with 5 mL of 20% TCA and 5 mL of ethanol: ethylacetate mixture (v/v = 1:1). The

final precipitate was resolved in 1 mL of 6 mol/L guanidine HCl, and the absorbance at 380 nm was determined for the sample treated with DNPH and HCl, which was subtracted as a background and compared with a known protein carbonyl standard.

#### **Nitric oxide (NO) assay**

The amount of stable nitrite (nitrite and nitrate), the end product of NO in gastric mucosa, was determined by colorimetric assay as described previously<sup>[20]</sup>. Briefly, 50  $\mu$ L of gastric mucosa homogenate was mixed with an equal volume of Griess reagent consisting of 1% sulfanilamide, 0.1% naphthyl ethylenediamine dihydrochloride and 2.5%  $\text{H}_3\text{PO}_4$ , and incubated at room temperature for 10 min. The absorbance was read at 540 nm on a microplate reader (Elx800, Bio-TEK Ins, USA). The amount of nitrite was calculated from a  $\text{NaNO}_2$  standard curve.

#### **Measurement of nitrotyrosine (NT) levels**

Gastric mucosa was homogenized on ice in the prepared solution (20 mmol/L Tris-HCl containing 1% NP-40, 100 mmol/L NaF, 137 mmol/L NaCl, 5 mmol/L EDTA, 0.1 mol/L PMSF, 1% proteinase inhibitor, and 10% glycerol, pH 7.5) for 30 min at 4°C. The homogenate was centrifuged at 12000 r/min for 20 min to remove cellular debris. Protein concentration was determined using a BCA protein assay reagent kit.

Nitrotyrosine levels were quantified as previously described<sup>[21]</sup>. In short, assay was performed in 96-well plates coated with 5 mg/L of nitrotyrosine-BTG conjugate, which was blocked with gelatin to prevent non-specific binding. A standard curve was plotted by incubating serial dilutions of NT with biotin labeled anti-nitrotyrosine Fab' in PBS containing 0.1% gelatin for 1 h. Subsequently, plates were incubated with a streptavidin peroxidase conjugate followed by o-phenylenediamine (OPD). The reaction was terminated after 20-30 min by addition of 4 mol/L  $\text{H}_2\text{SO}_4$ . Data on the standard curve were fitted to a logistic plot and the levels of NT were measured. All samples and standards were assayed in triplicate.

Anti-nitrotyrosine monoclonal antibody used in ELISA was a kind gift from Dr. Yang TB (Institute of Space Medico-Engineering, Beijing, China). The study of cross-reaction with nitrotyrosine-like compounds showed that the antibodies have a high specificity for NT<sup>[21]</sup>.

#### **Measurement of neuronal and inducible NO synthase (nNOS and iNOS) levels**

The stomach was homogenized on ice in a buffer containing 50 mmol/L Tris-Cl, 150 mmol/L NaCl, 0.02%  $\text{NaN}_2$ , 100 mg/L phenylmethanesulfonyl fluoride, 1 mg/L aprotinin, and 1% Triton X-100. Lysates were centrifuged at 12000 r/min for 25 min at 4°C. The supernatant was used for nNOS and inducible NOS (iNOS) determined by Western blot analysis.

#### **Western blot analysis**

Proteins were detected by the Bradford method using

bovine serum albumin as a standard. An equal amount of 40  $\mu$ g protein from each sample was run per lane on 10% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted to nitrocellulose membranes. The membranes were blocked by overnight incubation in 5% dry milk at 4°C, and thereafter incubated with primary antibodies (1:200-1000 dilution) for 3 h at room temperature. Each blot was probed with monoclonal anti-PCNA, anti-Cyclin D1 (Santa Cruz Biotech, USA), polyclonal anti- $\beta$ -actin (Upstate, USA), anti-nNOS, and anti-iNOS (Santa Cruz Biotech, USA). The membranes were washed and incubated with horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (1:2000 dilution) (Upstate, USA) for 1 h. Immune complexes were visualized with an ECL kit (Pierce; Rockford, IL, USA) according to the manufacturer's protocol. Signal intensity was quantified using a Bio-Rad image analysis system and the results were normalized to the signal intensity of  $\beta$ -actin for each blot.

#### **Immunohistochemical analysis**

Stomachs were excised from three rats in each group and fixed in 10% neutral buffered formalin, embedded in paraffin and sectioned at 5  $\mu$ m for immunohistochemical staining. Staining was performed according to the routine standard procedures. Briefly, the sections were deparaffinized in xylene, cleared in graded ethanol to PBS, then quenched in 3% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) containing 0.1% sodium azide to suppress the endogenous peroxidase activity and placed in 10 mmol/L citrate buffer (pH 6.0) for 15 min at 100°C for antigen retrieval. A routine streptavidin-biotin protocol using the DAKO LSAB + kits (Dako Japan, Kyoto, Japan) was applied. The tissue sections mounted on glass slides were incubated in PBS containing 0.5% BSA to reduce nonspecific protein binding, and sequentially incubated to react with monoclonal anti-PCNA primary antibody overnight at 4°C. The antibody was then linked with streptavidin conjugated to horseradish peroxidase (HRP). HRP sites were visualized with 3,3'-diaminobenzidine (DAB) and  $\text{H}_2\text{O}_2$ , counterstained with hematoxylin. The presence of PCNA was detected by light field microscopy as a dark brown reaction product in cell nuclei. Some sections were reacted with normal mouse IgG instead of the specific antibody as a negative control. An image analysis system (NYD100) was used for quantitative analysis of cell density (cell number/view field) of the PCNA-positive cells in the rat stomach. Four sections from four rats were used. PCNA-positive cells per section were counted in five randomly selected view fields at a magnification of  $\times 400$ . At least, 20 fields in each group were analyzed.

#### **Statistical analysis**

All experiments were done in triplicate and stomach tissues were excised from three rats in each group. One-way analysis of variance was used to estimate the overall significance followed by *post hoc* Tukey's test corrected for multiple comparisons. Data are presented as mean  $\pm$  SD.  $P < 0.05$  was considered statistically significant.

## RESULTS

### **Quercetin treatment could partially prevent ethanol-induced cell proliferation in gastric mucosa**

PCNA is a polypeptide that specifically increases in nuclei during G1 and S phases of the cell cycle. It is considered to be an essential cofactor for the activation of DNA polymerase during DNA replication. Therefore, PCNA-positive nuclei indicate that cells replicate DNA and undergo proliferation. It is well known that Cyclin D1 promotes G1 phase progression. The levels of PCNA and Cyclin D1 were higher in gastric mucosa exposed to 6% ethanol for 7 d than in normal control rats, while the expression of PCNA and Cyclin D1 was reduced after treatment with quercetin in this study (Figure 1). PCNA immunohistochemistry and computer image analysis showed, a significantly increased number of PCNA positive cells in the fundic gland of rats treated with ethanol for 7 d. The number of PCNA positive cells in ethanol + quercetin and quercetin treated rats was very analogous to that in the control rats (Figure 2, Table 1).

### **Quercetin treatment could prevent ethanol-induced lipid peroxidation and protein oxidation in gastric mucosa**

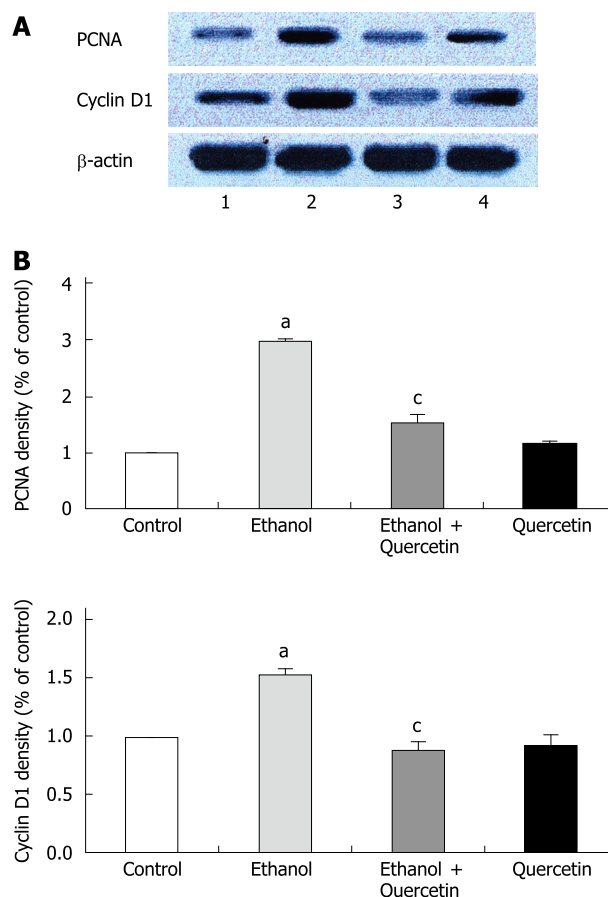
As TBARS shown in Figure 3, ethanol-induced ROS may increase lipid peroxidation. Quantitative measurement of TBARS in gastric mucosa revealed a significant effect of ethanol treatment on ethanol-induced lipid peroxidation and protein oxidation in gastric mucosa ( $1.772 \mu\text{mol/g protein}$ ) compared to the normal control rats ( $1.298 \mu\text{mol/g protein}$ ), which was reduced to  $1.500 \mu\text{mol/g protein}$  ( $P < 0.05$ ). TBARS was slightly decreased in the rats treated with quercetin (Figure 3A), suggesting that quercetin can decrease lipid peroxidation in gastric mucosa. The mean values of carbonyl contents in gastric tissue are shown in Figure 3B, revealing a similar pattern of TBARS in each group of rats.

### **Quercetin treatment could prevent ethanol-induced decrease in nitrite/nitrate content in gastric mucosa**

The nitrite/nitrate content in gastric mucosa was determined using the Griess method. As shown in Table 1, the nitrite/nitrate content in the group treated with 6% ethanol for 7 d was significantly lower than that in the control group ( $P < 0.01$ ) and significantly higher in rats treated with combined ethanol and quercetin than that in rats treated with ethanol only ( $P < 0.01$ ). The gastric nNOS level was slightly increased in rats treated with quercetin, suggesting that quercetin treatment can prevent ethanol-induced decrease of nitrite/nitrate content in rat gastric mucosa.

### **Quercetin treatment could prevent ethanol-induced decrease in nNOS levels**

NO produced by nNOS was detected by Western blot in gastric mucosa (Figure 4). Quantitative analysis revealed a significant effect of ethanol treatment on ethanol-induced decrease in nNOS levels. The gastric nNOS level

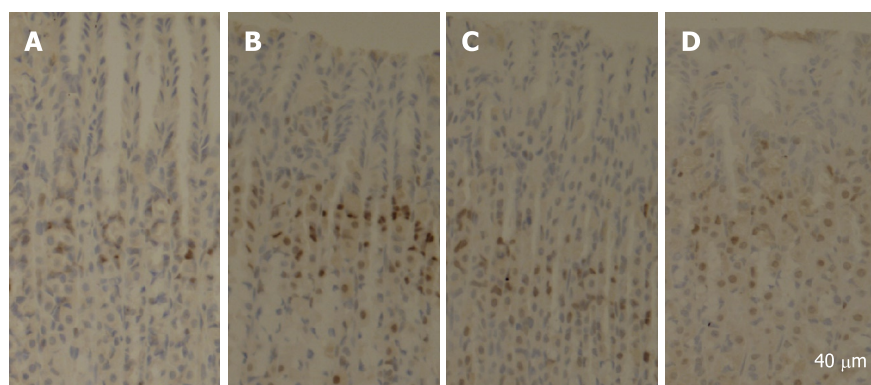


**Figure 1** Immunoblotting of nuclear extracts from gastric mucosa with antibodies to PCNA and Cyclin D1 in the 4 groups as indicated in lanes 1-4 (A) and values normalized by arbitrarily setting the densitometry of control to 1.0 (B).  $\beta$ -actin staining was performed to ensure an equal loading. The results indicated are in percentage above the control value and are representative of four independent experiments.  $^aP < 0.05$  vs control animals,  $^cP < 0.05$  vs ethanol-treated animals.

in rats treated with combined ethanol and quercetin was significantly higher than that in rats treated with ethanol only. The gastric nNOS level was slightly increased in rats treated with quercetin, suggesting that quercetin can prevent ethanol-induced decrease in nNOS, which is in agreement with the data on nitrite/nitrate (Table 1) in rat gastric mucosa. No iNOS expression was detected in each group.

### **Quercetin treatment could prevent ethanol-induced decrease in protein-bound 3-NT**

Ultimately, increased NO, nitrite/nitrate, and peroxynitrite resulted in production of protein-bound 3-NT in gastric mucosa (Table 1). Quantitative analysis revealed a significant effect of ethanol on ethanol-induced decrease in protein-bound 3-NT. Gastric 3-NT levels in rats treated with combined ethanol and quercetin were significantly higher than those in rats treated with ethanol only. The level of 3-NT was slightly increased in rats not treated with ethanol, suggesting that quercetin treatment can prevent ethanol-induced decrease in 3-NT, which is in agreement with the data on nitrite/nitrate (Table 1) and nNOS level in rat gastric mucosa (Figure 4).

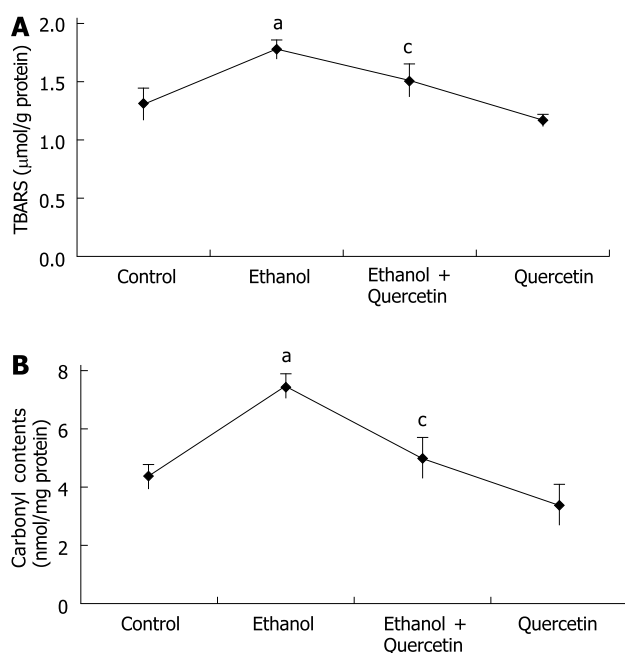


**Figure 2** Staining of PCNA from rats in the 4 groups, respectively (A-D). Stem cells at the neck position were positively stained, while other cells were negatively stained. A significantly increased number of PCNA positive cells were observed in the fundic gland of rats treated with ethanol for 7 d. **A:** Control; **B:** Ethanol; **C:** Ethanol + Quercetin; **D:** Quercetin.

**Table 1** Number of PCNA positive cells and levels of NO and NT in rat gastric mucosa (mean  $\pm$  SD)

	Control	Ethanol	Ethanol + Quercetin	Quercetin
PCNA	21.6 $\pm$ 0.8	42.3 $\pm$ 0.7 <sup>b</sup>	37.1 $\pm$ 0.4 <sup>a</sup>	18.6 $\pm$ 0.6
NOx (μmol/L)	11.334 $\pm$ 0.467	7.978 $\pm$ 0.334 <sup>b</sup>	9.889 $\pm$ 0.620 <sup>a</sup>	12.098 $\pm$ 0.516
Nitrotyrosine (μmol/L)	8.986 $\pm$ 1.351	6.854 $\pm$ 0.460 <sup>b</sup>	8.071 $\pm$ 1.208 <sup>a</sup>	10.875 $\pm$ 1.034

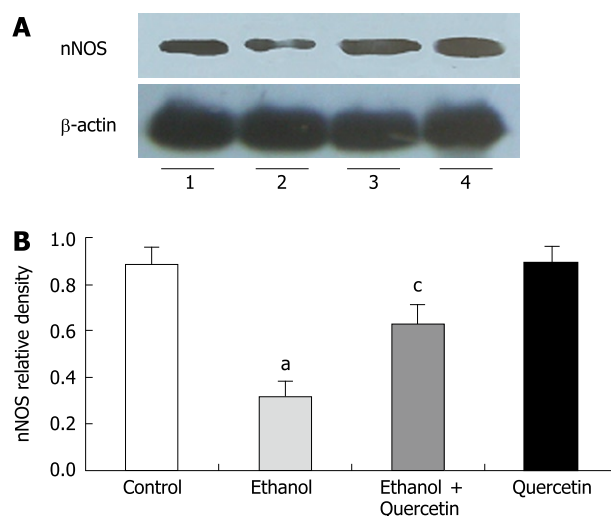
<sup>a</sup>*P* < 0.05 vs ethanol group, <sup>b</sup>*P* < 0.01 vs control group.



**Figure 3** Lipid peroxidation (A) and protein oxidation (B) determined in gastric mucosa of rats after treatment with different agents. The data are expressed as mean  $\pm$  SD of four independent experiments. <sup>a</sup>*P* < 0.05 vs control animals, <sup>c</sup>*P* < 0.05 vs ethanol-treated animals.

## DISCUSSION

ROS, such as superoxide anion radical ( $O_2^{\cdot-}$ ), hydroxyl radical ( $OH^{\cdot}$ ), lipid peroxidation and nitric oxide (NO), are involved both in the regulation of cell proliferation and apoptosis and in macromolecular damage to gastric cells, leading to increased oxidative stress and stress-induced senescence<sup>[22,23]</sup>. ROS are oxygen-containing



**Figure 4** Immunoblotting of gastric homogenate with the antibody to nNOS in different treatment groups as indicated in lanes 1-4 (A) and values normalized by arbitrarily setting the densitometry of actin (B). The results indicated are in percentage above the control value and are representative of the four independent experiments. <sup>a</sup>*P* < 0.05 vs control animals, <sup>c</sup>*P* < 0.05 vs ethanol-treated animals.

molecules having either unpaired electrons or ability to abstract electrons from other molecules. Lipids are modified by ROS and visualized as a thiobarbituric acid-reactive substance (TBARS). Oxidative damage to proteins generates increased carbonyl groups due to oxidation of sensitive amino acids, such as histidine, proline, arginine and lysine<sup>[24]</sup>. We measured the TBARS and protein carbonyls to serve as an indicator for intracellular oxidation in gastric mucosa. NO formation in cells is rapidly converted to nitrite. After reducing nitrate to nitrite with bacterial nitrate reductase, nitrite levels can be determined as an indicator for NO synthesis based on the Griess reaction<sup>[20]</sup>.  $O_2^{\cdot-}$  reacts with NO to produce peroxynitrite ( $ONOO^{\cdot}$ ), which is considered a more powerful oxidant than  $O_2^{\cdot-}$ <sup>[25]</sup>, entering the cells rapidly. A variety of nitrate macromolecules are chiefly at the aromatic rings<sup>[26]</sup>. The nitration of tyrosyl residues on proteins is considered the stable “foot print” of RNS stress both *in vitro* and *in vivo*<sup>[27]</sup>. In this study, we also measured the levels of  $NO_x$  and 3-NT to provide an index of NO in gastric mucosa. Using these indicators, the effect of quercetin on chronic ethanol-induced generation of ROS and NO was detected.



The present study demonstrated a clear enhancement of cell proliferation in gastric mucosa of animals subjected to ethanol treatment for 7 d, which is similar to that in our previous studies<sup>[7]</sup>. Since PCNA and Cyclin D1 were strongly up-regulated (Figure 1), and the number of PCNA positive cells was increased in gastric mucosa (Figure 2 and Table 1). This enhancement function was accompanied with an increase in ROS generation and abolished by co-administration of quercetin and ethanol, which was accompanied with a decrease in ROS level. Quercetin has the ability to directly block the cell cycle at the G1/S transition in colon and gastric cancer cells<sup>[28]</sup> as well as in human leukemic T cells<sup>[29]</sup>. However, the protein levels of PCNA and Cyclin D1 were similar to the control values irrespective of quercetin administration alone in our study. These results show that an excessive amount of ROS can induce enhanced cell proliferation in gastric mucosa of rats *in vivo*.

In addition to ROS, RNS in the form of NO has also been implicated in regulation of cellular proliferation, but its role as a proliferative signal is not well defined, because it appears to depend on the cell type responsible for its release and the NOS isoforms within cells, as well as on the concentration of released NO and the composition of intracellular milieu<sup>[30,31]</sup>. The neuronal and endothelial isoforms are thought to be responsible for production of low levels of NO<sup>[32]</sup> and both isoforms have been identified in gastric mucosa<sup>[33,34]</sup>. NO is a lipophilic radical, which can exert beneficial effects by reacting with O<sub>2</sub> when produced in a small amount and, in this manner, behaves as an antioxidant. A low level of NO could protect against ROS and inhibit gastric cancer cell proliferation<sup>[23]</sup>. NO donors retard gastric wound healing by inhibiting cell migration and proliferation and inducing cell apoptosis in a dose- and time-dependent manner<sup>[35]</sup>. However, excess NO produced by inducible NOS (iNOS) plays a potent role as a cytotoxic agent during infection and inflammation, with essential involvement of chronic inflammation, especially increased rates of cell proliferation, in *H pylori*-associated glandular stomach carcinogenesis<sup>[36]</sup>. Suppression of NO generation by iNOS inhibitors (aminoguanidine, AG) could also suppress cancer cell proliferation in gastric cancer xenografts<sup>[37]</sup>.

In the present study, iNOS expression was not detectable in gastric mucosa. After treatment with 6% ethanol for 7 d, the expression of nNOS and the levels of NOx (nitrite/nitrate) and NT in gastric homogenates were decreased, suggesting that the nNOS activity is decreased. These results are consistent with the reported data<sup>[38]</sup>. Surprisingly, the decreased nNOS activity could be abolished by co-administration of quercetin and ethanol. Without further examination, we cannot rule out the mechanism of quercetin-enhanced activity of nNOS. It was reported that resveratrol, another kind of flavonoids, can inhibit gastric cancer cell proliferation by stimulating the activity of NOS *in vitro*<sup>[23]</sup>, suggesting that quercetin may play a role as resveratrol in the inhibition of gastric cell proliferation *in vivo*.

In conclusion, our findings indicate that the antioxi-

dant action of quercetin resides depends in part, on its ability to stimulate nNOS and increase production of NO that would interact with endogenously produced reactive oxygen to inhibit hyper-proliferation of gastric mucosal cells in rats that have chronic ethanol consumption.

## COMMENTS

### Background

Chronic ethanol consumption resulting in hyper-regenerative gastrointestinal mucosa has an increased susceptibility to chemical carcinogens and thus influences carcinogenesis. Some studies indicate that ethanol-associated gastric cell proliferation may involve oxidative stress. It was reported that quercetin, a 3,3',4',5,7-pentahydroxy flavone, has effects on oxidative damage to ethanol-induced gastric lesions due to its antioxidant properties of potent anti-oxidant. We hypothesized that quercetin has an effect on gastric mucosal cell proliferation in rats that have chronic ethanol consumption, thus inhibiting gastric cancer.

### Research frontiers

In this study, we developed an animal model by continuous ethanol ingestion for 7 d. By using this model, we investigated the relationship between chronic ethanol intake and gastric mucosal cell proliferation, which is related to reactive oxygen species (ROS) and reactive nitrogen species (RNS).

### Innovations and breakthroughs

Our findings indicate that the antioxidant action of quercetin resides, depends in part, on its ability to stimulate nNOS and increase production of NO that would interact with endogenously produced reactive oxygen to inhibit gastric mucosal cell proliferation in rats that have chronic ethanol consumption.

### Applications

This animal model established by continuous ethanol ingestion for 7 d is a useful tool for studying the mechanism of gastric mucosal cell proliferation *in vivo*. We will investigate the signal transduction of ROS in gastric mucosal cell proliferation.

### Terminology

ROS are oxygen-containing molecules having either unpaired electrons or ability to abstract electrons from other molecules. Reactive RNS are forms of NO.

### Peer review

This is an interesting paper, in which the authors showed that ethanol could induce gastric mucosal cell proliferation in their animal model. The ROS/RNS pathway may be involved. Further study is needed to show signal transduction of ROS in gastric mucosal cell proliferation.

## REFERENCES

- Seitz HK, Maurer B, Stickel F. Alcohol consumption and cancer of the gastrointestinal tract. *Dig Dis* 2005; **23**: 297-303
- Franke A, Teyssen S, Singer MV. Alcohol-related diseases of the esophagus and stomach. *Dig Dis* 2005; **23**: 204-213
- Kountouras J, Chatzopoulos D, Zavos C. Reactive oxygen metabolites and upper gastrointestinal diseases. *Hepatogastroenterology* 2001; **48**: 743-751
- La Casa C, Villegas I, Alarcon de la Lastra C, Motilva V, Martin Calero MJ. Evidence for protective and antioxidant properties of rutin, a natural flavone, against ethanol induced gastric lesions. *J Ethnopharmacol* 2000; **71**: 45-53
- Santos FA, Rao VS. 1,8-cineol, a food flavoring agent, prevents ethanol-induced gastric injury in rats. *Dig Dis Sci* 2001; **46**: 331-337
- Bilici D, Suleyman H, Banoglu ZN, Kiziltunc A, Avci B, Ciftcioglu A, Bilici S. Melatonin prevents ethanol-induced gastric mucosal damage possibly due to its antioxidant effect. *Dig Dis Sci* 2002; **47**: 856-861
- Ge YB, Du J, Fan LL, Li YC, Gu L. Chronic ethanol feeding alters the epithelial cell proliferation and apoptosis in rat gastric mucosa. *Histol Histopathol* 2007; **22**: 185-190
- Hernandez-Munoz R, Montiel-Ruiz C, Vazquez-Martinez O. Gastric mucosal cell proliferation in ethanol-induced chronic mucosal injury is related to oxidative stress and lipid

- peroxidation in rats. *Lab Invest* 2000; **80**: 1161-1169
- 9 **Forman HJ**, Torres M, Fukuto J. Redox signaling. *Mol Cell Biochem* 2002; **234-235**: 49-62
  - 10 **Bronner C**, Landry Y. Kinetics of the inhibitory effect of flavonoids on histamine secretion from mast cells. *Agents Actions* 1985; **16**: 147-151
  - 11 **Reutrakul V**, Ningnuek N, Pohmakotr M, Yoosook C, Napaswad C, Kasisit J, Santisuk T, Tuchinda P. Anti HIV-1 flavonoid glycosides from *Ochna integerrima*. *Planta Med* 2007; **73**: 683-688
  - 12 **Hanasaki Y**, Ogawa S, Fukui S. The correlation between active oxygens scavenging and antioxidative effects of flavonoids. *Free Radic Biol Med* 1994; **16**: 845-850
  - 13 **Plumb GW**, Price KR, Williamson G. Antioxidant properties of flavonol glycosides from green beans. *Redox Rep* 1999; **4**: 123-127
  - 14 **Fiorani M**, De Sanctis R, Menghinello P, Cucchiari L, Cellini B, Dacha M. Quercetin prevents glutathione depletion induced by dehydroascorbic acid in rabbit red blood cells. *Free Radic Res* 2001; **34**: 639-648
  - 15 **Morand C**, Crespy V, Manach C, Besson C, Demigne C, Remesy C. Plasma metabolites of quercetin and their antioxidant properties. *Am J Physiol* 1998; **275**: R212-R219
  - 16 **Kahraman A**, Erkasap N, Koken T, Serteser M, Aktepe F, Erkasap S. The antioxidative and antihistaminic properties of quercetin in ethanol-induced gastric lesions. *Toxicology* 2003; **183**: 133-142
  - 17 **Buege JA**, Aust SD. Microsomal lipid peroxidation. *Methods Enzymol* 1978; **52**: 302-310
  - 18 **Ambrosio G**, Flaherty JT, Duilio C, Tritto I, Santoro G, Elia PP, Condorelli M, Chiariello M. Oxygen radicals generated at reflow induce peroxidation of membrane lipids in reperfused hearts. *J Clin Invest* 1991; **87**: 2056-2066
  - 19 **Fagan JM**, Slecicka BG, Sohar I. Quantitation of oxidative damage to tissue proteins. *Int J Biochem Cell Biol* 1999; **31**: 751-757
  - 20 **Grisham MB**, Johnson GG, Gautreaux MD, Berg RD. Measurement of nitrate and nitrite in extracellular fluids: A window to systemic nitric oxide metabolism. In: Everse J, Grisham MB (editors). *Methods: A Companion to Methods in Enzymology*. San Diego: Academic Press, 1995: 84-90
  - 21 **Qu LN**, Yang TB, Yuan YH, Zhong P, Yang B, Zhao H. A novel competitive ELISA for both free and protein-bound nitrotyrosine. *Hybrid Hybridomics* 2003; **22**: 401-406
  - 22 **Kim H**. Oxidative stress in *Helicobacter pylori*-induced gastric cell injury. *Inflammopharmacology* 2005; **13**: 63-74
  - 23 **Holian O**, Wahid S, Atten MJ, Attar BM. Inhibition of gastric cancer cell proliferation by resveratrol: role of nitric oxide. *Am J Physiol Gastrointest Liver Physiol* 2002; **282**: G809-G816
  - 24 **Young J**, McKinney SB, Ross BM, Wahle KW, Boyle SP. Biomarkers of oxidative stress in schizophrenic and control subjects. *Prostaglandins Leukot Essent Fatty Acids* 2007; **76**: 73-85
  - 25 **Huie RE**, Padmaja S. The reaction of NO with superoxide. *Free Radic Res Commun* 1993; **18**: 195-199
  - 26 **Beckman JS**, Chen J, Ischiropoulos H, Crow JP. Oxidative chemistry of peroxynitrite. *Methods Enzymol* 1994; **233**: 229-240
  - 27 **Radi R**, Peluffo G, Alvarez MN, Naviliat M, Cayota A. Unraveling peroxynitrite formation in biological systems. *Free Radic Biol Med* 2001; **30**: 463-488
  - 28 **Yoshida M**, Sakai T, Hosokawa N, Marui N, Matsumoto K, Fujioka A, Nishino H, Aoike A. The effect of quercetin on cell cycle progression and growth of human gastric cancer cells. *FEBS Lett* 1990; **260**: 10-13
  - 29 **Yoshida M**, Yamamoto M, Nikaido T. Quercetin arrests human leukemic T-cells in late G1 phase of the cell cycle. *Cancer Res* 1992; **52**: 6676-6681
  - 30 **Lane P**, Gross SS. Cell signaling by nitric oxide. *Semin Nephrol* 1999; **19**: 215-229
  - 31 **Wallace JL**, Miller MJ. Nitric oxide in mucosal defense: a little goes a long way. *Gastroenterology* 2000; **119**: 512-520
  - 32 **Stuehr DJ**. Mammalian nitric oxide synthases. *Biochim Biophys Acta* 1999; **1411**: 217-230
  - 33 **Beck KF**, Eberhardt W, Frank S, Huwiler A, Messmer UK, Muhl H, Pfeilschifter J. Inducible NO synthase: role in cellular signalling. *J Exp Biol* 1999; **202**: 645-653
  - 34 **Akiba Y**, Nakamura M, Nagata H, Kaunitz JD, Ishii H. Acid-sensing pathways in rat gastrointestinal mucosa. *J Gastroenterol* 2002; **37** Suppl 14: 133-138
  - 35 **Kiviluoto T**, Watanabe S, Hirose M, Sato N, Mustonen H, Puolakkainen P, Ronty M, Ranta-Knuuttila T, Kivilaakso E. Nitric oxide donors retard wound healing in cultured rabbit gastric epithelial cell monolayers. *Am J Physiol Gastrointest Liver Physiol* 2001; **281**: G1151-G1157
  - 36 **Cao X**, Tsukamoto T, Nozaki K, Tanaka H, Cao L, Toyoda T, Takasu S, Ban H, Kumagai T, Tatsumatsu M. Severity of gastritis determines glandular stomach carcinogenesis in *Helicobacter pylori*-infected Mongolian gerbils. *Cancer Sci* 2007; **98**: 478-483
  - 37 **Wang GY**, Ji B, Wang X, Gu JH. Anti-cancer effect of iNOS inhibitor and its correlation with angiogenesis in gastric cancer. *World J Gastroenterol* 2005; **11**: 3830-3833
  - 38 **Nam SY**, Kim N, Lee CS, Choi KD, Lee HS, Jung HC, Song IS. Gastric mucosal protection via enhancement of MUC5AC and MUC6 by geranylgeranylacetone. *Dig Dis Sci* 2005; **50**: 2110-2120

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# Proteasome inhibitor ameliorates severe acute pancreatitis and associated lung injury of rats

Xi Chen, Shun-Le Li, Tao Wu, Ji-Dong Liu

Xi Chen, Shun-Le Li, Tao Wu, Ji-Dong Liu, Department of General Surgery, The Second Affiliated Hospital of Medical College of Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China

**Author contributions:** Chen X and Li SL contributed equally to this work; Chen X and Li SL designed the research; Li SL and Liu JD performed the research; Wu T contributed to new reagents/analytic tools; Chen X, Li SL and Liu JD analyzed the data; Chen X and Li SL wrote the paper.

**Correspondence to:** Xi Chen, Department of General Surgery, The Second Affiliated Hospital of Medical College of Xi'an Jiaotong University, Xi'an 710004, Shaanxi Province, China. 2002chenxi@163.com

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**Peer reviewers:** Parimal Chowdhury, Professor, Department of Physiology and Biophysics, College of Medicine, University of Arkansas for Medical Sciences, 4301 W Markham Street Little Rock, Arkansas 72205, United States; Yoshiharu Motoo, MD, PhD, FACP, FACG, Professor and Chairman, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

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## Abstract

**AIM:** To observe the effect of proteasome inhibitor MG-132 on severe acute pancreatitis (SAP) and associated lung injury of rats.

**METHODS:** Male adult SD rats were randomly divided into SAP group, sham-operation group, and MG-132 treatment group. A model of SAP was established by injection of 5% sodium taurocholate into the biliary-pancreatic duct of rats. The MG-132 group was pretreated with 10 mg/kg MG-132 intraperitoneally (ip) 30 min before the induction of pancreatitis. The changes in serum amylase, myeloperoxidase (MPO) activity of pancreatic and pulmonary tissue were measured. The TNF- $\alpha$  level in pancreatic cytosolic fractions was assayed with an enzyme-linked immunosorbent assay (ELISA) kit. Meanwhile, the pathological changes in both pancreatic and pulmonary tissues were also observed.

**RESULTS:** MG-132 significantly decreased serum amylase, pancreatic weight/body ratio, pancreatic TNF- $\alpha$  level, pancreatic and pulmonary MPO activity ( $P < 0.05$ ). Histopathological examinations revealed that pancreatic and pulmonary samples from rats pretreated with MG-132 demonstrated milder edema, cellular damage, and inflammatory activity ( $P < 0.05$ ).

**CONCLUSION:** The proteasome inhibitor MG-132 shows a protective effect on severe acute pancreatitis and associated lung injury of rats.

## INTRODUCTION

Acute pancreatitis (AP) is a common clinical disease and its incidence has been increasing in recent years. Although most patients experience mild and self-limited AP, some patients have severe acute pancreatitis (SAP)<sup>[1-3]</sup>. Abnormal activation of digestive enzymes within pancreatic acinar cells is thought to be a critical initiating event<sup>[4]</sup>. Pancreatic injury leads to a localized and a subsequent systemic inflammatory response which determines the severity of pancreatitis. It may lead to the development of multiple organ dysfunction syndrome (MODS), which is responsible for the mortality rate associated with this disease. The major component of MODS is lung injury, clinically manifested as acute respiratory distress syndrome<sup>[5]</sup>. So how to ameliorate the injury of pancreatic acinar cells and downstream events is the way to influence the severity of pancreatitis.

MG-132 (Z-Leu-Leu-Leu-aldehyde) is a member of the peptide aldehyde proteasome inhibitors, including calpains, cathepsins and the proteasome<sup>[6,7]</sup>. Both calpain I and proteasome can degrade I $\kappa$ B and enhance the activity of NF- $\kappa$ B<sup>[8]</sup>. Cathepsins, especially cathepsin B, has been confirmed to be a key agent for the abnormal activation of digest enzymes within pancreatic acinar cells<sup>[9]</sup>. MG-132 can also inhibit the activation of NF- $\kappa$ B by blocking I $\kappa$ B degradation and enhance the expression of heat shock proteins (HSP) that suppress the inflammatory response<sup>[10]</sup>. In the present study, we used a model of severe acute pancreatitis (SAP) established

by retrograde injection of 5% sodium taurocholate (1 mL/kg) into the pancreatic duct to observe the effect of the proteasome inhibitor MG-132 on severe acute pancreatitis and associated lung injury of rats.

## MATERIALS AND METHODS

### *Animals and materials*

Male Sprague-Dawley rats, weighing 250-300 g, were obtained from Experimental Animals Center of Medical College of Xi'an Jiaotong University. The animals were fasted overnight with free access to water and standard rat chow diet before the experiment. Five percent sodium taurocholate was purchased from Sigma, USA. MG-132 was purchased from Calbiochem, Germany. TNF- $\alpha$  ELISA kit was obtained from Genzyme, USA. Serum amylase kit and MPO kit were purchased from Nanjing Jiancheng Company, China.

### *Induction of acute pancreatitis*

The rats were randomly divided into control group, SAP group and MG-132 treatment group (SAP + MG-132), 10 in each group. The animals were anesthetized with ketamine and subjected to a midline laparotomy. A blunt needle was inserted transduodenally into the common biliary-pancreatic duct as previously described<sup>[11]</sup>. The hepatic duct was closed at the hilum of the liver with a bulldog clamp to prevent backflow. Sodium taurocholate (5% in saline) was infused using a fine needle inserted into 5 mm of the common biliary-pancreatic duct and each rat received a total volume of 1 mL/kg body weight for 1 min. After 5 min, the needle and clamp were removed, and the laparotomy incision was closed. Animals in the treatment group were injected intraperitoneally (ip) with 10 mg/kg MG-132 dissolved in 0.25 mL dimethyl sulfoxide (DMSO). Thirty minutes later, pancreatitis was induced as above. Animals in the control group underwent a sham operation consisting of laparotomy and puncture of the duodenum, under an identical anesthesia. Six hours after duct infusion or sham operation, the animals were killed by depletion and samples were taken for study. Blood was collected by cardiac puncture using heparinized syringes, centrifuged at 4000 r/min for 10 min, and stored at 4°C for further analysis. The pancreas and lungs were carefully isolated and weighed for subsequent experiments. Tissues for histological examination were isolated, fixed in 10% formalin and embedded in paraffin for sectioning.

### *Measurement of the ratio of pancreas weight to body weight*

Changes in pancreatic weight were assessed for pancreatic interstitial edema. The whole pancreas was removed and weighed. Weight of each pancreatic sample was used to estimate the water content in pancreas as previously described<sup>[12]</sup>, which was presented as a ratio of pancreas/body weight for evaluating the consequence of pancreatic edema.

### *Water content in lung*

For quantification of lung edema, the left lung was resected,

blotted dry, and weighed (wet weight). Thereafter, the left lung was desiccated for 24 h at 80°C and weighed again (dry weight). Water content in the lung was determined by calculating the wet weight/dry weight ratio as previously described<sup>[13]</sup>.

### *Serum amylase and MPO activity*

Serum amylase was assayed with an amylase kit with a kinetic spectrophotometric method according to the manufacturer's instructions. Briefly, the methodology is based on the enzymatic degradation of ethylidene-*p*-nitrophenol-G7 by amylase coupled with glucosidase, thus producing *p*-nitrophenol which exhibits strong absorbance at 405 nm. Enzymatic activity was expressed as units/liter.

Pancreatic and lung myeloperoxidase (MPO) activity, a quantitative indicator of neutrophil infiltration, was assessed according to the instructions from Nanjing Jiancheng Company. Briefly, tissues were thawed, homogenized in a 20 mmol/L phosphate buffer (pH 7.4), centrifuged at 13000 r/min for 10 min at 4°C. The resulting pellet was re-suspended in 50 mmol/L phosphate buffer (pH 6.0) containing 0.5% hexadecyl trimethylammonium bromide. The suspension was subjected to four cycles of freezing and thawing. The sample was then centrifuged at 10000 r/min for 5 min at 4°C. The supernatant was used for MPO assay. The absorbance at 450 nm of the resulting mixture was determined. The MPO activity was expressed as U/g tissue.

### *TNF- $\alpha$ level in pancreas*

TNF- $\alpha$  level in pancreatic cytosolic fractions was measured with a commercial ELISA kit according to the instructions of its manufacturer. The absorbance was read on a microplate reader and concentrations were calculated according to the standard curve.

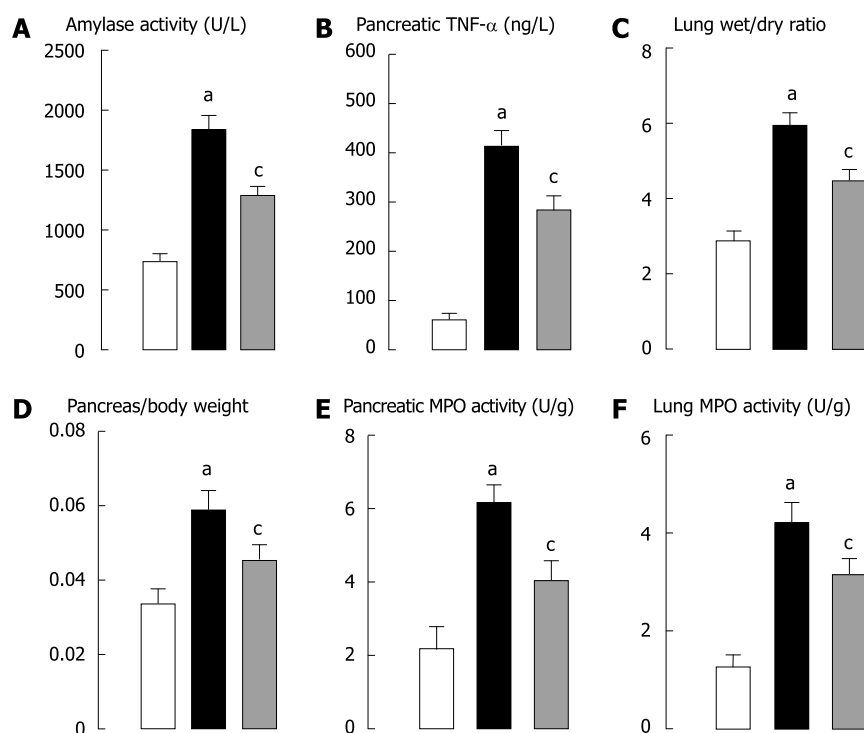
### *Histological examination*

Paraffin-embedded tissues were sectioned, stained with hematoxylin and eosin, and assessed by two different attending physicians unaware of the experimental protocol, at Department of Pathology of Xi'an Jiaotong University. The pancreas damage evaluation system was used as previously described<sup>[14]</sup>, the sections were examined and scored on a scale of 0-3 with 0 being normal and 3 being severe. Six characteristics were included, namely the presence of edema, acinar necrosis, inflammatory infiltrate, hemorrhage, fat necrosis, and perivascular inflammation. Five parameters were used as criteria for lung injury, manifested as alveolar thickening, vascular congestion, hemorrhage, edema, and leukocyte infiltration. Each observer was required to give a score from 0 to 2 for each slide according to the criteria mentioned above. A score of 0 indicates that there was no histological damage. A score of 1 indicates only mild or intermediate histological damage in the slides, and a score of 2 was given to the tissue sections with severe morphological deterioration in most of the areas observed.

### *Statistical analysis*

All results are expressed as mean  $\pm$  SE. Statistical





**Figure 1** Effect of MG-132 on serum amylase level (A), pancreatic TNF- $\alpha$  level (B), lung water content (C), pancreas/body weight ratio (D), and MPO activity in pancreas (E) and lung (F). White bars represent the control group (a sham operation consisting of laparotomy and puncture of the duodenum), black bars represent the SAP group (with retrograde injection of sodium taurocholate into pancreatic duct) and grey bars represent the MG-132 group (10 mg/kg MG-132 ip 30 min before the induction of pancreatitis). <sup>a</sup> $P < 0.05$  vs sham group, <sup>c</sup> $P < 0.05$  vs SAP group.

analysis of data was accomplished by analysis of variance (ANOVA).  $P < 0.05$  was considered statistically significant.

## RESULTS

### Effect of MG-132 on serum amylase activity

Serum amylase activity, a most common indicator for assessing pancreatitis, was markedly increased in the SAP animals (Figure 1A). The effect of MG-132 on pancreatitis was statistically significant.

### Effect of MG-132 on pancreatic TNF- $\alpha$ level

In the SAP animals, the concentration of TNF- $\alpha$  was increased (Figure 1B), which could be ameliorated in rats treated with MG-132, showing that TNF- $\alpha$  could improve pancreatitis.

### Effect of MG-132 on lung wet/dry weight ratio

The ratio of lung wet/dry weight, a commonly used indicator for estimating the water content in acute lung injury was significantly increased in the SAP group compared with the sham group (Figure 1C). Treatment with MG-132 could reduce the water content lung.

### Effect of MG-132 on the ratio of pancreas to body weight

Pancreatic edema, one of the major criteria for assessing pancreatitis was found in our experiment. Injection of 5% sodium taurocholate into the biliary-pancreatic duct of rats could significantly increase the ratio of pancreas to body weight (Figure 1D). Treatment with MG-132 showed a beneficial effect on pancreatic edema.

### Effect of MG-132 on pancreatic and lung MPO activity

SAP is associated with a rise in both pancreatic and lung MPO activity, indicating the presence of sequestered

neutrophils<sup>[15]</sup>. Pre-treatment of the animals with MG-132 significantly reduced the MPO activity both in pancreas and in lung (Figure 1E and F).

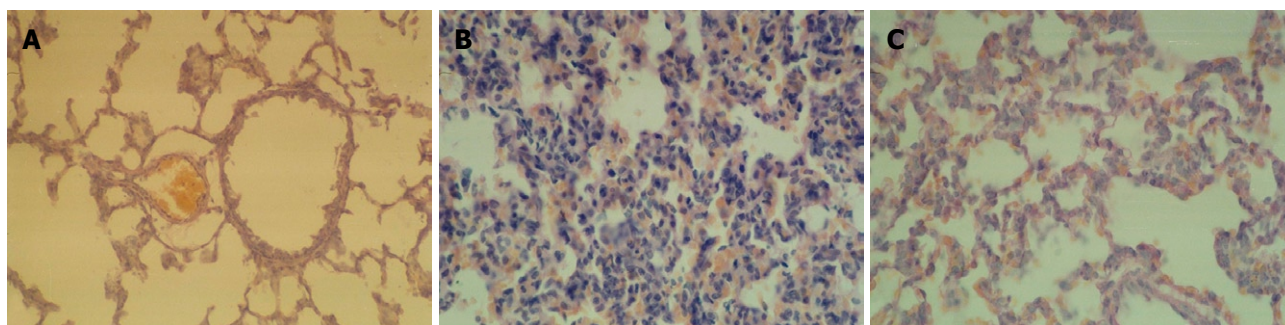
### Effect of MG-132 on pancreatic and lung histology

To assess the effects of MG-132 on local pancreatic injury, the morphology of pancreas was examined and compared with the treatment group. The results showed that the SAP group exhibited severe edema and a high degree of destruction of histoarchitecture of the acini cells. The architecture and integrity of acini cells were improved in the MG-132 group.

Normal lung tissue morphology (Figure 2A) was observed in the sham group. Histological examination of the sections confirmed lung injury with significant alveolate thickening, vasocongestion and infiltration with leukocytes observed in the SAP group (Figure 2B). In contrast, the lung injury was significantly ameliorated in the animals treated with MG-132 (Figure 2C). The scores of histological evaluation of pancreatitis and lung injury are summarized in Table 1.

## DISCUSSION

Acute pancreatitis is a life-threatening disease with a high mortality rate, especially in the setting of systemic inflammatory response and multiple organ failure when severe infection of necrosis occurs<sup>[16]</sup>. Under physiological conditions, digestive enzymes are synthesized by and stored in pancreatic acinar cells as inactive proenzymes known as zymogens which are secreted into the duodenum where enterokinase initiates their activation. The pathogenesis of acute pancreatitis remains obscure. However, it is believed that the premature activation of zymogens within acinar cells is a critical initiating event,



**Figure 2** Representative images illustrating histologically observed morphology of pancreas in pulmonary sections (HE, × 200).

**Table 1** Effect of MG-132 on histological damage to pancreas and lung

	Sham	SAP	MG-132
Pancreas	0.36 ± 0.04	13.82 ± 1.12 <sup>a</sup>	8.33 ± 0.52 <sup>c</sup>
Lung	0.23 ± 0.05	7.63 ± 0.65 <sup>a</sup>	4.46 ± 0.31 <sup>c</sup>

<sup>a</sup>*P* < 0.05 vs sham group, <sup>c</sup>*P* < 0.05 vs SAP group.

thus leading to auto-digestion of the gland. Afflicted acinar cells release factors that lead to recruitment of inflammatory cells and generation of multiple mediators, such as reactive oxygen species and cytokines<sup>[17]</sup>. Two potential key elements involved in this process are cathepsin B and NF-κB.

Cathepsin B is a lysosomal hydrolase, which activates human trypsinogen *in vitro* and is redistributed in a zymogen-granule enriched subcellular compartment during the early course of experimental pancreatitis<sup>[18,19]</sup>. It was reported that inhibition of lysosomal protease cathepsin B can suppress pancreatic inflammation<sup>[20,21]</sup>. Studies in cathepsin B gene knocked-out mice showed that the premature and intracellular activation of trypsinogen largely depends on the presence of cathepsin B<sup>[22]</sup>.

NF-κB is a member of the transcription factors which, under normal conditions, are coupled to an inhibitor (IκB) in cytoplasm<sup>[23,24]</sup>. In response to stress, a cascade of phosphorylation events results in IκB phosphorylation and degradation by proteasome. NF-κB with subsequent up-regulation of the expression of genes coding for a variety inflammatory factors including cytokines and chemokines such as TNF-α, IL-1, IL-6, IL-8. In acute pancreatitis<sup>[25]</sup>, NF-κB activation can be inhibited by blocking the degradation of IκB, which has been shown to ameliorate the severity of pancreatitis<sup>[26]</sup>.

During acute pancreatitis, lung injury is associated with the accumulation of neutrophils within the interstitial and alveolar spaces. Leukocyte sequestration within an inflamed area is a multistep process that begins with leukocyte activation<sup>[27]</sup>, followed by the rolling of inflammatory cells and the adhesion of circulating activated inflammatory cells to the endothelium via adhesion molecules, such as intercellular adhesion molecule-1 (ICAM-1)<sup>[28]</sup>. Cytokines, such as IL-1 and TNF-α, can also increase lung injury and spread to other

distant organs, indicating that NF-κB plays key role in this proinflammatory process<sup>[29-31]</sup>.

MG-132 is a cell-permeable aldehyde proteasome inhibitor, which has been shown to suppress the inflammatory cascade by decreasing NF-κB activity through blocking IκB degradation and increasing cellular HSP level<sup>[32]</sup>. MG-132 can also suppress pancreatic inflammation by inhibiting calpains and cathepsin B.

Since the SAP model established by injecting sodium taurocholate is most similar to the situation in human severe acute pancreatitis<sup>[33]</sup>, we chose it to examine the effect of proteasome inhibitor MG-132 on pancreatitis and lung injury in order to simulate the situation in humans.

In the present study, administration of MG-132 significantly suppressed the elevation of serum amylase, TNF-α and pancreatic MPO activity. Significant improvements were also observed in pancreatic histology after treatment with MG-132. Edema, acinar cell and fat necrosis, perivascular inflammation occurring in almost all inflammatory processes in any organ, were resolved in pancreatic tissues from the animals treated with MG-132.

The group pre-treated with MG-132, as compared with the sham and SAP groups, showed a significant reduction in the lung water content and MPO activity. These results are in close agreement with the histological analysis, suggesting that alveolar thickening, vasocongestion and recruitment of leukocytes in lung tissue can be suppressed with MG-132.

In conclusion, MG-132, a proteasome inhibitor, can ameliorate sodium taurocholate-induced SAP and its associated lung injury.

## COMMENTS

### Background

Severe acute pancreatitis (SAP) is still a fatal disease and its pathogenesis has not been fully understood. Pathological activation of digestive zymogens within pancreatic acinar cells is considered the key initiator of AP. The effect of MG-132 was investigated in experimental severe acute pancreatitis in the present study.

### Research frontiers

Proteasome inhibitors have a broad inhibitory action on pancreatic enzymes and production of proinflammatory cytokines. Therefore, they are expected to prevent necrotic changes in the pancreas and reduce the mortality rate.

### Innovations and breakthroughs

The SAP model established by retrograde injection of 5% sodium taurocholate (1 mL/kg) into the pancreatic duct was used to observe the effect of the

proteasome inhibitor MG-132 on severe acute pancreatitis and its associated lung injury of rats. Taurocholate-induced pancreatitis is a reliable model of severe acute pancreatitis rats with significantly greater pancreatic damage and systemic inflammatory response in comparison with cerulein-induced pancreatitis. Pancreatic and lung injury was a distant organ injury which is the key determinant of mortality in AP patients.

### Applications

MG-132 shows its protective effect on SAP induced by sodium taurocholate and its associated lung injury of rats. Moreover, proteasome inhibitors may promote further studies on the treatment of AP.

### Peer review

This paper describes the effect of MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal), a proteasome inhibitor, on SAP and its associated lung injury of rats. The model selected is appropriate and induces SAP and lung injury. Further researches are needed to explore its mechanism.

## REFERENCES

- Pandolf SJ. Acute pancreatitis. *Curr Opin Gastroenterol* 2006; **22**: 481-486
- Yadav D, Lowenfels AB. Trends in the epidemiology of the first attack of acute pancreatitis: a systematic review. *Pancreas* 2006; **33**: 323-330
- Bhatia M, Wong FL, Cao Y, Lau HY, Huang J, Puneet P, Chevali L. Pathophysiology of acute pancreatitis. *Pancreatol* 2005; **5**: 132-144
- Sherwood MW, Prior IA, Voronina SG, Barrow SL, Woodsmith JD, Gerasimenko OV, Petersen OH, Tepikin AV. Activation of trypsinogen in large endocytic vacuoles of pancreatic acinar cells. *Proc Natl Acad Sci USA* 2007; **104**: 5674-5679
- Malangoni MA, Martin AS. Outcome of severe acute pancreatitis. *Am J Surg* 2005; **189**: 273-277
- Elliott PJ, Zollner TM, Boehncke WH. Proteasome inhibition: a new anti-inflammatory strategy. *J Mol Med* 2003; **81**: 235-245
- Lee DH, Goldberg AL. Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol* 1998; **8**: 397-403
- Christman JW, Lancaster LH, Blackwell TS. Nuclear factor kappa B: a pivotal role in the systemic inflammatory response syndrome and new target for therapy. *Intensive Care Med* 1998; **24**: 1131-1138
- Saluja AK, Donovan EA, Yamanaka K, Yamaguchi Y, Hofbauer B, Steer ML. Cerulein-induced in vitro activation of trypsinogen in rat pancreatic acini is mediated by cathepsin B. *Gastroenterology* 1997; **113**: 304-310
- Salinthone S, Singer CA, Gerthoffer WT. Inflammatory gene expression by human colonic smooth muscle cells. *Am J Physiol Gastrointest Liver Physiol* 2004; **287**: G627-G637
- Aho HJ, Koskensalo SM, Nevalainen TJ. Experimental pancreatitis in the rat. Sodium taurocholate-induced acute haemorrhagic pancreatitis. *Scand J Gastroenterol* 1980; **15**: 411-416
- Rongione AJ, Kusske AM, Kwan K, Ashley SW, Reber HA, McFadden DW. Interleukin 10 reduces the severity of acute pancreatitis in rats. *Gastroenterology* 1997; **112**: 960-967
- Tsang SW, Ip SP, Leung PS. Prophylactic and therapeutic treatments with AT 1 and AT 2 receptor antagonists and their effects on changes in the severity of pancreatitis. *Int J Biochem Cell Biol* 2004; **36**: 330-339
- Kusske AM, Rongione AJ, Ashley SW, McFadden DW, Reber HA. Interleukin-10 prevents death in lethal necrotizing pancreatitis in mice. *Surgery* 1996; **120**: 284-288; discussion 289
- Schmidt J, Lewandrowski K, Warshaw AL, Compton CC, Rattner DW. Morphometric characteristics and homogeneity of a new model of acute pancreatitis in the rat. *Int J Pancreatol* 1992; **12**: 41-51
- Mullane KM, Kraemer R, Smith B. Myeloperoxidase activity as a quantitative assessment of neutrophil infiltration into ischemic myocardium. *J Pharmacol Methods* 1985; **14**: 157-167
- Shi C, Zhao X, Wang X, Andersson R. Role of nuclear factor-kappaB, reactive oxygen species and cellular signaling in the early phase of acute pancreatitis. *Scand J Gastroenterol* 2005; **40**: 103-108
- Baron TH, Morgan DE. Acute necrotizing pancreatitis. *N Engl J Med* 1999; **340**: 1412-1417
- Teich N, Bodeker H, Keim V. Cathepsin B cleavage of the trypsinogen activation peptide. *BMC Gastroenterol* 2002; **2**: 16
- Saluja AK, Donovan EA, Yamanaka K, Yamaguchi Y, Hofbauer B, Steer ML. Cerulein-induced in vitro activation of trypsinogen in rat pancreatic acini is mediated by cathepsin B. *Gastroenterology* 1997; **113**: 304-310
- Van Acker GJ, Saluja AK, Bhagat L, Singh VP, Song AM, Steer ML. Cathepsin B inhibition prevents trypsinogen activation and reduces pancreatitis severity. *Am J Physiol Gastrointest Liver Physiol* 2002; **283**: G794-G800
- Halangk W, Lerch MM, Brandt-Nedelev B, Roth W, Ruthenbuerger M, Reinheckel T, Domschke W, Lippert H, Peters C, Deussing J. Role of cathepsin B in intracellular trypsinogen activation and the onset of acute pancreatitis. *J Clin Invest* 2000; **106**: 773-781
- Hietaranta AJ, Saluja AK, Bhagat L, Singh VP, Song AM, Steer ML. Relationship between NF-kappaB and trypsinogen activation in rat pancreas after supramaximal caerulein stimulation. *Biochem Biophys Res Commun* 2001; **280**: 388-395
- Hietaranta A, Mustonen H, Puolakkainen P, Haapiainen R, Kempainen E. Proinflammatory effects of pancreatic elastase are mediated through TLR4 and NF-kappaB. *Biochem Biophys Res Commun* 2004; **323**: 192-196
- Makhija R, Kingsnorth AN. Cytokine storm in acute pancreatitis. *J Hepatobiliary Pancreat Surg* 2002; **9**: 401-410
- Altavilla D, Famulari C, Passaniti M, Campo GM, Macri A, Seminara P, Marini H, Calo M, Santamaria LB, Bono D, Venuti FS, Mioni C, Leone S, Guarini S, Squadrito F. Lipid peroxidation inhibition reduces NF-kappaB activation and attenuates cerulein-induced pancreatitis. *Free Radic Res* 2003; **37**: 425-435
- Lau HY, Wong FL, Bhatia M. A key role of neurokinin 1 receptors in acute pancreatitis and associated lung injury. *Biochem Biophys Res Commun* 2005; **327**: 509-515
- Bhatia M, Mochhala S. Role of inflammatory mediators in the pathophysiology of acute respiratory distress syndrome. *J Pathol* 2004; **202**: 145-156
- O'Reilly DA, Roberts JR, Cartmell MT, Demaine AG, Kingsnorth AN. Heat shock factor-1 and nuclear factor-kappaB are systemically activated in human acute pancreatitis. *JOP* 2006; **7**: 174-184
- Shi C, Zhao X, Wang X, Andersson R. Role of nuclear factor-kappaB, reactive oxygen species and cellular signaling in the early phase of acute pancreatitis. *Scand J Gastroenterol* 2005; **40**: 103-108
- Long J, Song N, Liu XP, Guo KJ, Guo RX. Nuclear factor-kappaB activation on the reactive oxygen species in acute necrotizing pancreatic rats. *World J Gastroenterol* 2005; **11**: 4277-4280
- Lee DH, Goldberg AL. Proteasome inhibitors cause induction of heat shock proteins and trehalose, which together confer thermotolerance in *Saccharomyces cerevisiae*. *Mol Cell Biol* 1998; **18**: 30-38
- Chan YC, Leung PS. Acute pancreatitis: animal models and recent advances in basic research. *Pancreas* 2007; **34**: 1-14

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RAPID COMMUNICATION

## Change of intestinal mucosa barrier function in the progress of non-alcoholic steatohepatitis in rats

Sheng Li, Wan-Chun Wu, Chi-Yi He, Zhen Han, Dao-You Jin, Lin Wang

Sheng Li, Wan-Chun Wu, Chi-Yi He, Zhen Han, Dao-You Jin, Lin Wang, Department of Gastroenterology, Yijishan Hospital, Wannan Medical College, Wuhu 241001, Anhui Province, China

**Author contributions:** Li S and Wu WC designed and performed the research; He CY, Han Z and Jin DY designed the research; Wang L performed the research; Li S and Wu WC analyzed the data; Li S and Wu WC wrote the paper.

**Correspondence to:** Wan-Chun Wu, Department of Gastroenterology, Yijishan Hospital, Wuhu 241001, Anhui Province, China. [wwch5182000@yahoo.com.cn](mailto:wwch5182000@yahoo.com.cn)

Telephone: +86-553-5739106 Fax: +86-553-5738401

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### Abstract

**AIM:** To explore the change of intestinal mucosa barrier function in the progress of non-alcoholic steatohepatitis (NASH) in rats.

**METHODS:** Thirty-two Sprague-Dawley (SD) rats were randomly divided into control group and model group. Rats in the control group were given normal diet, and rats in the model group were given fat-rich diet. Eight rats in each group were killed at end of the 8th and 12th wk, respectively. The levels of endotoxin, D-xylose, TG, TC, ALT and AST, intestinal tissue SOD and MDA as well as intestinal mucus secretory IgA (sIgA) were measured. The pathology of liver was observed by HE staining.

**RESULTS:** At end of the 8th wk, there was no marked difference in the levels of endotoxin, D-xylose and sIgA between the two groups. At end of the 12th wk, rats in the model group developed steatohepatitis and had a higher serum level of endotoxin ( $P = 0.01$ ) and D-xylose ( $P = 0.00$ ) and a lower serum level of sIgA ( $P = 0.007$ ).

**CONCLUSION:** Intestinal mucosa barrier malfunction may exist in NASH rats and may be an important promoter of NASH in rats.

### INTRODUCTION

Non-alcoholic steatohepatitis (NASH) is a type of non-alcoholic fatty liver disease (NAFLD), and may progress to hepatic fibrosis and cirrhosis<sup>[1-3]</sup>. The pathogenesis of NASH remains unclear. Nowadays, lipid metabolism abnormality, insulin resistance and oxidative stress and lipid peroxidation reaction<sup>[4-8]</sup> are thought to play an important role in the pathogenesis of NASH<sup>[9,10]</sup>. It was reported that the change of intestinal environment may play a role in NASH, which may be a cause of enterogenous endotoxemia<sup>[11,12]</sup>. Since the relationship between intestinal mucosa barrier function and NASH is uncertain, we established an animal model of NASH by giving fat-rich diet to explore the change of intestinal mucosa barrier function in the progress of NASH.

### MATERIALS AND METHODS

#### Materials

Thirty-two healthy female mice, provided by Nanjing Qinglongshan Experimental Animal Center, were used in this study. D-xylose, SOD and MDA kit were purchased from Nanjing Jiancheng Bioengineering Institute (NJBI). Quantitative chromogenic end-point tachyplesus amebocyte lysate kit was purchased from Xiamen Houshiji, Ltd. Secretory IgA (sIgA) kit was purchased from Beijing North Institute of Biological Technology (BNIBT).

#### Methods

**Establishment of animal mode:** 32 female Sprague-Dawley (SD) rats, weighing 130-150 g, after a week of adaptive feeding, were randomly divided into model group and control group (16 in each group). Rats in the control group were given normal diet and rats in the model group ( $n = 16$ ) were given fat-rich diet containing 88% normal diet, 10% lard and 2% cholesterol. All rats were maintained at controlled room temperature in a

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**Key words:** Non-alcoholic steatohepatitis; Intestinal mucosa barrier; Endotoxin; Secretory IgA

**Peer reviewer:** Anthony J Bauer, PhD, Department of Medicine/Gastroenterology, University of Pittsburgh, 3550 Terrace Street, S-849 Scaife Hall, Pittsburgh 15261, United States



12-h light/dark cycle with free access to laboratory feed and water. Eight rats in each group were killed at wk 8, 12 respectively during the study. All rats had no access to food and water for 12 h, but received intra-gastric 5% D-xylose (0.5 mL/100 g, BW) and 0.3% pentobarbital (0.15-0.2 mL/kg) *via* abdominal cavity, 25 min and a short wile, respectively, before they were killed.

**Histological evaluation:** Liver specimen was obtained from the central part of liver, observed by HE staining, and evaluated according to the guidelines for diagnosis and treatment of nonalcoholic fatty liver diseases revised by Fatty Liver and Alcoholic Liver Study Group of the Chinese Liver Disease Association<sup>[13]</sup>.

**Measurement of ALT, AST, TG and TC:** Two milliliters blood was taken from abdominal aorta and serum was taken after centrifugation at 4000 r/min for 10 min. The levels of ALT, AST, TG and TC were measured with an automatic biochemical analyzer.

**Measurement of D-xylose:** Two milliliters blood was taken from abdominal aorta, and collected into a tube (containing heparin) immediately and plasma was taken after the blood was centrifuged at 4000 r/min for 10 min. The Level of D-xylose in plasma was measured with a D-xylose kit.

**Measurement of endotoxin:** One milliliter blood was taken from portal vein and collected into an apyrogenic tube (containing heparin) immediately. Plasma was taken after the blood was centrifuged at 3000 r/min for 10 min (environmental temperature: 0°C). The levels of endotoxin were measured by limulus amebocyte lysate test.

**Detection of sIgA:** sIgA was detected as previously described<sup>[14]</sup>. A 10 cm long tissue was obtained from the small intestine, dissected and washed with normal saline carefully. Intestine mucus was collected into an Eppendorf tube, and centrifuged at 3000 r/min for 10 min (environmental temperature: 0°C) after 1 mL 0.01 mol/L PBS was added. The supernatant was harvested. The level of sIgA was measured by double antibody sandwich immunoradiometric assay. The total protein of intestine mucus was assayed by Bradford brilliant blue method simultaneously. The sIgA content in total protein of one milligram small intestine mucus was detected.

**Detection of SOD and MDA in small intestine tissue:** The small intestine tissue was weighed to prepare 10% tissue homogenate by adding normal saline according to weighing ratio. The homogenate was centrifuged at 3000 r/min for 10 min (environmental temperature: 0°C). The supernatant was harvested to make 1% tissue homogenate by adding normal saline. The levels of SOD and MDA in tissue homogenate were measured.

### Statistical analysis

All statistical analyses were performed using SPSS 11.5

**Table 1** Level of serum TG, TC, ALT and AST (mean  $\pm$  SD,  $n = 8$ )

Group	Time (wk)	TG (mmol/L)	TC (mmol/L)	ALT (U/L)	AST (U/L)
Control	8	0.72 $\pm$ 0.17	1.21 $\pm$ 0.29	39.00 $\pm$ 7.46	134.88 $\pm$ 35.11
Model	8	0.79 $\pm$ 0.20	1.78 $\pm$ 0.35 <sup>a</sup>	61.75 $\pm$ 15.85 <sup>a</sup>	96.63 $\pm$ 52.80 <sup>a</sup>
Control	12	0.76 $\pm$ 0.17	1.26 $\pm$ 0.25	41.88 $\pm$ 6.27	138.00 $\pm$ 36.70
Model	12	0.85 $\pm$ 0.18	1.99 $\pm$ 0.26 <sup>a</sup>	87.75 $\pm$ 26.89 <sup>a</sup>	248.88 $\pm$ 53.09

<sup>a</sup> $P < 0.05$  vs control group.

software package. All data were expressed as mean  $\pm$  SD. Group comparison was done by one-factor analysis of variance.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Liver histology

The structure of hepatic lobules and the morphology of liver cells were normal in the control group. Lipid droplets were observed in 50%-75% of hepatic cells in the model group after 8 wk of fat-rich diet, predominantly bullules, consistent with the diagnostic criteria for simple fatty liver disease. Fatty degeneration in hepatic cells exceeded 75% and patch necrosis, mild to moderate chronic inflammation could be seen after 12 wk of fat-rich diet, consistent with the diagnostic criteria for steatohepatitis.

### Contents of serum TG, TC, ALT and AST

Serum TC, ALT and AST levels were higher in the model group than in the control group in the 8th, and 12th wk. There was a statistical difference between the two groups ( $P < 0.05$ ). The serum TG level was slightly higher in the model group than in the control group, but there was no statistical difference between the two groups (Table 1).

### Level of serum D-xylose, endotoxin and intestine mucus sIgA

At end of the 8th wk, there was no significant difference in the levels of endotoxin, D-xylose and sIgA between the two groups. At end of the 12th wk, rats in the model group developed steatohepatitis and had a higher serum level of endotoxin and D-xylose ( $P < 0.05$ ), but a lower level of sIgA ( $P < 0.05$ ) (Table 2).

### Level of SOD and MDA in small intestine tissue

The level of SOD in small intestine tissue was lower in the model group than in the control group in the 8th and 12th wk. There was a statistical difference between the two groups ( $P < 0.05$ ). The level of MDA in small intestine tissue was higher in the model group than in control group in the 8th and 12th wk. There was a statistical difference between the two groups ( $P < 0.05$ ) (Table 3).

### Correlation analysis of serum D-xylose, endotoxin and intestine mucus sIgA

A line tendency could be observed in scatter plots bellow. The serum level of endotoxin in portal vein was positively

**Table 2** Level of serum D-xylose, endotoxin and intestine mucus sIgA (mean  $\pm$  SD,  $n = 8$ )

Group	Time (wk)	D-xylose (mmol/L)	sIgA ( $\mu$ g/mg)	Endotoxin (EU/mL)
Control	8	0.65 $\pm$ 0.21	1.72 $\pm$ 0.67	0.267 $\pm$ 0.022
Model	8	0.71 $\pm$ 0.17	1.55 $\pm$ 0.58	0.272 $\pm$ 0.021
Control	12	0.72 $\pm$ 0.23	1.64 $\pm$ 0.60	0.270 $\pm$ 0.023
Model	12	1.33 $\pm$ 0.37 <sup>a</sup>	0.78 $\pm$ 0.27 <sup>a</sup>	0.302 $\pm$ 0.020 <sup>a</sup>

<sup>a</sup> $P < 0.05$  vs control group.**Table 3** Level of SOD and MDA in small intestine tissue SOD and MDA (mean  $\pm$  SD,  $n = 8$ )

Group	Time (wk)	SOD (U/mgprot)	MDA (nmol/mgprot)
Control	8	83.29 $\pm$ 10.56	0.55 $\pm$ 0.06
Model	8	71.61 $\pm$ 9.28 <sup>a</sup>	0.72 $\pm$ 0.05 <sup>a</sup>
Control	12	80.79 $\pm$ 7.76	0.59 $\pm$ 0.04
Model	12	61.26 $\pm$ 7.01 <sup>c</sup>	0.93 $\pm$ 0.08 <sup>c</sup>

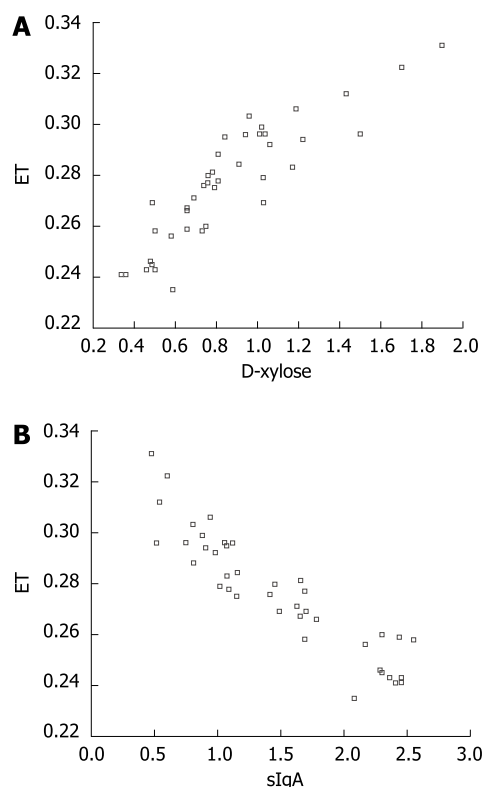
<sup>a</sup> $P < 0.05$ , <sup>c</sup> $P < 0.05$  vs control group.

correlated with that of D-xylose in abdominal aorta ( $r = 0.846$ ,  $n = 8$ ,  $P < 0.01$ ) and negatively correlated that of sIgA in intestine mucus ( $r = -0.873$ ,  $n = 8$ ,  $P < 0.01$ ) (Figure 1A and B).

## DISCUSSION

At present, the specific pathogenesis and progress of NASH remain unclear. It was reported that there is enterogenous endotoxemia in NASH, suggesting that NASH is closely related to endotoxin<sup>[15-17]</sup>. Wigg *et al*<sup>[11]</sup> reported that small intestinal bacterial overgrowth is present in 50% of patients with non-alcoholic steatosis not accompanying increased intestinal permeability or elevated endotoxin levels. Brun P *et al*<sup>[12]</sup> showed that obese mice with NASH have higher intestinal mucosa permeability and circulating level of endotoxemia in portal vein than the control mice, suggesting that genetically obese mice display an enhanced intestinal permeability, leading to severe endotoxemia in portal vein. Therefore, whether there is an increased intestinal permeability and a change in intestinal mucosa barrier function in NASH patients needs to be further explored.

Intestinal mucosa barriers include mechanical barrier, chemical barrier, immunologic barrier and biology barrier<sup>[18-21]</sup>, any damage of these barriers will damage intestinal mucosa barrier function. In this study, we used plasma D-xylose, endotoxin and intestine mucus sIgA to evaluate the intestinal mucosa barrier function in rats with NASH and to observe its change in NASH rats. Intestine mucus sIgA is a major ingredient of intestinal immunologic barrier, mostly secreted by plasmocytes of intestinal mucosa, and may restrain intestinal bacteria to adhere to intestinal mucosa surface, to counteract toxin, enzyme and virus in the intestinal tract, thus playing an important role in intestinal immunity<sup>[22-24]</sup>. SOD is the most important anti-oxidation enzyme in anti-oxidation defense system and MDA is the end product

**Figure 1** Relationships scatter plot. A: ET and D-xylose; B: ET and sIgA.

of lipid peroxidation, which can cause tissue injury. SOD activity and MDA level can reflect the degree of lipid peroxidation and oxidative stress. It was reported that NASH is closely related to lipid peroxidation and oxidative stress<sup>[25-27]</sup>. In the present study, we successfully established the NASH model by giving fat-rich diet, and observed the change of intestinal mucosa barrier function in simple fatty liver disease and NASH. The results showed that there was no statistical difference in serum D-xylose, endotoxin and intestine mucus sIgA between the two groups at the 8th wk, suggesting that there might be no damage to the intestinal mucosa barrier at the stage of simple fatty liver disease. However, the SOD activity was decreased in intestinal tissue, while the level of MDA was increased, suggesting that lipid peroxidation and anti-oxidation are imbalanced. There was a significant difference in serum D-xylose, endotoxin and intestine mucus sIgA between the two groups ( $P < 0.05$ ) at the 12th wk. Serum D-xylose and endotoxin levels were higher in the model group than in the control group, while the intestine mucus sIgA levels were lower in the model group, suggesting that the intestinal mucosa barrier is damaged at the stage of NASH and that the SOD activity is further decreased in the intestinal tissue while MDA level is further elevated and lipid peroxidation reaction is further aggravated. The fact that serum endotoxin level in portal vein was positively correlated with that of serum D-xylose in abdominal aorta, but negatively correlated with that of sIgA in intestine mucus, suggesting that the damage to intestinal mucosa barrier may cause enterogenous endotoxemia.

In our study, no damage to intestinal mucosa barrier occurred at the early stage of nonalcoholic fatty liver disease. With the progress from simple fatty liver disease to NASH, severe damage to intestinal mucosa barrier occurred. The pathogenesis of intestinal mucosa barrier damage is unclear. It may be due to the increased lipid peroxidation reaction in intestinal tissue and intestinal mucosa damage caused by endotoxin<sup>[28]</sup>. It needs to be further studied. Increased sIgA levels in intestine mucus would lead to the ability of intestinal bacterium to inhibit adherence to intestinal mucosa surface and decrease counteracting toxin, so bacteria and endotoxin are increased in the intestinal tract. Wigg *et al*<sup>[11]</sup> reported that bacteria grow in small intestine of patients with non-alcoholic steatosis, suggesting that and decreased sIgA promotes overgrowth of bacteria in small intestine. We suppose that small intestinal bacterial overgrowth in small intestine can produce more endotoxin in enteric cavity, thus damaging intestinal mucosa barrier and absorbing more endotoxin, finally leading to enterogenous endotoxemia. It was reported that endotoxin can not only injure hepatic cells but also activate Kupffer cells by combining receptor CD14 and signal receptor TLR4. The activated Kupffer cells release a series of bioactive substances, such as TNF- $\alpha$ , causing hepatic injury, thus aggravating the effect of endotoxin and promoting development of NASH<sup>[29-31]</sup>.

## COMMENTS

### Background

The pathogenesis of non-alcoholic steatohepatitis (NASH) remains unclear. Insulin resistance, obesity-related inflammation, oxidative stress, microcirculation disturbance, and malnutrition are thought to play a key role in the pathogenesis of NASH. Studies have also demonstrated that change in intestinal environment may also play a role in the pathogenesis of NASH, and may be a cause of enterogenous endotoxemia. It has been found that a higher intestinal permeability may also play a role in the process of NASH. However, it is not accepted that there exists enterogenous endotoxemia in NASH.

### Research frontiers

Great effort has been made to clarify the pathogenesis of NASH. The source and pathogenesis of endotoxin in the process are two hot spots.

### Innovations and breakthroughs

In this study, the relationship between intestinal mucosa barrier function and NASH was studied.

### Applications

The intestinal mucosa barrier malfunction may lead to NASH. There might be a vicious circle between intestinal mucosa barrier malfunction and NASH.

### Terminology

NASH is a kind of liver disease which resembles alcoholic liver disease accompanying steatosis, inflammation, necrosis, and fibrosis. Intestinal mucosa barriers include mechanical barrier, chemical barrier, immunologic barrier and biology barrier.

### Peer review

This paper explores the change of intestinal mucosa barrier function in the progress of NASH in rats. The well designed study demonstrated that the intestinal mucosa barrier malfunction may exist in NASH rats, and may be an important promoter of NASH in rats.

## REFERENCES

- Day CP. Non-alcoholic steatohepatitis (NASH): where are we now and where are we going? *Gut* 2002; **50**: 585-588
- Powell EE, Cooksley WG, Hanson R, Searle J, Halliday JW, Powell LW. The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. *Hepatology* 1990; **11**: 74-80
- Harrison SA, Torgerson S, Hayashi PH. The natural history of nonalcoholic fatty liver disease: a clinical histopathological study. *Am J Gastroenterol* 2003; **98**: 2042-2047
- Koruk M, Savas MC, Yilmaz O, Taysi S, Karakok M, Gundogdu C, Yilmaz A. Serum lipids, lipoproteins and apolipoproteins levels in patients with nonalcoholic steatohepatitis. *J Clin Gastroenterol* 2003; **37**: 177-182
- Baskol G, Baskol M, Kocer D. Oxidative stress and antioxidant defenses in serum of patients with non-alcoholic steatohepatitis. *Clin Biochem* 2007; **40**: 776-780
- Kojima H, Sakurai S, Uemura M, Fukui H, Morimoto H, Tamagawa Y. Mitochondrial abnormality and oxidative stress in nonalcoholic steatohepatitis. *Alcohol Clin Exp Res* 2007; **31**: S61-S66
- Mitsuyoshi H, Itoh Y, Okanoue T. [Role of oxidative stress in non-alcoholic steatohepatitis] *Nippon Rinsho* 2006; **64**: 1077-1082
- Leclercq IA. Pathogenesis of steatohepatitis: insights from the study of animal models. *Acta Gastroenterol Belg* 2007; **70**: 25-31
- Angulo P, Lindor KD. Non-alcoholic fatty liver disease. *J Gastroenterol Hepatol* 2002; **17** Suppl: S186-S190
- Choudhury J, Sanyal AJ. Insulin resistance in NASH. *Front Biosci* 2005; **10**: 1520-1533
- Wigg AJ, Roberts-Thomson IC, Dymock RB, McCarthy PJ, Grose RH, Cummins AG. The role of small intestinal bacterial overgrowth, intestinal permeability, endotoxaemia, and tumour necrosis factor alpha in the pathogenesis of non-alcoholic steatohepatitis. *Gut* 2001; **48**: 206-211
- Brun P, Castagliuolo I, Di Leo V, Buda A, Pinzani M, Palu G, Martines D. Increased intestinal permeability in obese mice: new evidence in the pathogenesis of nonalcoholic steatohepatitis. *Am J Physiol Gastrointest Liver Physiol* 2007; **292**: G518-G525
- Fatty Liver and Alcoholic Liver Disease Study Group of the Chinese Liver Disease Association. [Guidelines for diagnosis and treatment of nonalcoholic fatty liver diseases] *Zhonghua Ganzangbing Zazhi* 2006; **14**: 161-163
- Zhang YP, Shi ZR. Effects of probiotics on the bacterial groups of intestinal and sIgA in severely burned rats. *Zhongguo Weishengwu Zazhi* 2004; **5**: 257-259
- Kirsch R, Clarkson V, Verdonk RC, Marais AD, Shephard EG, Ryffel B, de la M Hall P. Rodent nutritional model of steatohepatitis: effects of endotoxin (lipopolysaccharide) and tumor necrosis factor alpha deficiency. *J Gastroenterol Hepatol* 2006; **21**: 174-182
- Li X, Han de W, Zhao LF, Yin L. [Effect of Endotoxin on the expression of peroxisome proliferator-activated receptor alpha in the development of nonalcoholic steatohepatitis in rats] *Zhonghua Ganzangbing Zazhi* 2005; **13**: 89-91
- Fan JG, Xu ZJ, Wang GL, Ding XD, Tian LY, Zheng XY. [Change of serum endotoxin level in the progress of nonalcoholic steatohepatitis in rats] *Zhonghua Ganzangbing Zazhi* 2003; **11**: 73-76
- Baumgart DC, Dignass AU. Intestinal barrier function. *Curr Opin Clin Nutr Metab Care* 2002; **5**: 685-694
- Sakaguchi T, Brand S, Reinecker HC. Mucosal barrier and immune mediators. *Curr Opin Gastroenterol* 2001; **17**: 573-577
- Tlaskalova-Hogenova H, Farre-Castany MA, Stepankova R, Kozakova H, Tuckova L, Funda DP, Barot R, Cukrowska B, Sinkora J, Mandel L. The gut as a lymphoepithelial organ: the role of intestinal epithelial cells in mucosal immunity. *Folia Microbiol (Praha)* 1995; **40**: 385-391
- Sydora BC, Martin SM, Lupicki M, Dieleman LA, Doyle J, Walker JW, Fedorak RN. Bacterial antigens alone can influence intestinal barrier integrity, but live bacteria are required for initiation of intestinal inflammation and injury. *Inflamm Bowel Dis* 2006; **12**: 429-436
- Mazanec MB, Nedrud JG, Kaetzel CS, Lamm ME. A three-tiered view of the role of IgA in mucosal defense. *Immunol*

- Today* 1993; **14**: 430-435
- 23 **Takahashi I**, Kiyono H. Gut as the largest immunologic tissue. *JPEN J Parenter Enteral Nutr* 1999; **23**: S7-S12
- 24 **Lamm ME**. Interaction of antigens and antibodies at mucosal surfaces. *Annu Rev Microbiol* 1997; **51**: 311-340
- 25 **Day CP**, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998; **114**: 842-845
- 26 **Koruk M**, Taysi S, Savas MC, Yilmaz O, Akcay F, Karakok M. Oxidative stress and enzymatic antioxidant status in patients with nonalcoholic steatohepatitis. *Ann Clin Lab Sci* 2004; **34**: 57-62
- 27 **Baskol G**, Baskol M, Kocer D. Oxidative stress and antioxidant defenses in serum of patients with non-alcoholic steatohepatitis. *Clin Biochem* 2007; **40**: 776-780
- 28 **Mercer DW**, Smith GS, Cross JM, Russell DH, Chang L, Cacioppo J. Effects of lipopolysaccharide on intestinal injury; potential role of nitric oxide and lipid peroxidation. *J Surg Res* 1996; **63**: 185-192
- 29 **Rivera CA**, Adegboyega P, van Rooijen N, Tagalicud A, Allman M, Wallace M. Toll-like receptor-4 signaling and Kupffer cells play pivotal roles in the pathogenesis of non-alcoholic steatohepatitis. *J Hepatol* 2007; **47**: 571-579
- 30 **Tomita K**, Tamiya G, Ando S, Ohsumi K, Chiyo T, Mizutani A, Kitamura N, Toda K, Kaneko T, Horie Y, Han JY, Kato S, Shimoda M, Oike Y, Tomizawa M, Makino S, Ohkura T, Saito H, Kumagai N, Nagata H, Ishii H, Hibi T. Tumour necrosis factor alpha signalling through activation of Kupffer cells plays an essential role in liver fibrosis of non-alcoholic steatohepatitis in mice. *Gut* 2006; **55**: 415-424
- 31 **Poniachik J**, Csendes A, Diaz JC, Rojas J, Burdiles P, Maluenda F, Smok G, Rodrigo R, Videla LA. Increased production of IL-1alpha and TNF-alpha in lipopolysaccharide-stimulated blood from obese patients with non-alcoholic fatty liver disease. *Cytokine* 2006; **33**: 252-257

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## Multiple giant diverticula of the foregut causing upper gastrointestinal obstruction

Genoveffa Balducci, Mario Dente, Giulia Cosenza, Paolo Mercantini, Pier Federico Salvi

Genoveffa Balducci, Mario Dente, Giulia Cosenza, Paolo Mercantini, Pier Federico Salvi, Departments of Surgery II School of Medicine, University of Rome "La Sapienza", Sant'Andrea Hospital, Via di Grottarossa 1035-39, Rome 00189, Italy

**Author contributions:** Balducci G and Dente M mainly wrote the manuscript and performed all the procedures; Balducci G provided the idea of management and revised the manuscript; Balducci G, Dente M, Cosenza G, Mercantini P, and Salvi PF revised the manuscript.

**Correspondence to:** Mario Dente, Dr, Departments of Surgery II School of Medicine, University of Rome "La Sapienza", Sant'Andrea Hospital, Via di Grottarossa 1035-39, Rome 00189, Italy. [mario.dente@hotmail.it](mailto:mario.dente@hotmail.it)

Telephone: +39-633-775693 Fax: +39-633-775322

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### Abstract

Small bowel diverticulosis represents an uncommon disorder (except for Meckel diverticulum) often misdiagnosed since it causes non-specific gastrointestinal symptoms. Most of times the diagnosis is carried out in case of related complications, such as diverticulitis, hemorrhage, perforation or obstruction. Intestinal obstruction can be caused by inflammatory stenosis due to repeated episodes of diverticulitis, volvulus, intussusception or jejunal stones. Herein we report a case of multiple jejunal diverticula causing chronic gastrointestinal obstruction.

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**Key words:** Jejunal diverticula; Chronic symptoms; Gastrointestinal obstruction; Jejunal resection

**Peer reviewer:** Richard A Kozarek, MD, Department of Gastroenterology, Virginia Mason Medical Center, 1100 Ninth Avenue, PO Box 900, Seattle 98111-0900, United States

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### INTRODUCTION

Multiple diverticulosis of the foregut is uncommon<sup>[1]</sup>. Although it is often asymptomatic, it can lead to severe complications, such as obstruction, hemorrhage, diverticulitis and perforation. Obstruction can be caused by inflammatory stenosis due to repeated episodes of diverticulitis, volvulus or intussusception, voluminous jejunal stones or dyskinesia of the small bowel<sup>[2-4]</sup>. We herein report a case of chronic gastrointestinal obstruction in a patient with a previous diagnosis of jejunal multiple giant diverticula.

### CASE REPORT

A 49-year-old woman was admitted to our department, because of abdominal pain and vomiting together with a history of repeated episodes of obstructive gastrointestinal symptoms in the last two years.

In a previous diagnostic work out, she underwent contrast barium that showed multiple giant diverticula in the proximal small bowel tract (Figure 1).

At physical examination, she was dehydrated and her abdomen was distended but soft. A plain X-ray abdominal film showed distended small bowel loops and multiple gas-fluid levels

The actual clinical condition, the long duration of symptoms and the previous diagnosis were all considered indications for surgery.

At the operation, diffuse giant diverticula were observed in the duodenum (Figure 2) and proximal jejunum so that duodenal diverticulectomy and jejunal resection were performed.

No postoperative complication was observed. During the 4-mo follow-up, the patient remained free of GI symptoms.

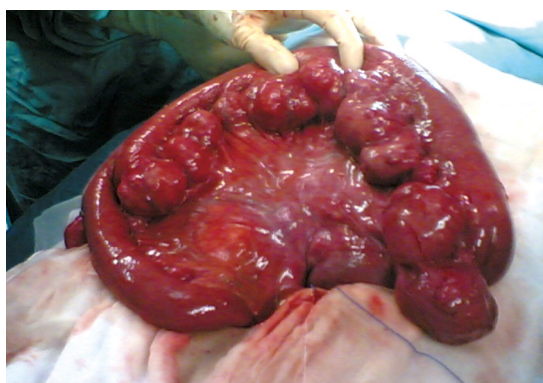
### DISCUSSION

Jejunal diverticulosis is a rare entity with an incidence rate ranging from 0.3%-1.3% in autopsy series to 2.3% of radiographic findings<sup>[1]</sup>.

Like colonic diverticula, small bowel diverticula other than Meckel's, are false diverticula resulting from mucosal herniation at the point where blood vessels penetrate the intestinal wall. This also explains their



**Figure 1** Contrast barium study showing multiple giant jejunal diverticula.



**Figure 2** All diverticula arising at the mesenteric border.

typical location at the mesenteric side<sup>[2]</sup>.

Aetiology of jejunal diverticula is still unclear since an anatomic wall defect seems not to be the only factor. Increased intraluminal pressure can play a main role as happens in case of colonic diverticulosis. Small bowel diverticula in fact can be seen in patients older than 50 years with peristaltic disorders, such as progressive systemic sclerosis, visceral myopathy and visceral neuropathies leading to increase of intraluminal pressure<sup>[3]</sup>.

This condition is often misdiagnosed as it is often asymptomatic or causes minor, non-specific gastrointestinal symptoms. Nevertheless, it could lead to severe complications, such as hemorrhage, diverticulitis, perforation and obstruction.

The small number of symptomatic patients may explain chronic post prandial abdominal pain, nausea and vomiting, borborygmi, alternating diarrhea and constipation, and weight loss, and present with anemia, steatorrhea, tenderness, and fever<sup>[1,4]</sup>. All these symptoms reflect inflammation, malabsorption, hemorrhage or mechanical obstruction.

Hemorrhage and perforation are the consequence of progressive ulceration in case of acute diverticulitis. While perforation is the result of progressive ulceration in case of acute diverticulitis, hemorrhage could be caused by diverticulitis with ulceration, or diverticulosis associated with trauma and irritation, or congenital arteriovenous malformations<sup>[5]</sup>.

Obstruction can be caused by inflammatory stenosis due to repeated episodes of diverticulitis, volvulus or

intussusception and voluminous jejunal stones. The latter seem to be caused by the intradiverticular milieu including alteration of the chemical environment and malabsorption, both of which are related to intestinal stasis and stagnant diverticula. Dyskinesia of the small bowel, in fact, causes an intraluminal stasis with bacterial overgrowth leading to deconjugation of bile acids. Deconjugated bile acid, together with cholic acid formed from bile salts and precipitate in aggregates, starts enterolith formation<sup>[6,7]</sup>.

In our patient, intermittent occlusive symptoms were probably caused by hyperdistension of the voluminous diverticula (Figure 2), resulting in external obstruction of jejunal loops.

Asymptomatic diverticula are found only in case of radiography or surgery performed for unrelated causes.

The diagnostic work up in symptomatic patients can start with plain abdominal X-ray film that could show distension of jejunal loops and gas-fluid levels into voluminous diverticula. Upper gastrointestinal X-ray study by barium contrast clearly shows the presence of multiple diverticula.

In case of acute abdomen due to diverticular perforation or intestinal obstruction, X-ray studies show typical signs of these conditions giving no information about the cause that will be recognized at surgery.

The treatment of choice for jejunal diverticulosis, often performed emergently, is resection of all the affected jejunum even in case of perforation or peridiverticular stenosis, in order to avoid further complications<sup>[4,8]</sup>.

In case of obstruction due to an enterolith, some authors suggest conservative management by performing the manual breakage of all stones, intradiverticular and blocking ones, pushing their fragments to the colon<sup>[1,4,8,9]</sup>.

This treatment is to be discouraged because of the persisting risk of stone formation and diverticular complications.

In contrast to jejunal diverticulosis, duodenal diverticula have been treated with simple diverticulectomy. The decision to operate a duodenal diverticulum, however, should be made with great caution because postoperative complications such as fistula formation and pancreatitis are not rare given the peripapillary location of many of these diverticula.

## REFERENCES

- 1 **Kassahun WT**, Fangmann J, Harms J, Bartels M, Hauss J. Complicated small-bowel diverticulosis: a case report and review of the literature. *World J Gastroenterol* 2007; **13**: 2240-2242
- 2 **Hamada N**, Ishizaki N, Shirahama K, Nakamura N, Murata R, Kadono J, Shimazaki T, Sameshima T, Misono T, Taira A. Multiple duodeno-jejunal diverticula causing massive intestinal bleeding. *J Gastroenterol* 2000; **35**: 159-162
- 3 **Krishnamurthy S**, Kelly MM, Rohrmann CA, Schuffler MD. Jejunal diverticulosis. A heterogenous disorder caused by a variety of abnormalities of smooth muscle or myenteric plexus. *Gastroenterology* 1983; **85**: 538-547
- 4 **Lempinen M**, Salmela K, Kempainen E. Jejunal diverticulosis: a potentially dangerous entity. *Scand J Gastroenterol* 2004; **39**: 905-909

- 5 **Rodriguez HE**, Ziauddin MF, Quiros ED, Brown AM, Podbielski FJ. Jejunal diverticulosis and gastrointestinal bleeding. *J Clin Gastroenterol* 2001; **33**: 412-414
- 6 **Crace PP**, Grisham A, Kerlakian G. Jejunal diverticular disease with unborn enterolith presenting as a small bowel obstruction: a case report. *Am Surg* 2007; **73**: 703-705
- 7 **Hofmann AF**, Mysels KJ. Bile acid solubility and precipitation in vitro and in vivo: the role of conjugation, pH, and Ca<sup>2+</sup> ions. *J Lipid Res* 1992; **33**: 617-626
- 8 **Steen Voorde P**, Schaardenburgh P, Viersma JH. Enterolith ileus as a complication of jejunal diverticulosis: two case reports and a review of the literature. *Dig Surg* 2003; **20**: 57-60
- 9 **Hayee B**, Khan HN, Al-Mishlab T, McPartlin JF. A case of enterolith small bowel obstruction and jejunal diverticulosis. *World J Gastroenterol* 2003; **9**: 883-884

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## CASE REPORT

# Acute ischemic colitis during scuba diving: Report of a unique case

Konstantinos Goumas, Androniki Poulou, Ioannis Tyrmipas, Dimitrios Dandakis, Stavros Bartzokis, Magdalini Tsamouri, Kalipso Barbati, Dimitrios Soutos

Konstantinos Goumas, Androniki Poulou, Ioannis Tyrmipas, Dimitrios Dandakis, Stavros Bartzokis, Dimitrios Soutos, Department of Gastroenterology, Red Cross Hospital of Athens, Athens, Greece

Magdalini Tsamouri, Kalipso Barbati, Department of Pathology, Red Cross Hospital of Athens, Athens, Greece

**Author contributions:** Goumas K organised the whole manuscript and supervised the process, Poulou A performed the colonoscopy, Tyrmipas I and Dandakis D organised the patient data and figures, Bartzokis S helped write and correct the manuscript, Barbati K and Tsamouri M were the pathologists that examined patient biopsies and Soutos D is the director of the Gastroenterology department.

**Correspondence to:** Dimitrios Dandakis, Department of Gastroenterology, Red Cross Hospital of Athens, Ochis 12, 11522 Athens, Greece. [ddandakis@yahoo.com](mailto:ddandakis@yahoo.com)

Telephone: +30-6944798901 Fax: +30-210-8215277

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## INTRODUCTION

Diving is a difficult underwater activity in which environmental conditions can affect body structure and function. Barotrauma is caused by compression during descent or expansion during ascent, of the gas filled spaces of the body and it may be associated with pneumothorax, pneumomediastinum and embolism. Decompression sickness may occur when gas, which has dissolved in tissues at depth, eventually produces bubbles which occasionally result in severe cardiorespiratory and neurological emergencies<sup>[1]</sup>.

Approximately 13% of divers complain of gastrointestinal disturbances upon ascent, while most of them present as aerophagy<sup>[2]</sup>. Rarer, more severe diving-associated gastrointestinal manifestations have been reported in the literature, including a few cases of gastrointestinal barotraumas, mainly gastric rupture<sup>[3-6]</sup> and a case of small bowel infarction due to thrombosis of mesenteric veins<sup>[7]</sup>. We describe a clinical case of ischemic colitis in a 27-year-old male admitted to our emergency department, who manifested abdominal pain while he was in the process of scuba diving 20 meters undersea, followed by bloody diarrhoea as soon as he ascended to sea level. Taking into account his past medical history, the thorough impeccable clinical and laboratory examinations and presence of no other factors predisposing to ischemia of the colon, we assume that a possible relationship between the diving conditions and the pathogenesis of ischemic colitis may exist. This case, is as far as we know, the first report relating scuba diving with acute ischemic colitis.

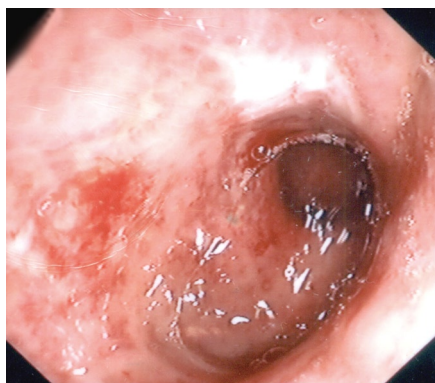
## Abstract

The presentation of clinical symptoms due to decompression during diving, varies significantly, as mainly minor disturbances for the gastrointestinal tract in particular have been reported. The following case debates whether diving can cause severe symptoms from the gastrointestinal system. We describe a clinical case of ischemic colitis presented in a 27-year-old male, who manifested abdominal pain while in the process of scuba diving 20 meters undersea, followed by bloody diarrhoea as soon as he ascended to sea level. Taking into account his past medical history, the thorough, impeccable clinical and laboratory examinations and presence of no other factors predisposing to ischemia of the colon, we assume that a possible relationship between diving conditions and the pathogenesis of ischemic colitis may exist. This unusual case might represent a hematologic manifestation of decompression sickness, due to increased coagulability and/or transient air emboli, occurring during a routine scuba diving ascent to sea level.

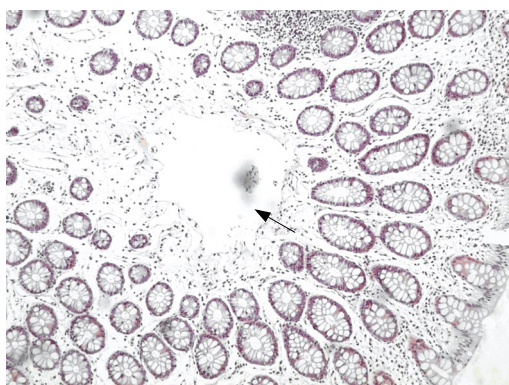
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**Key words:** Air emboli; Barotraumas; Coagulability;





**Figure 1** Endoscopic image of patient's ischemic colitis.

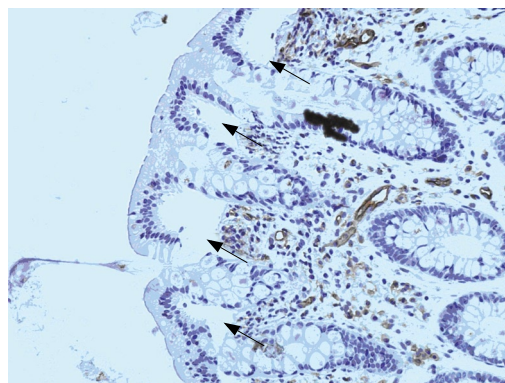


**Figure 2** HE staining of colonic mucosa with an air bubble in the lamina propria (arrow).

## CASE REPORT

A 27-year-old male novice scuba diver came to the emergency department of our hospital, accompanied by his father, suffering from lower abdominal pain with concurrent bloody diarrhoea. He reported that 2 h earlier, he was submerged for approximately 5 min at a depth of 20 meters when he experienced an acute and intense lower abdominal pain with a simultaneous urge to defecate. Further, he insisted that although under stress, his ascent had been completely normal, under control and in accordance with a scheduled dive plan. Immediately after reaching the surface, he had two loose bowel movements followed by bloody diarrhoea and tenesmus. Our patient was a non-smoker, was taking no medication, and his past medical history was limited to a known Gilbert's syndrome. When admitted to the accident and emergency department his vital signs were: blood pressure of 120/80 mmHg, pulse rate of 72 bpm and respiratory rate of approximately 12 breaths/min. Physical examination revealed a moderate abdominal tenderness at the left lower quadrant and a mild abdominal distention, while per rectum examination confirmed the presence of fresh blood in the lumen with no other apparent physical signs of clinical importance detected.

Laboratory results were: Hematocrit (HCT): 47.1%, white cell counts (WCC): 7.800/ $\mu$ L with 63%



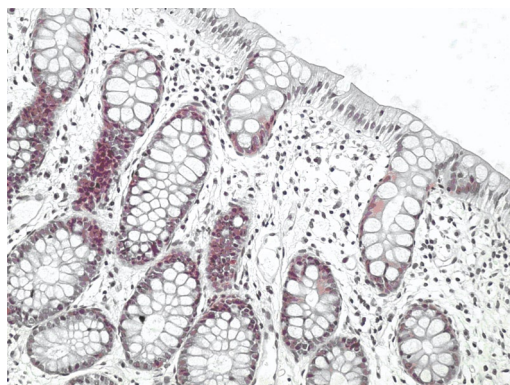
**Figure 3** Immunohistochemistry for CD31 revealing lack of staining in the areas of air bubbles in the lamina propria (arrows).

neutrophils, 27% lymphocytes, 7% monocytes and 3% eosinophils, platelet (PLT): 236.000/ $\mu$ L, total-bilirubin: 2.73 mg/dL, Direct-bilirubin: 0.25 mg/dL and serum lactic dehydrogenase: 223 U/L. Coagulation tests including prothrombin time (PT), international normalized ratio (INR), activated partial thromboplastin time (APTT), fibrinogen, protein S, protein C, antithrombin III and V leiden factor were in the normal range. Urea and electrolytes, antiphospholipid antibodies, serum amylase, blood gases and all other tests including urine analysis were also normal. Chest and abdominal X-rays plus ultrasound examination of both upper and lower abdomen did not reveal any pathological findings. Colonoscopy, with inspection of the terminal ileum, performed 24 h later, revealed an edematous mucosa of the sigmoid colon in extent of 20 cm, with redness, superficial ulcerations and submucosal haemorrhages (Figure 1). The histological study of specimens taken from the affected area showed findings compatible with ischemic colitis, while the pathologist noticed presence of air in the lamina propria but not intravascularly (Figures 2 and 3). No air was detected in the colon wall by magnetic resonance tomography performed 2 d after admission.

The patient recovered uneventfully with bloody diarrhoea ceasing a few hours after admission and abdominal pain progressively diminishing till disappearance in 48 h. The patient was discharged with complete comeback 4 d after admission. The follow-up colonoscopy two mo later showed complete endoscopic and histologic healing of the mucosa (Figure 4) with the patient free of any clinical symptoms.

## DISCUSSION

Abdominal discomfort and belching due to air swallowing are quite frequent manifestations during diving, with more severe gastrointestinal complications scarcely described. Gastric rupture due to expansion of intra-gastric air during a quick ascent to sea level has been described in a few case reports<sup>[3-6]</sup>. Massive pneumoperitoneum without rupture of an abdominal hollow viscous organ, possibly caused by lung



**Figure 4** HE staining of normal histologic appearance of our patient's colonic mucosa, 2 mo after the ischemic colitis episode.

barotrauma has also been reported<sup>[8]</sup>. Gertler *et al* have presented a case of mesenteric vein thrombosis as a unique complication of decompression sickness<sup>[7]</sup>. Finally, we must emphasize that a confirmed case of acute ischemic colitis in association with scuba diving has never been reported.

In our case, the patient's clinical manifestations of ischemic colitis initiated with acute abdominal pain while at a depth of 20 meters under sea, progressing to bloody diarrhoea on the surface. Several environmental parameters during diving can alter body function and structure<sup>[1]</sup>, as for approximately every 10 meters of descent in sea water ambient pressure increases by 100 kPa (1 bar). The air trapped in body cavities including the gastrointestinal tract, is therefore subjected to compression during descent and expansion during ascent to sea level, which may lead to tissue damage such as gastric rupture<sup>[3-6]</sup>. Decompression sickness occurs when the partial pressure of gas trapped in the hollow cavities of the body raises in direct proportion to the increase in ambient pressure. This leads to large volumes of inert gas, mainly nitrogen, which dissolve in tissues while at depth transforming to bubbles during ascent to sea level. In addition breathing workload increases due to a combination of increased gas density, increased hydrostatic pressure and altered respiratory mechanisms. Finally, a number of unpredictable events such as malfunction of a diving equipment or technical issues, as well as panic attacks or hypothermia of the diver, may increase the existing physical and/or psychological stress. In our case, except for the arduous and stress inducing conditions during underwater activity, other factors predisposing to ischemic colitis were not identified.

Our patient's past medical history was free of thrombotic events whilst complete examination and evaluation of the patient, including specific blood tests, did not reveal any type of coagulopathy. On the other hand, the pathophysiologic mechanism of decompression sickness could predispose to vascular obstruction and venous infarction<sup>[9]</sup>. The partial pressure of body gases increases during a scuba dive, resulting in a time-dependent concentration of mainly nitrogen in body fluids and tissues. Bubbles can be formed during

ascent due to rapid decrease of barometric pressure. Bubbles are formed predominately in the venous circulation, although in overwhelming decompression sickness they can be found in the arterial circulation also<sup>[10]</sup>, causing vascular obstruction due to coalition. Although bubble formation is considered as the causative mechanism of decompression sickness, a series of hematological disorders leading to a hypercoagulable state have been described<sup>[9,11]</sup>. *In vitro* experiments concluded that increased vascular permeability, vascular obstruction due to fat emboli and interaction of the bubble surface with the cellular elements of the blood, may contribute to the pathogenesis of decompression sickness<sup>[9,12]</sup>. Mesenteric vein thrombosis<sup>[7]</sup>, retinal artery occlusion<sup>[13]</sup> and vascular obstruction due to fat emboli<sup>[14]</sup>, have previously been reported as unique complications of decompression sickness. The presence of trapped air in the lamina propria, likely of intravascular origin, demonstrated in biopsies taken from the colonic area with ischemic lesions, supports the thesis that decompression sickness was the main cause of our patient's colonic ischemia. Vascular bubbles and bowel wall congestion are the visceral changes described in decompression sickness<sup>[15]</sup>. It has recently been reported that magnetic resonance imaging (MRI) may be superior to autopsy in the demonstration of gas in intraparenchymal blood vessels of internal organs<sup>[16]</sup>. Our patient's MRI scan didn't reveal any intravascular air bubbles, which can be attributed to the 48 h delay between the scan and the onset of the acute episode. Finally, a procedure that may lead to rapid alleviation of decompression sickness symptoms<sup>[3,6,8]</sup> is recompression, which in our case was not implemented due to the rapid clinical improvement and complete recovery of the patient.

In conclusion, this unusual case emphasizes the probability that scuba diving can cause colonic ischemia, even in young patients with no known coagulation disorders or other factors predisposing to colonic ischemia.

## REFERENCES

- 1 **Goddard D**, Currie G, Denison D, Farrell P, Ross J, Stephenson R, Watt S, Wilmshurst P. British Thoracic Society guidelines on respiratory aspects of fitness for diving. *Thorax* 2003; **58**: 3-13
- 2 **Lundgren CE**, Ornhaugen H. Nausea and abdominal discomfort--possible relation to aerophagia during diving: an epidemiologic study. *Undersea Biomed Res* 1975; **2**: 155-160
- 3 **Hunter JD**, Roobottom CA, Bryson PJ, Brown C. Conservative management of gastric rupture following scuba diving. *J Accid Emerg Med* 1998; **15**: 116-117
- 4 **Hayden JD**, Davies JB, Martin IG. Diaphragmatic rupture resulting from gastrointestinal barotrauma in a scuba diver. *Br J Sports Med* 1998; **32**: 75-76
- 5 **Haller C**, Guenot C, Azagury D, Rosso R. [Intestinal barotrauma after diving--mechanical ileus in incarceration of the last loop of the small intestine between a mobile cecum and sigmoid] *Swiss Surg* 2003; **9**: 181-183
- 6 **Petri NM**, Vranjkovic-Petri L, Aras N, Druzijanic N. Gastric rupture in a diver due to rapid ascent. *Croat Med J* 2002; **43**: 42-44

- 7 **Gertler SL**, Stein J, Simon T, Miyai K. Mesenteric venous thrombosis as sole complication of decompression sickness. *Dig Dis Sci* 1984; **29**: 91-95
- 8 **Oh ST**, Kim W, Jeon HM, Kim JS, Kim KW, Yoo SJ, Kim EK. Massive pneumoperitoneum after scuba diving. *J Korean Med Sci* 2003; **18**: 281-283
- 9 **Philp RB**. A review of blood changes associated with compression-decompression: relationship to decompression sickness. *Undersea Biomed Res* 1974; **1**: 117-150
- 10 **Catchpole HR**, Gersh I. Pathogenic factors and pathological consequences of decompression sickness. *Physiol Rev* 1947; **27**: 360-397
- 11 **Philp RB**, Ackles KN, Inwood MJ, Livingstone SD, Achimastos A, Binns-Smith M, Radomski MW. Changes in the hemostatic system and in blood and urine chemistry of human subjects following decompression from a hyperbaric environment. *Aerosp Med* 1972; **43**: 498-505
- 12 **Levin LL**, Stewart GJ, Lynch PR, Bove AA. Blood and blood vessel wall changes induced by decompression sickness in dogs. *J Appl Physiol* 1981; **50**: 944-949
- 13 **Hsu AA**, Wong TM, How J, Tan J, Tan KT. Retinal artery occlusion in a diver. *Singapore Med J* 1992; **33**: 299-301
- 14 **Kitano M**, Hayashi K. Acute decompression sickness--report of an autopsy case with widespread fat embolism. *Acta Pathol Jpn* 1981; **31**: 269-276
- 15 **Waller SO**. Autopsy features in scuba diving fatalities. *Med J Aust* 1970; **1**: 1106-1108
- 16 **Plattner T**, Thali MJ, Yen K, Sonnenschein M, Stoupis C, Vock P, Zwuygart-Brugger K, Kilchor T, Dirnhofer R. Virtopsy-postmortem multislice computed tomography (MSCT) and magnetic resonance imaging (MRI) in a fatal scuba diving incident. *J Forensic Sci* 2003; **48**: 1347-1355

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## CASE REPORT

# A symptomatic cyst of the ligamentum teres of the liver: A case report

Emmanuel E Lagoudianakis, Nikolaos Michalopoulos, Haridimos Markogiannakis, Artemisia Papadima, Konstantinos Filis, Panagiotis Kekis, Vaggelogiannis Katergiannakis, Andreas Manouras

Emmanuel E Lagoudianakis, Nikolaos Michalopoulos, Haridimos Markogiannakis, Artemisia Papadima, Konstantinos Filis, Panagiotis Kekis, Vaggelogiannis Katergiannakis, Andreas Manouras, Department of Propaedeutic Surgery, Hippocrateion Hospital, Athens Medical School, University of Athens, Athens 11527, Greece

**Author contributions:** Lagoudianakis EE and Michalopoulos N wrote the paper; Markogiannakis H, Papadima A, Filis K and Kekis P performed the research; Katergiannakis V and Manouras A analyzed the data and designed the research.

**Correspondence to:** Andreas Manouras, MD, PhD, Associate Professor of General Surgery, 1st Department of Propaedeutic Surgery, Hippocrateion Hospital, Athens Medical School, University of Athens, Q. Sophias 114, Athens 11527, Greece. [amanouras@hippocratio.gr](mailto:amanouras@hippocratio.gr)

Telephone: +30-69-77304422 Fax: +30-210-7707574

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A symptomatic cyst of the ligamentum teres of the liver: A case report. *World J Gastroenterol* 2008; 14(20): 3266-3268 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3266.asp>

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## INTRODUCTION

Ligamentum teres (or round ligament) of the liver is a cord-like ligament found within the falciform ligament on the inner surface of the anterior abdominal wall and represents a remnant of the umbilical vein, which is a connecting venous structure between the placenta and the umbilical portion of the left portal vein. It is located at the dorsal free margin of the falciform ligament.

Lesions of the liver ligaments are extremely rare and cysts of the falciform ligament have been previously reported<sup>[1-3]</sup>.

The aetiology of these cysts is not well understood yet and their clinical manifestations vary a lot. They may appear completely asymptomatic or may produce palpable masses and pain. The differential diagnosis in such cases includes benign or malignant tumors arising from the liver ligaments or the abdominal wall, fatty masses, disseminated cancer and hepatic lesions.

CT scan, although necessary, may not well define the nature and origin of these masses. Definite diagnosis is made based on laparotomy and pathologic examination of the surgical specimen.

We report a case of a symptomatic patient with a cyst of the ligamentum teres of the liver treated with total excision. The pathologic features of this cyst are also presented.

## CASE REPORT

A 57-year old woman was referred to our department due to right upper quadrant pain and episodes of vomiting during the last 2 years. The pain was mild, experienced at irregular periods of time and had no relation to meals. The patient did not have any previous medical history. She was a non-smoker and did not receive any medication in a regular basis. Physical examination was unremarkable. Complete blood cell count, electrolytes,

## Abstract

Cysts of the liver ligaments are extremely rare and cysts of the ligamentum teres of the liver have been sporadically reported in the literature during the last century. The present report describes a case of a symptomatic patient with a cyst of the ligamentum teres of the liver. The patient presented with right upper quadrant pain and indigestion during the last 2 years. Ultrasound and computed tomography scans revealed a water-density mass attached to the anterior abdominal wall, but definite diagnosis could not be reached. The cyst was completely excised during laparotomy. Cysts of the ligamentum teres of the liver, although infrequent, may produce clinical symptoms and require excision. Ultrasound and computed tomography scan preoperatively cannot rule out malignancy, thus exploratory laparotomy and total resection of these lesions are necessary.

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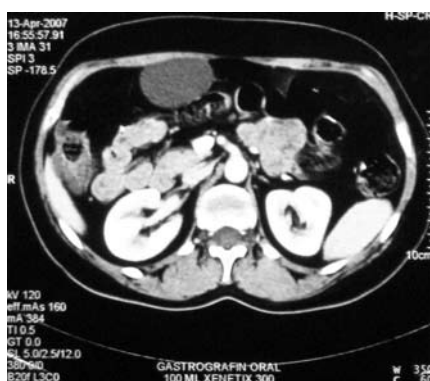
**Key words:** Ligamentum teres mass; Liver cyst; Right upper quadrant mass; Congenital liver cyst

**Peer reviewer:** Gianluigi Giannelli, MD, Dipartimento di Clinica Medica, Immunologia e Malattie Infettive, Sezione di Medicina Interna, Policlinico, Piazza G. Cesare 11, Bari 70124, Italy





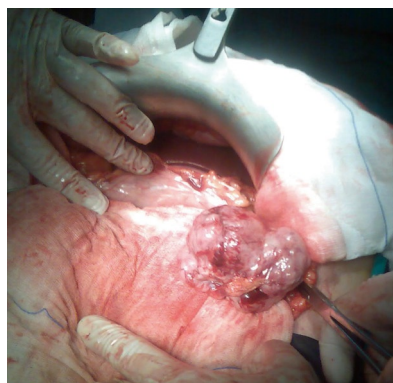
**Figure 1** Ultrasound appearance of the cystic abdominal mass.



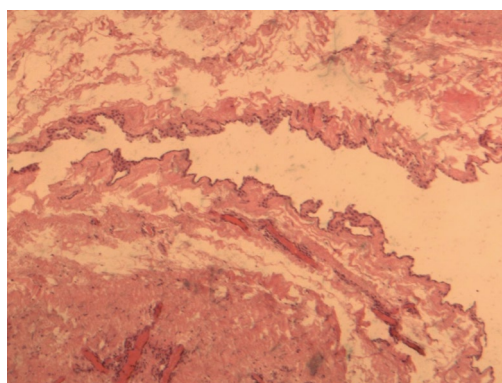
**Figure 2** Abdominal CT scan with intravenous contrast media used showing a water-density mass attached to the anterior abdominal wall. A well circumscribed area of low attenuation in contact with the abdominal wall is identified.

eosinophil count, serum biochemistry and urinalysis were within normal limits. Levels of serous neoplastic markers, such as carbohydrate antigen 19-9 (CA 19-9), carcinoembryonic antigen (CEA), carbohydrate antigen 125 (CA 125), and  $\alpha$ -fetoprotein (AFP) were normal as well. Anti-echinococcal IgM and IgG antibodies and viral markers for hepatitis B and C were negative. Chest X-ray was normal. Plain radiographs of the abdomen did not reveal any pathological entity. The patient underwent ultrasound examination (US) that showed a 4.7 cm  $\times$  3.5 cm cystic mass on the anterior surface of the liver (Figure 1). CT scanning demonstrated the presence of a 5 cm  $\times$  4 cm  $\times$  7 cm solitary water-density mass, in contact with the right rectus abdominis muscle, showing no enhancement after intravenous contrast media injection (Figure 2). No ascites, lymphnodes or other intra-abdominal masses were found either in the liver or in the peritoneum.

The cyst was removed without rupture by a midline abdominal incision (Figure 3). The cyst's origin was at the attachment between the ligamentum teres and the anterior abdominal wall. It was neither drained nor aspirated during the procedure. An appropriate dissection plane between the cyst and the ligament could easily be found as it was not hard and adhesive. The exploration of the rest of the peritoneal cavity did not reveal any other lesions. Macroscopic examination showed a circumscribed serous cyst, 5 cm in diameter, with a thick fibrous wall of 1 mm. Microscopically, the wall of the



**Figure 3** The cyst is shown originating from the ligamentum teres.



**Figure 4** Hematoxylin-eosin staining of the cyst wall shows the single layered cuboidal epithelium ( $\times$  40).

lesion was composed of a single layered cuboidal epithelium. No signs of malignancy were identified (Figure 4). The patient's postoperative course was uneventful. The patient was discharged on the second postoperative day. After 6 mo, the patient was well and in good condition with no further symptoms.

## DISCUSSION

Unusual lesions reported in the falciform ligament and ligamentum teres of the liver are lipoma<sup>[4-6]</sup>, paraganglioma<sup>[7]</sup>, lymphangioma<sup>[8]</sup> and leiomyosarcoma<sup>[9-14]</sup>.

Moreover, simple serous cysts of the falciform ligament<sup>[1-3,15]</sup> and the ligamentum teres of the liver have been sporadically reported in the literature<sup>[16-20]</sup>.

The etiology of liver ligament cysts is diverse and the causes have been classified into primary and secondary<sup>[1]</sup>. Primary cysts arise during development from congenital defects of mesenteric origin<sup>[21]</sup>. Secondary cysts are the result of infections (echinococcus, abscesses), trauma (hematomas and biliary leaks) and neoplasms with cystic degeneration. Partial obliteration of the umbilical veins has also been reported to cause falciform ligament cysts<sup>[20]</sup>. Pathology report suggested the congenital origin of the cyst in our case.

The main symptoms of these patients are unclear and have been reported to include vague abdominal pain and indigestion. Physical examination may sometimes demonstrate a palpable right upper quadrant mass as well.

Our patient was treated with laparotomy to reach a definite diagnosis. Intraoperative findings included a solitary cyst, 5 cm × 5 cm in size, originating from the round ligament of the liver, which was attached to the right rectus abdominis muscle. It was easily dissected since it contained no hard adhesions to the abdominal wall and totally removed. Laparotomy allowed complete inspection of the abdomen to exclude other intra-abdominal masses or lymphnodes.

Ultrasound and computed tomography scans are essential to identify the solid or cystic nature of such lesions but cannot provide a definite diagnosis. In a similar previous report, a falciform ligament cyst has been suggested at CT by a water-density mass at the caudal aspect of the left intersegmental fissure<sup>[3]</sup>. Nevertheless, a significant number of other benign or malignant lesions have been previously presented as mentioned above.

As a result, the differential diagnosis of a right upper quadrant mass includes several clinical entities and radiological findings can only imply but not ensure a definite diagnosis.

## REFERENCES

- 1 **Bryan DH**, Pillarisetty S. Cyst of the falciform ligament of the liver: a rare cause of right upper quadrant pain. *Am Surg* 1992; **58**: 779-781
- 2 **Brock JS**, Pachter HL, Schreiber J, Hofstetter SR. Surgical diseases of the falciform ligament. *Am J Gastroenterol* 1992; **87**: 757-758
- 3 **Enterline DS**, Rauch RE, Silverman PM, Korobkin M, Akwari OE. Cyst of the falciform ligament of the liver. *AJR Am J Roentgenol* 1984; **142**: 327-328
- 4 **Farkas E**, Besznyak I, Koves I. [Giant lipoma of the ligamentum teres hepatic] *Orv Hetil* 1991; **132**: 637-638
- 5 **Kakitsubata Y**, Nakamura R, Shiba T, Sugimura H, Suzuki Y, Kakitsubata S, Watanabe K, Kawana T, Iwamura T. Lipoma of the falciform ligament: US, CT, and MRI appearances. *Clin Imaging* 1993; **17**: 27-29
- 6 **Honda H**, Watanabe K, Mihara K, Hoshi H, Sakihama M. Lipoma of the hepatic falciform ligament. *J Comput Assist Tomogr* 1983; **7**: 170
- 7 **Delbridge L**, Connolly J. Paraganglioma of the falciform ligament: a case report. *Aust N Z J Surg* 1982; **52**: 315-317
- 8 **Morgan K**, Ricketts RR. Lymphangioma of the falciform ligament--a case report. *J Pediatr Surg* 2004; **39**: 1276-1279
- 9 **Okajima K**, Kobayashi M, Mannami T, Takeuchi Y. [Primary leiomyosarcoma of the peritoneum--report of a rare case arising in the falciforme ligament of the liver] *Gan No Rinsho* 1968; **14**: 500-507
- 10 **Morita Y**, Saito H, Okushiba S, Shinohara M, Satoh N, Uchino J. [A case report of leiomyosarcoma of the hepatic falciform ligament--clinical significance of the hepatic falciform artery] *Rinsho Hoshasen* 1987; **32**: 1613-1616
- 11 **Yamaguchi J**, Azuma T, Fujioka H, Tanaka K, Furui J, Tomioka T, Kanematsu T. Leiomyosarcoma occurring in the ligamentum teres of the liver: a case report and a review of seven reported cases. *Hepatogastroenterology* 1996; **43**: 1051-1056
- 12 **Tomaszewski MM**, Kuenster JT, Hartman K. Leiomyosarcoma of ligamentum teres of liver: case report. *Pediatr Pathol* 1986; **5**: 147-156
- 13 **Adachi M**, Sugita T, Maehara M, Sugaya H, Ihori M, Hisauchi T, Harada T, Kogure H, Tajima Y. A case report of leiomyosarcoma originating in the ligamentum teres of the liver. *Gastroenterol Jpn* 1979; **14**: 238-242
- 14 **Mital RN**, Bazaz-Malik G. Leiomyosarcoma of ligamentum teres of the liver. *Am J Gastroenterol* 1971; **56**: 48-51
- 15 **Gondring WH**. Solitary cyst of the falciform ligament of the liver; report of a case and review of the literature. *Am J Surg* 1965; **109**: 526-529
- 16 **Henderson MS**. Cyst of the Round Ligament of the Liver. *Ann Surg* 1909; **50**: 550-551
- 17 **Seniutovich VF**, Genyk SN, Krysa VM. [Serous cyst of the round ligament of the liver] *Khirurgiia* (Mosk) 1976; **128**
- 18 **Krylov LB**, Shurkalin BK. [A solitary cyst of the round ligament of the liver] *Khirurgiia* (Mosk) 1968; **44**: 140-141
- 19 **Smirnov VE**, Pravdin IS. [Serous cyst of the round ligament of the liver] *Vestn Khir Im I I Grek* 1989; **143**: 60-61
- 20 **Karabin JE**. Cyst in the ligamentum teres of the liver, remnant of the umbilical vein. *Am J Surg* 1951; **82**: 531-532
- 21 **Tessari R**. [Congenital cysts of the falciform ligament of the liver] *Minerva Med* 1970; **61**: 625-632

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## Combined choriocarcinoma, neuroendocrine cell carcinoma and tubular adenocarcinoma in the stomach

Yasumitsu Hirano, Takuo Hara, Hiroshi Nozawa, Kaeko Oyama, Naohiro Ohta, Kenji Omura, Go Watanabe, Hideki Niwa

Yasumitsu Hirano, Kenji Omura, Go Watanabe, Department of General and Cardiothoracic Surgery, Kanazawa University Graduate school of Medical Science 13-1, Takara-machi, Kanazawa 920-8641, Japan

Takuo Hara, Hiroshi Nozawa, Kaeko Oyama, Naohiro Ohta, Department of Surgery, Kouseiren Takaoka Hospital 5-10, Eiraku-chou, Takaoka 933-8555, Japan

Hideki Niwa, Department of Pathology, Kouseiren Takaoka Hospital 5-10, Eiraku-chou, Takaoka 933-8555, Japan

**Author contributions:** Hirano Y, Hara T, Nozawa H, Oyama K and Ohta N contributed equally to this case; Niwa H contributed to pathological diagnosis; and Hirano Y, Hara T, Omura K and Watanabe G wrote the paper.

**Correspondence to:** Yasumitsu Hirano, MD, PhD, Department of General and Cardiothoracic Surgery, Kanazawa University Graduate School of Medical Science 13-1, Takara-machi, Kanazawa 920-8641, Japan. [yasumitsu@ira.yamaguchi-u.ac.jp](mailto:yasumitsu@ira.yamaguchi-u.ac.jp)  
Telephone: +81-76-265-2354 Fax: +81-76-222-6833

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**Peer reviewers:** Takayuki Yamamoto, MD, Inflammatory Bowel Disease Center, Yokkaichi Social Insurance Hospital, 10-8 Hazuyamacho, Yokkaichi 510-0016, Japan; Hiroki Nakamura, MD, Department of Gastroenterology and Hepatology, 1-1-1, Minami Kogushi, Ube, Yamaguchi 755-8505, Japan

Hirano Y, Hara T, Nozawa H, Oyama K, Ohta N, Omura K, Watanabe G, Niwa H. Combined choriocarcinoma, neuroendocrine cell carcinoma and tubular adenocarcinoma in the stomach. *World J Gastroenterol* 2008; 14(20): 3269-3272 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3269.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3269>

### Abstract

We described a patient with adenocarcinoma of the stomach combined with choriocarcinoma and neuroendocrine cell carcinoma. An 85-year-old man visited our hospital because of appetite loss. Gastric fiberoscopy revealed a large tumor occupying the cardinal region and anterior wall of the gastric body. The patient underwent total gastrectomy with lymphnode dissection and partial resection of the liver. Choriocarcinoma, small cell carcinoma and tubular adenocarcinoma existed in the gastric tumor. The choriocarcinomatous foci contained cells positive for beta-subunit of human chorionic gonadotropin (B-hCG) and human placental lactogen mainly in syncytiotrophoblastic cells. The small cell carcinomatous foci contained cells positive for synaptophysin, neuron-specific enolase (NSE), and chromogranin A. The prognosis for gastric adenocarcinoma with choriocarcinoma and neuroendocrine cell carcinoma is exceedingly poor. This patient died about 2 mo after the first complaint from hepatic failure. This is the first reported case of gastric cancer with these three pathological features.

### INTRODUCTION

Primary carcinoma of the stomach is almost always adenocarcinoma or signet ring cell carcinoma and there have been few reports of choriocarcinoma<sup>[1-5]</sup> or neuroendocrine cell carcinoma<sup>[6-9]</sup>. We report a patient with adenocarcinoma of the stomach combined with choriocarcinoma and neuroendocrine cell carcinoma. This is the first reported case of gastric cancer with these three pathological features.

### CASE REPORT

An 85-year-old man was admitted to Kouseiren Takaoka Hospital because of appetite loss in March 2004. He had been treated for hypertension and gout in another hospital. His family history was negative for family and hereditary disease. On examination, the patient was pale because of severe anemia, and had an ill-defined mobile left hypochondrial mass, approximately 10 cm in size. Findings for the chest and heart were normal. Lymphadenopathy, hepatomegaly, and splenomegaly were not observed and the testes and breasts were normal. Blood examination showed severe anemia, leukocytosis, and platelet count was increased. The level of serum carcinoembryonic antigen (CEA) was slightly elevated (Table 1). Radiographic examination of the



Table 1 Laboratory data of the patient on admission

	Data
CBC	
WBC	12100/ $\mu$ L
RBC	$234 \times 10^4$ / $\mu$ L
Hb	6.3 g/dL
Ht	20.8%
Plts	$51.9 \times 10^4$ / $\mu$ L
Blood chemistry	
T-Bil	0.4 mg/dL
D-Bil	0.4 mg/dL
AST	22 IU/L
ALT	13 IU/L
LDH	260 IU/L
ALP	365 IU/L
ZTT	10.9 K-U
TTT	3.6 M-U
Ch-E	61 IU/L
$\gamma$ -GTP	27 IU/L
T-AMY	189 IU/L
CPK	41 IU/L
Na	135 mEq/L
K	4.2 mEq/L
Cl	102 mEq/L
Ca	8.4 mg/dL
Fe	16 $\mu$ g/dL
BUN	20.3 mg/dL
Cr	1.3 mg/dL
UA	4.2 mg/dL
Tch	141 mg/dL
TG	92 mg/dL
FBS	123 mg/dL
TP	6.2 g/dL
Alb	3.1 g/dL
Tumor marker	
AFP	7.5 ng/mL
CEA	5.4 ng/mL
CA19-9	< 2.0 ng/mL

upper gastrointestinal tract demonstrated a Borrmann type 1 tumor in the cardia. Gastric fiberoscopy revealed a large tumor occupying the cardial region and anterior wall of the gastric body accompanied by areas of hemorrhage. Tumor invasion to the esophagus was highly suspected. Biopsy specimens were interpreted as showing adenocarcinoma without features of choriocarcinoma or neuroendocrine cell carcinoma. Contrast-enhanced computed tomography (CT) of the abdomen showed a 7-cm low-density tumor suspected to be regional lymph node metastasis. Liver and lung metastases and abnormality with his testes and breast were not detected radiologically. The patient underwent total gastrectomy on March 15, 2004, with the preoperative diagnosis of primary gastric carcinoma. Liver metastasis, peritoneal dissemination, and ascites were not investigated, and distant metastasis to other organs was not present. There was an invasive tumor encircling the gastric body and cardia and this tumor was invading the liver. Total gastrectomy with lymphnode dissection and partial resection of the liver were performed. The Roux-en-Y method of reconstruction was performed after resection.

### Gross findings

The resected specimen included an elevated tumor with

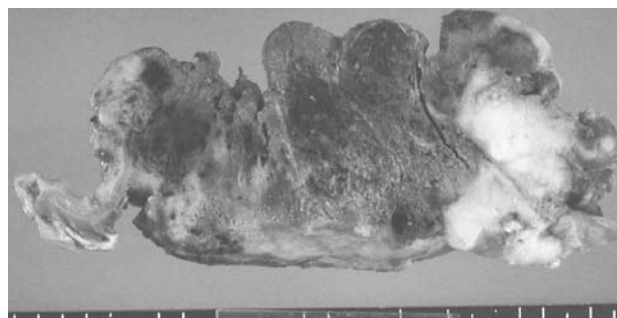


Figure 1 Cut surfaces of the tumor demonstrate two different features, a hemorrhagic brown area and a whitish-yellow area.

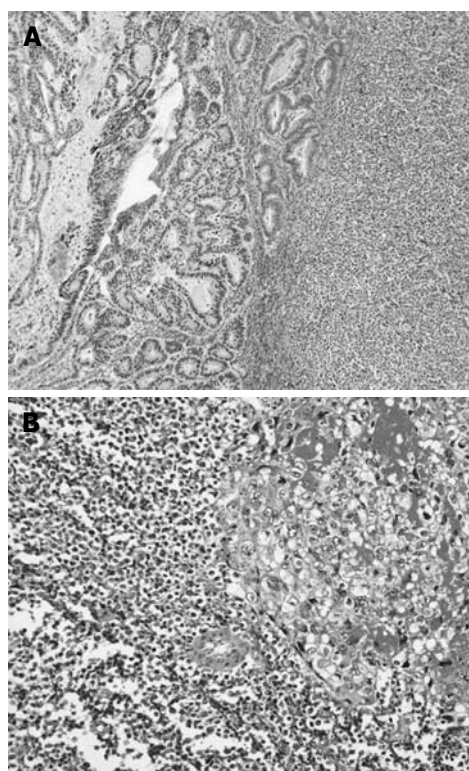


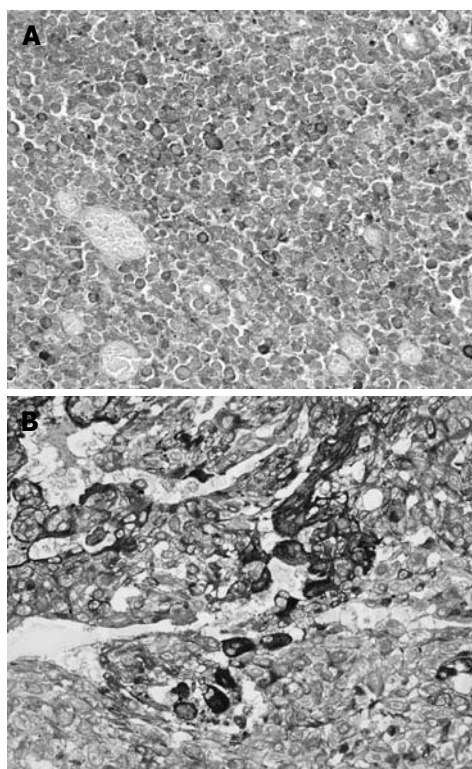
Figure 2 A: The hemorrhagic brown area is composed of choriocarcinoma; B: The whitish-yellow area contains small cell carcinoma.

ulcer measuring 12.0 cm  $\times$  11.5 cm in the cardia, the body of the stomach, and abdominal esophagus, and the tumor had invaded the liver. Cut surfaces of the tumor showed two different features, a hemorrhagic brown area and a whitish-yellow area. Most of the tumor was composed of the hemorrhagic brown area (Figure 1).

### Histopathological findings

The hemorrhagic brown area was composed of choriocarcinoma, and consisted mostly of clusters of cytotrophoblastic cells separated by steaming masses of syncytiotrophoblasts (Figure 2A). Cytotrophoblastic cells were small cells with large, oval nuclei, and syncytiotrophoblasts were large cells with bizzare nuclei. The whitish-yellow area contained small cell carcinoma, consisting of small amounts of cytoplasm with large nuclei (Figure 2B) and tubular adenocarcinoma.





**Figure 3** A: The choriocarcinomatous foci contain cells positive for B-hCG; B: The small cell carcinomatous foci contain cells positive for chromogranin A.

### Immunohistochemical findings

The choriocarcinomatous foci contained cells positive for beta-subunit of human chorionic gonadotropin (B-hCG) and human placental lactogen mainly in syncytiotrophoblastic cells (Figure 3A). These findings enabled us to diagnose these cells as choriocarcinoma. The small cell carcinomatous foci contained cells positive for synaptophysin, neuron-specific enolase (NSE), and chromogranin A (Figure 3B). From these results, we diagnosed them as neuroendocrine cell carcinoma.

### Outcome

The patient was discharged uneventfully 3 wk after surgery. He presented to our hospital with general malaise 2 wk after discharge. CT revealed multiple liver tumors, and his serum hCG level was 67 000 IU/mL. The liver tumor progressed, the patient died eventually from hepatic failure 6 wk after operation.

## DISCUSSION

Choriocarcinoma can be gonadal or extragonadal in origin, and most often arises in the uterus in association with pregnancy<sup>[10]</sup>. The most common sites for extragonadal tumors are the mediastinum, ovary and testis<sup>[11]</sup>. There are many reported cases with metastatic choriocarcinoma to the stomach<sup>[12]</sup>, but primary choriocarcinomas of the stomach are extremely rare. Primary neuroendocrine carcinomas are known to arise in the stomach, although they are also rare. Motoyama *et al.*<sup>[13]</sup> reported a case of combined choriocarcinoma, hepatoid carcinoma, small cell carcinoma, and tubular

adenocarcinoma in the esophagus in 1995, but there has been no reported case of combined choriocarcinoma, neuroendocrine carcinoma and tubular adenocarcinoma in the stomach in the English-language literature.

There are several theories of the histopathogenesis of primary choriocarcinoma of the stomach. These hypotheses include origin from a gonadal angle displaced in the abdomen<sup>[14]</sup>, histological resemblance to choriocarcinoma<sup>[10]</sup>, origin from an underlying gastric teratoma<sup>[15]</sup>, and the retrodifferentiation or opisthoplatia of carcinoma cells to the level of the embryonal ectoderm with the ability to form trophoblasts<sup>[16]</sup>. The finding that gastric choriocarcinomas are frequently accompanied by adenocarcinoma is supported by this retrodifferentiation theory. In the present case, choriocarcinomas, neuroendocrine carcinomas and tubular adenocarcinoma existed in the same tumor of the stomach, and this finding suggests that choriocarcinoma and neuroendocrine carcinoma represent aberrant differentiation in common adenocarcinoma.

In the present case, we failed to diagnose adenocarcinoma combined with choriocarcinoma and neuroendocrine carcinoma before operation based on pathological examination of biopsy specimens. Therefore, larger biopsy specimens from the whole tumor should be taken when encountering large and hemorrhagic tumors so that pathologic components are not missed.

It is well known that choriocarcinomas and neuroendocrine carcinomas readily metastasize to distant organs and carry a poor prognosis because effective regimens have not been established. Further studies to establish new regimens are required.

## REFERENCES

- 1 Imai Y, Kawabe T, Takahashi M, Matsumura M, Komatsu Y, Hamada E, Niwa Y, Kurita M, Shiina S, Shimada T. A case of primary gastric choriocarcinoma and a review of the Japanese literature. *J Gastroenterol* 1994; **29**: 642-646
- 2 Liu Z, Mira JL, Cruz-Caudillo JC. Primary gastric choriocarcinoma: a case report and review of the literature. *Arch Pathol Lab Med* 2001; **125**: 1601-1604
- 3 Kobayashi A, Hasebe T, Endo Y, Sasaki S, Konishi M, Sugito M, Kinoshita T, Saito N, Ochiai A. Primary gastric choriocarcinoma: two case reports and a pooled analysis of 53 cases. *Gastric Cancer* 2005; **8**: 178-185
- 4 Dye DW, Broadwater R, Lamps LW. Uncommon malignancies: case 2. Gastric choriocarcinoma. *J Clin Oncol* 2005; **23**: 6251-6253
- 5 Liu Z, Mira JL, Cruz-Caudillo JC. Primary gastric choriocarcinoma: a case report and review of the literature. *Arch Pathol Lab Med* 2001; **125**: 1601-1604
- 6 Kuroda N, Oonishi K, Iwamura S, Ohara M, Hirouchi T, Mizumo K, Miyazaki E, Enzan H. Gastric carcinosarcoma with neuroendocrine differentiation as the carcinoma component and leiomyosarcomatous and myofibroblastic differentiation as the sarcomatous component. *APMIS* 2006; **114**: 234-238
- 7 Shpaner A, Yusuf TE. Primary gastric small-cell neuroendocrine carcinoma. *Endoscopy* 2007; **39** Suppl 1: E310-E311
- 8 Fujiyoshi Y, Eimoto T. Chromogranin A expression correlates with tumour cell type and prognosis in signet ring cell carcinoma of the stomach. *Histopathology* 2008; **52**:

- 305-313
- 9 **Anjaneyulu**, Rao SC, Rao RV. Primary choriocarcinoma of stomach. *Indian J Pathol Microbiol* 2000; **43**: 471-474
- 10 **Noguchi T**, Takeno S, Sato T, Takahashi Y, Uchida Y, Yokoyama S. A patient with primary gastric choriocarcinoma who received a correct preoperative diagnosis and achieved prolonged survival. *Gastric Cancer* 2002; **5**: 112-117
- 11 **Moran CA**, Suster S. Primary mediastinal choriocarcinomas: a clinicopathologic and immunohistochemical study of eight cases. *Am J Surg Pathol* 1997; **21**: 1007-1012
- 12 **Lombard F**, Burtin P, Ketani S, Delaby J, Cales P, Boyer J. Mediastinal posterior choriocarcinoma with hemorrhagic gastric metastasis: endosonographic features. *Gastrointest Endosc* 1992; **38**: 187-190
- 13 **Motoyama T**, Higuchi M, Taguchi J. Combined choriocarcinoma, hepatoid adenocarcinoma, small cell carcinoma and tubular adenocarcinoma in the oesophagus. *Virchows Arch* 1995; **427**: 451-454
- 14 **Nakao A**, Sakagami K, Uda M, Mitsuoka S, Yamashita N, Ito H. Gastric carcinoma with predominant choriocarcinomatous component. *Int J Clin Oncol* 1998; **3**: 403-405
- 15 **Regan JF**, Cremin JH. Chorionepithelioma of the stomach. *Am J Surg* 1960; **100**: 224-233
- 16 **Hartz PH**, Ramirez CA. Coexistence of carcinoma and chorioepithelioma in the stomach of a young man. *Cancer* 1953; **6**: 319-326

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## Pneumatosis cystoides intestinalis after fluorouracil chemotherapy for rectal cancer

Kenji Mimatsu, Takatsugu Oida, Atsushi Kawasaki, Hisao Kano, Youichi Kuboi, Osamu Aramaki, Sadao Amano

Kenji Mimatsu, Takatsugu Oida, Atsushi Kawasaki, Hisao Kano, Youichi Kuboi, Osamu Aramaki, Department of Surgery, Social Insurance Yokohama Central Hospital, Kanagawa 231-8553, Japan

Sadao Amano, Department of Surgery, Nihon University School of Medicine, Tokyo 173-0032, Japan

**Author contributions:** Mimatsu K, Oida T, Kawasaki A, Kano H, Kuboi Y and Aramaki O carried out the operation and were consultant overseeing the patient's care; Mimatsu K wrote the manuscript; Amano S was responsible for drafting the manuscript and revising it critically.

**Correspondence to:** Kenji Mimatsu, MD, Department of Surgery, Social Insurance Yokohama Central Hospital, 268 Yamashita-cho Naka-ku Yokohama, Kanagawa 231-8553, Japan. [mimatsu.kenji@yokochu.jp](mailto:mimatsu.kenji@yokochu.jp)

Telephone: +81-45-6411921 Fax: +81-45-6719872

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### INTRODUCTION

Fluorouracil (FU) is one of the most commonly used chemotherapeutic agents in clinical oncology regimens. With regard to colorectal cancer, treatment involving FU with leucovorin (LV) can improve the survival, tumor response and quality of life<sup>[1]</sup> of patients. We report a case of pneumatosis cystoides intestinalis (PCI) in a patient who received adjuvant chemotherapy with 5-FU and l-LV<sup>[2]</sup>. To our knowledge, FU-related or FU-induced PCI has not been reported previously. This case will add to the reported series of patients with FU-induced small bowel toxicity<sup>[3,4]</sup> and chemotherapy-related PCI<sup>[5-9]</sup>.

### CASE REPORT

A 76-year-old male underwent anterior resection for stage III rectal cancer. He received an adjuvant chemotherapy protocol comprising intravenous bolus injection of 600 mg/m<sup>2</sup> 5-FU at 1 h after the initiation of 2 h-long 250 mg/m<sup>2</sup> l-LV infusion, once a week for 6 wk, followed by 2 wk of rest<sup>[2]</sup>. After 1 cycle of this treatment, the patient presented with diarrhea and abdominal pain. Although his abdomen was distended, he did not exhibit any peritoneal signs. He was afebrile and had no neutropenia. His stool culture was negative. An abdominal radiogram revealed the presence of free air under the diaphragm and intramural gas in the entire intestine (Figure 1). Abdominal computed tomography (CT) revealed the presence of free air in the intestinal wall, retroperitoneal space (Figure 2A), and falciform ligament (Figure 2B). Since bowel perforation was strongly suspected, an emergency operation was performed. Laparotomy revealed pneumatosis of the intestine (Figure 3) and colon, and pneumoretroperitoneum without evidence of perforation. Therefore, gastrostomy was performed to reduce the pressure in the bowel. PCI was

### Abstract

Pneumatosis cystoides intestinalis (PCI) is a relatively rare condition characterized by intraluminal gas in the gastrointestinal tract. Several chemotherapeutic agents have been reported to be associated with PCI, although fluorouracil-related PCI is extremely rare. We report a case of a 76-year old man who received adjuvant chemotherapy for rectal cancer with fluorouracil (FU) and leucovorin (LV). After 1 cycle of the treatment, he presented with diarrhea and abdominal pain. Abdominal radiogram revealed the presence of free air under the diaphragm and intramural gas in the intestine. Laparotomy was performed, showing a suspected diagnosis of perforation in the gastrointestinal tract. Intraoperative findings revealed pneumatosis of the intestine without evidence of perforation. He was treated supportively and his symptoms improved. In conclusion, we should consider the possibility of PCI occurring in patients with malignancies during chemotherapy treatment.

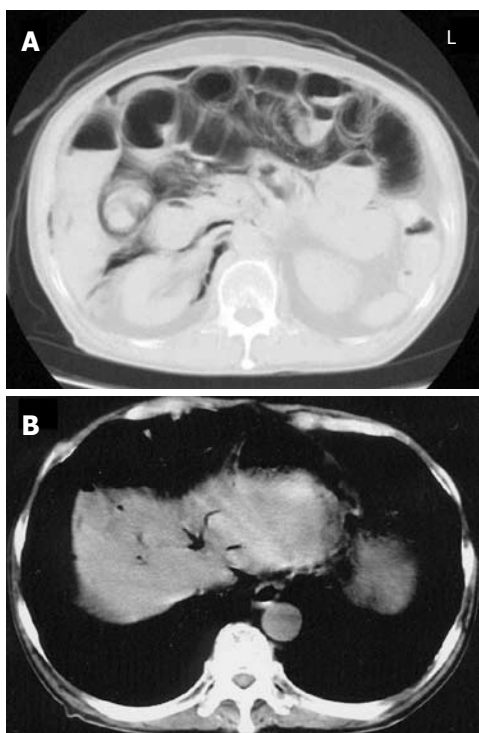
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**Key words:** Pneumatosis cystoides intestinalis; Chemotherapy; Fluorouracil; Colorectal cancer

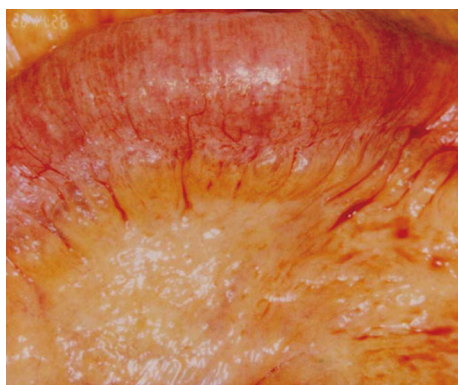
**Peer reviewer:** Damian Casadesus Rodriguez, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado,



**Figure 1** Abdominal radiogram showing intraluminal gases in the entire small intestine and free air under the diaphragm.



**Figure 2** Abdominal CT scan showing excessive intraluminal gases in the entire small intestine and free air in the retroperitoneal space (A), and free air in the falciform ligament (B).



**Figure 3** Expanded intraluminal air spaces in the small intestine and mesenterium during intra-operation.

disappeared within 2 wk after parenteral nutrition, antibiotic treatment and oxygen therapy. Enema showed no

incidence of anastomotic stenosis and he administered oral uracil-ftorafur, and no recurrence of PCI was observed during the 1-year follow-up.

## DISCUSSION

PCI is relatively rare condition characterized by multiple intraluminal gases existing in any part of the gastrointestinal tract. The mechanism and etiology of PCI are not fully understood. According to most hypotheses, mechanical and bacterial factors are the predominant causes for PCI<sup>[10-12]</sup>. However, in this present case, no mechanical or bacterial factors, including bowel ischemia, bowel obstruction<sup>[13,14]</sup>, inflammatory bowel disease and infectious colitis, for the gas production in the intestinal wall were observed.

Several chemotherapeutic agents have been reported to be associated with PCI, including cyclophosphamide, cytarabine, vincristine, doxorubicin, daunorubicin, etoposide, docetaxel, irinotecan and cisplatin<sup>[5-9]</sup>. Although fluorouracil-related PCI has not been previously described, the cytotoxic effect of chemotherapy on the epithelial bowel can also play a role in the pathogenesis of PCI<sup>[7]</sup>. Because the intestinal mucosa is highly proliferative, mucosal damage occurs easily during chemotherapy<sup>[6]</sup>. Moreover, the chemotherapeutic agent might interfere with the mucosal integrity of the intestinal tract, resulting in extensive intramural air<sup>[8]</sup>. Tamura *et al*<sup>[15]</sup> reported that PCI following chemotherapy might be due to depletion of submucosal lymphoid tissue or leukemic infiltrates, such as denuded Peyer's patches producing mucosal defects, thereby permitting entry of gas into the bowel wall. It was reported that chemotherapy-related PCI occurs due to immunosuppressive treatment for hematological malignancies<sup>[5,6]</sup>. Neutropenia is an important factor for the development of PCI<sup>[5-9]</sup>. However, the current patient did not suffer from neutropenia before or when PCI was diagnosed.

Several studies have reported severe erosion and superficial ulceration in the ileum after chemotherapy comprising 5-FU and LV in colon cancer patients<sup>[3,4]</sup>. The mechanisms are thought to be multifactorial, including alteration in the local mucosal blood flow and thrombogenic and vasospastic effects of 5-FU on the vascular epithelium<sup>[5]</sup>. The mechanism underlying 5-FU-induced PCI is thought to be multifactorial, including bowel toxicity caused by 5-FU itself.

In conclusion, although PCI is a rare complication of chemotherapy, the possibility of PCI occurring in patients undergoing chemotherapy should be kept in mind.

## ACKNOWLEDGMENTS

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## REFERENCES

- 1 Poon MA, O'Connell MJ, Wieand HS, Krook JE, Gerstner JB, Tschetter LK, Levitt R, Kardinal CG, Mailliard JA.



- Biochemical modulation of fluorouracil with leucovorin: confirmatory evidence of improved therapeutic efficacy in advanced colorectal cancer. *J Clin Oncol* 1991; **9**: 1967-1972
- 2 **Yoshino M**, Ota K, Kurihara M, Akazawa S, Tominaga T, Sasaki T, Konishi T, Kodaira S, Kumai K, Sugano K. [Late phase II trial of high-dose I-leucovorin and 5-fluorouracil in advanced colorectal carcinoma. I-Leucovorin and 5-FU Study Group (Japan Eastern Group)] *Gan To Kagaku Ryoho* 1995; **22**: 785-792
- 3 **Fata F**, Ron IG, Kemeny N, O'Reilly E, Klimstra D, Kelsen DP. 5-fluorouracil-induced small bowel toxicity in patients with colorectal carcinoma. *Cancer* 1999; **86**: 1129-1134
- 4 **Bucaloiu ID**, Dubagunta S, Pachipala KK, Kamal N, Fata F. Small-cell cancers, and an unusual reaction to chemotherapy: Case 4. Fluorouracil-related small bowel vasculitis. *J Clin Oncol* 2003; **21**: 2442-2443
- 5 **Galm O**, Fabry U, Adam G, Osieka R. Pneumatosis intestinalis following cytotoxic or immunosuppressive treatment. *Digestion* 2001; **64**: 128-132
- 6 **Hashimoto S**, Saitoh H, Wada K, Kobayashi T, Furushima H, Kawai H, Shinbo T, Funakoshi K, Takahashi H, Shibata A. Pneumatosis cystoides intestinalis after chemotherapy for hematological malignancies: report of 4 cases. *Intern Med* 1995; **34**: 212-215
- 7 **Candelaria M**, Bourlon-Cuellar R, Zubieta JL, Noel-Ettiene LM, Sanchez-Sanchez JM. Gastrointestinal pneumatosis after docetaxel chemotherapy. *J Clin Gastroenterol* 2002; **34**: 444-445
- 8 **Shih IL**, Lu YS, Wang HP, Liu KL. Pneumatosis coli after etoposide chemotherapy for breast cancer. *J Clin Oncol* 2007; **25**: 1623-1625
- 9 **Kung D**, Ruan DT, Chan RK, Ericsson ML, Saund MS. Pneumatosis intestinalis and portal venous gas without bowel ischemia in a patient treated with irinotecan and cisplatin. *Dig Dis Sci* 2008; **53**: 217-219
- 10 **Heng Y**, Schuffler MD, Haggitt RC, Rohrmann CA. Pneumatosis intestinalis: a review. *Am J Gastroenterol* 1995; **90**: 1747-1758
- 11 **Shindelman LE**, Geller SA, Wisch N, Bauer JJ. Pneumatosis cystoides intestinalis: a complication of systemic chemotherapy. *Am J Gastroenterol* 1981; **75**: 270-274
- 12 **Rogy MA**, Mirza DF, Kovats E, Rauhs R. Pneumatosis cystoides intestinalis (PCI). *Int J Colorectal Dis* 1990; **5**: 120-124
- 13 **Horiuchi A**, Akamatsu T, Mukawa K, Ochi Y, Arakura N, Kiyosawa K. Case report: Pneumatosis cystoides intestinalis associated with post-surgical bowel anastomosis: a report of three cases and review of the Japanese literature. *J Gastroenterol Hepatol* 1998; **13**: 534-537
- 14 **Knechtle SJ**, Davidoff AM, Rice RP. Pneumatosis intestinalis. Surgical management and clinical outcome. *Ann Surg* 1990; **212**: 160-165
- 15 **Tamura N**, Kojo H, Miyoshi Y, Fukumoto S, Hirayama C. Pneumatosis cystoides intestinalis: Report of 3 cases with special reference to its non-surgical treatment. *Z Gastroenterol* 1980; **18**: 617-624

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**Rakesh Aggarwal, Additional Professor**

Department of Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, India

**Dr. Sk Md Fazle Akbar, Assistant Professor**

Third Department of Internal Medicine, Ehime University School of Medicine, Shigenobu-Cho, Ehime 791-0295, Japan

**Marc Basson, MD, PhD, MBA, Chief of Surgery**

John D. Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48301, United States

**David Cronin II, MD, PhD, FACS, Associate Professor**

Department of Surgery, Yale University School of Medicine, 330 Cedar Street, FMB 116, P. O. Box 208062, New Haven, Connecticut 06520-8062, United States

**Massimo Colombo, Professor**

1st Division of Gastroenterology, Fondazione IRCCS Maggiore Hospital, Policlinico, Mangiagalli e Regina Elena, University of Milan, Via F. Sforza 35, 20122 Milan, Italy

**Arno J Dormann, PhD, MD**

Habil, Medizinische Klinik, Krankenhaus Holweide, Kliniken der Stadt Köln gGmbH, Neufelder St. 32, 51067 Köln, Germany

**Jose L del Pozo, MD, PhD**

Infectious Diseases Division, Mayo Clinic College of Medicine, 200 1st St SW, Rochester, MN 55905, United States

**Abdellah Essaid, Professor**

Hospital Ibn Sina, Rabat 10100, Morocco

**Abdel-Rahman El-Zayadi, Professor**

Department of Hepatology and Gastroenterology, Ain Shams University and Cairo Liver Center, 5, El-Gergawy St. Dokki, Giza 12311, Egypt

**James E East, BSc, MBChB, MRCP**

St. Mary's Hospital, Endoscopy Unit, Clarence Wing, 3rd Floor, Praed Street, London, W2 1NY, United Kingdom

**Alessandro Fichera, MD, FACS, FASCRS, Assistant Professor**

Department of Surgery - University of Chicago, 5841 S. Maryland Ave, MC 5031, Chicago, IL 60637, United States

**Dr. Mitsuhiro Fujishiro**

Department of Gastroenterology, Faculty of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo, Japan

**Diego Garcia-Compean, MD, Professor**

Faculty of Medicine, University Hospital, Department of Gastroenterology, Autonomous University of Nuevo Leon, Ave Madero y Gonzalitos, 64700 Monterrey, NL, México

**Toru Ishikawa, MD**

Department of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan

**Syed MW Jafri, Professor**

Medicine/Gastroenterology, Aga Khan University, POB 3500, Karachi 74800, Pakistan

**Ioannis E Koutroubakis, MD, PhD, Assistant Professor of Medicine**

University Hospital Heraklion, Department of Gastroenterology, PO Box 1352, 71110 Heraklion, Crete, Greece

**KShiu-Ming Kuo, MD**

University at Buffalo, 15 Farber Hall, 3435 Main Street, Buffalo 14214, United States

**Ezio Laconi, MD, PhD, Professor of General Pathology**

Department of Sciences and Biomedical Technologies, Unit of Experimental Pathology, University of Cagliari, Via Porcell, 4 - IV Piano, 09125 - Cagliari, Italy

**James YW Lau**

Department of Surgery, Prince of Wales Hospital, the Chinese University of Hong Kong, Hong Kong, China

**Laura Lladó, PhD**

Department of Surgery, Liver Transplant Unit, Hospital Universitari de Bellvitge, IDIBELL, 08907 Barcelona, Spain

**Mercedes Susan Mandell, MD, PhD**

Department of Anesthesiology, University of Colorado Health Sciences Ctr., 12401 E. 17th Ave, B113 Aurora, CO 80045, United States

**Natalia A Osna**

Liver Study Unit, Research Service (151), VA Medical Center, 4101 Woolworth Avenue, Omaha NE 68105, United States

**Robert D Odze, MD, FRCPc, Chief**

Gastrointestinal Pathology Service, Associate Professor of Pathology, Brigham and Women's Hospital, Harvard Medical School, Boston MA, United States

**George Papatheodoridis, MD, Assistant Professor**

2nd Department of Internal Medicine, Athens University Medical School, Hippokratia General Hospital of Athens, 114 Vas. Sophias Ave., 115 27 Athens, Greece

**Gustav Paumgartner, Professor**

University of Munich, Klinikum Grosshadern, Marchioninstr. 15, Munich, D-81377, Germany

**Kostas Pantopoulos, Associate Professor**

Department of Medicine, McGill University, Lady Davis Institute for Medical Research, 3755 Cote Ste-Catherine Road, Montreal, Quebec, H3T 1E2, Canada

**James M Scheiman, Professor**

Division of Gastroenterology, University of Michigan Medical Center, 3912 Taubman Center, Box 0362, Ann Arbor, Michigan 48109-0362, United States

**Akihito Tsubota, Assistant Professor**

Institute of Clinical Medicine and Research, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan

**Debbie Trinder, PhD**

School of Medicine and Pharmacology, University of Western Australia, Fremantle Hospital, PO Box 480, Fremantle 6959, Western Australia, Australia

**Jan Wu, Associate Professor of Medicine**

Internal Medicine/Transplant Research Program, University of California, Davis Medical Center, 4635 2nd Ave. Suite 1001, Sacramento CA 95817, United States

**Hiroshi Yoshida, MD**

First Department of Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan

**Ta-Sen Yeh, MD, PhD**

Department of Surgery, Chang Gung Memorial Hospital, 5 Fu-Hsing Street, Taoyuan, Taiwan, China

**Yuan Yuan, Professor**

Cancer Institute of China Medical University, 155 North Nanjing Street, Heping District, Shenyang 110001, Liaoning Province, China



## Meetings

### Events Calendar 2008-2009

**FALK SYMPOSIA 2008**  
 January 24-25, Frankfurt, Germany  
 Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008  
 February 14-16, Paris, France  
 EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
 8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
 3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation  
 Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

February 29, Québec, Canada  
 Canadian Association of Gastroenterology  
 E-mail: [general@cag-acg.org](mailto:general@cag-acg.org)

March 10-13, Birmingham, UK  
 British Society of Gastroenterology Annual Meeting  
 E-mail: [BSG@mailbox.ulcc.ac.uk](mailto:BSG@mailbox.ulcc.ac.uk)

March 14-15, HangZhou, China  
 Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
 Asian Pacific Association for the Study of the Liver  
 18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
 Shanghai-Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
 OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas  
 E-mail: [robert.giuli@oeso.org](mailto:robert.giuli@oeso.org)

April 9-12, Los Angeles, USA  
 SAGES 2008 Annual Meeting - part of Surgical Spring Week  
[www.sages.org/08program/html/](http://www.sages.org/08program/html/)

April 18-22, Buenos Aires, Argentina  
 9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
 Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
 43<sup>rd</sup> Annual Meeting of the European Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary

Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA  
 Digestive Disease Week 2008

May 21-22, California, USA  
 ASGE Annual Postgraduate Course  
 Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion  
 E-mail: [education@#97;sge.org](mailto:education@#97;sge.org)

June 4-7, Helsinki, Finland  
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June 6-8, Prague, Czech Republic  
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June 10-13, Istanbul, Turkey  
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*In press*

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; 40: 679-686 [PMID: 12411462]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; 169: 2257-2261 [PMID: 12771764]

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- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; 325: 184 [PMID: 12142303]

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- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; 42 Suppl 2: S93-99 [PMID: 12028325]

*Issue with no volume*

- 8 Banit DM, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (401): 230-238 [PMID: 12151900]

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- 9 Outreach: Bringing HIV-positive individuals into care. *HRS/A Careaction* 2002; 1-6 [PMID: 12154804]

## Books

*Personal author(s)*

- 10 Sherlock S, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 Lam SK. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 Breedlove GK, Schorffheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

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- 14 Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

## Electronic journal (list all authors)

Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

## Patent (list all authors)

- 16 Pagedas AC, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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<sup>[1]</sup>Passed away on October 20, 2007

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# Surgical treatment for rectal cancer: An international perspective on what the medical gastroenterologist needs to know

Rolv-Ole Lindsetmo, Yong-Geul Joh, Conor P Delaney

Rolv-Ole Lindsetmo, Department of Gastrointestinal Surgery, University Hospital of North Norway and Institute of Clinical Medicine, University of Tromsø, Tromsø N-9036, Norway  
Yong-Geul Joh, Department of Surgery, Hansol Hospital, Seoul 138-844, South Korea

Conor P Delaney, Division of Colorectal Surgery, University Hospital Case Medical Center, Cleveland, Ohio 44106-5047, United States

**Author contributions:** Lindsetmo RO wrote the paper; Joh YG and Delaney CP made critical revisions and additions to the manuscript.

**Correspondence to:** Conor P Delaney, MD, PhD, Division of Colorectal Surgery, University Hospitals Case Medical Center, 11100 Euclid Avenue, Cleveland, Ohio 44106-5047, United States. [conor.delaney@uhhospitals.org](mailto:conor.delaney@uhhospitals.org)

Telephone: +1-216-8448087 Fax: +1-216-8445957

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## Abstract

Rectal cancer accounts for one third of all colorectal cancers. The age adjusted death rates from colorectal cancer have declined over recent decades due to a combination of colorectal cancer screening, improved diagnostic tests, improved standardized surgical technique, improved medical support, neoadjuvant chemotherapies and radiation treatment or combinations of these. Because of complex treatment algorithms, use of multidisciplinary teams in the management of rectal cancer patients has also been popularized. Medical gastroenterologists performing colonoscopies are frequently the first health care provider to raise the suspicion of a rectal cancer. Although the diagnosis depends on histological confirmation, the endoscopic presentation is almost diagnostic in many cases. In order to meet the patient's immediate needs for information, it is important that the endoscopist has knowledge about the investigations and treatment options that will be required for their patient. The aim of this paper is to describe the modern preoperative investigations and operative procedures commonly offered to rectal cancer patients taking into account perspectives of three colorectal surgeons, practicing in the USA, Europe and Asia.

## INTRODUCTION

Rectal cancer accounts for one third of all colorectal cancers and in the USA 41 420 new rectal cancer cases were estimated in 2007<sup>[1]</sup>. The age adjusted death rate from colorectal cancer has declined over recent decades due to a combination of colorectal cancer screening, improved diagnostic tests, improved standardized surgical technique, improved medical support, neoadjuvant chemotherapies and radiation treatment or combinations of these<sup>[2]</sup>. Because of complex treatment algorithms, use of multidisciplinary teams in the management of rectal cancer patients has also been popularized<sup>[3]</sup>.

Medical gastroenterologists performing colonoscopies are frequently the first health care provider to raise the suspicion of a rectal cancer. Although the diagnosis depends on histological confirmation, the endoscopic presentation is almost diagnostic in many cases. In order to meet the patient's immediate needs for information, it is important that the endoscopist has knowledge about the investigations and treatment options that will be required for their patient.

The aim of this paper is to describe the modern preoperative investigations and operative procedures commonly offered to rectal cancer patients taking into account perspectives of three colorectal surgeons, practicing in the USA, Europe and Asia.



## PREOPERATIVE INVESTIGATION AND STAGING

Perhaps the most basic and informative test in patients with low rectal cancer is a digital examination. For many tumors, this will immediately give the experienced surgeon enough information to determine what treatment will be required. As well as a general health evaluation, such as appropriate cardiopulmonary investigations, the preoperative evaluation includes rigid proctoscopy, endoscopic rectal ultrasound, total colonoscopy, pelvic MRI, CT-scans of the abdomen, liver and lungs. These investigations will help the surgeon and his multidisciplinary team to determine: (1) The patient's health condition and comorbidities; (2) The stage of the rectal cancer; and (3) Which treatment option is best suited to meet the patient's preferences and at the same time be oncologically appropriate.

## MEDICAL HISTORY AND PHYSICAL EXAMINATION

The three decisions that should be made initially include: whether the tumor is suitable for local therapy; whether preoperative therapy is required; and whether a permanent stoma is necessary. Severe comorbidities and poor health status can be a relative contraindication to abdominal surgery, whether open or even laparoscopic. A local or palliative approach may then be more reasonable. Accurate preoperative tumor staging is of extreme importance as it determines the indications for neoadjuvant therapy and the possibilities for a local resection versus a radical abdominal procedure. This must be balanced against the patient's preferences while at the same time giving the patients and their family the option to choose an individualized treatment plan with optimal chance for cure.

A patient history of previous pelvic or abdominal surgery will increase the difficulty of a laparoscopic approach, and thereby increases the likelihood of a decision for open rectal surgery. Abdominal wall scars should be noted as they might preclude the optimal stoma placement. Morbid obesity, especially in males, because of more intra-abdominal fat and narrow pelvis compared to females, will also favor open rectal surgery compared to laparoscopic surgery.

If the patient has a low rectal cancer, careful palpation of the groin lymph nodes is mandatory. Finding of one or several enlarged, hard and painless lymph nodes in the groin will ultimately lead to focus on palliative treatment once the finding is verified by MRI or biopsy. Excision (removing whole lymph nodes) should be considered after preoperative irradiation therapy including the affected groin.

Information about the benefits and limitations of the various surgical methods available, including the laparoscopic approach compared to the open operation should be given by the operating surgeon. However, most patients would also expect the medical endoscopist

to have a brief overview and knowledge of the most common preoperative investigations and operative procedures performed in the treatment of rectal cancer. Frequently, the endoscopist receives questions about chemoradiation therapy or is involved in the diagnosis and treatment of its side-effects.

## DIGITAL RECTAL EXAMINATION (DRE)

Despite the limited sensitivity and specificity of DRE, until recently the whole treatment plan was based upon its performance. Important information can still be gained from a correctly performed digital examination. What is the condition of the anal sphincters? Can the tumor be reached? If yes, is it occlusive? How much of the circumference is involved? Is it fixed to the surrounding tissue or can it be freely moved? What is the distance from the dentate line to the lower border of the tumor? Can the upper edge be reached?

By this simple examination the size, mobility and location of the cancer can be assessed. Before any decisions about treatment are made, the information gathered from DRE has to be confirmed by more objective means.

## ENDOSCOPIC INVESTIGATIONS

A colonoscopy is used to rule out the presence of synchronous polyps and cancers in the rest of the colon with a reasonably high accuracy<sup>[4]</sup>. The findings of multiple polyps in a patient under the age of 50 should alarm the endoscopist of a hereditary colorectal cancer. A detailed family history of cancer is warranted and referral to genetic consultation should be considered. In patients with familial cancer syndromes, the planned operation is a total colectomy. A colectomy with ileorectal anastomosis is used for patients with hereditary non-polyposis colon cancer (HNPCC), and those with familial adenomatous polyposis (FAP) and fewer than 20 rectal polyps. Patients with FAP and more than 20 rectal polyps should undergo proctocolectomy and ileoanal anastomosis.

When the patient with rectal cancer meets the surgeon at the outpatient clinic, both transanal endoscopic rectal ultrasound (TRUS) and rigid proctoscopy will be performed. The diagnostic accuracy for TRUS is dependant on the experience of the operator, and the stage and location of the tumor. Because of limited reach, large tumors in the upper rectum are not suitable for rectal ultrasound. Occluding tumors that cannot be passed with the transducer are also not amenable for this examination. TRUS is most accurate for early rectal cancers in the distal half of the rectum, and is particularly valuable in assessing the T-stage. The limited penetration depth of 7 MHz ultrasound waves makes it difficult to access the N-stage with high precision, with most studies showing accuracy of 70%-75%<sup>[5]</sup>. Three dimensional rectal ultrasound imaging seems to improve the staging properties<sup>[5]</sup>. Thus, for making a decision about whether local resection is possible the results of TRUS are of significant importance.

## MRI OF PELVIS

Because standard protocols can be used, and because it is less operator dependent, MRI has become the standard for preoperative stage assessment of rectal tumors<sup>[5]</sup>. With its high resolution and accuracy MRI can give information about T-stage and N-stage as well as distance to planned resection margins, especially lateral or circumferential margins within the pelvic cavity. MRI may also be used for the assessment of response to preoperative neoadjuvant chemoradiation treatment (CRT).

## CT SCAN OF LIVER AND LUNGS

CT scans of liver and lungs are performed to rule out the presence of metastatic disease. Resectable liver metastasis can be removed in a one stage operation or as a second operation 3 mo after the primary rectal cancer surgery. Multiple metastases in both liver lobes or hilar lymph node involvement are signs of incurable disease. However, some of the new forms of chemotherapy have such excellent response rates that these patients may become surgical candidates after reassessment.

## BLOOD TESTS

After the diagnosis of colorectal cancer, the carcinoembryonic antigen (CEA) level is measured in a simple blood test. The result of the CEA does not have any implications for the treatment, but increased levels are associated with poorer prognosis<sup>[6]</sup>. After resection of the cancer, elevated CEA levels should return to normal or metastatic disease should be suspected. CEA levels > 50 are very suggestive of liver metastases. In the surveillance program a three-fold increase in CEA level should alert the surgeon to search for local recurrence or metastatic disease<sup>[7]</sup>.

Other blood tests such as electrolytes, hemoglobin, and albumin are frequently taken to assess the patient's general condition. A low serum albumin indicates poor nutritional status or deranged liver function and is associated with increased frequency of postoperative complications including anastomotic leaks.

## MULTIDISCIPLINARY TEAMS

The complexity of individualized and highly specialized preoperative investigations and neoadjuvant treatment plans has evolved into the need for multidisciplinary teams. These teams are now being used in many institutions to ensure patients are appropriately placed on multidisciplinary care pathways. The results of the preoperative investigations and the clinical information about the patient are reviewed in the presence of dedicated specialists in medical oncology, gastrointestinal radiology and colorectal surgery. In the same meeting the pathology report of previous cases can be presented by a pathologist. The accuracy of the preoperative investigations, critical reevaluation of indications for adjuvant treatment, adjuvant treatment response as well as a judg-

ment of the quality of the surgery performed can be discussed in relation to the pathological TMN stage and resection margins presented in the pathology report.

## NEOADJUVANT TREATMENT

There is an important debate going on among surgical and oncological experts in rectal cancer treatment regarding the use of pre- or post operative radiation with or without chemotherapy in order to reduce rates of local recurrence and improve survival. Best evidence seems to support preoperative radiation in order to reduce local recurrence and at the same time reduce the side effects of radiation<sup>[8-10]</sup>. Adding chemotherapeutic agents to increase tumor radiosensitivity has been shown to be beneficial in improving local control, but was reported to have no effect upon survival<sup>[11]</sup>. Most centers nowadays have included preoperative chemoradiation therapy in their multimodality treatment options. However, there are still discussions about what gives best oncological results: short term radiation with 25Gy given in daily fractions of 5Gy and surgery the following week, or long term radiation treatment with chemotherapy in daily fractions of 1.8Gy five days per week, 50.4Gy in total, followed by surgery 4 to 6 wk later<sup>[12]</sup>. The latter treatment option probably has the advantage of down staging of the tumor and thereby increases the possibilities of a sphincter saving procedure, particularly in advanced low rectal cancers<sup>[13]</sup>. The connection between preoperative chemoradiation and achievement of uninvolved circumferential resection margin (CRM) is uncertain<sup>[14,15]</sup>.

The long term follow up of the European Organisation for Research and Treatment of Cancer (EORTC) trial 22921 that compared adjuvant fluorouracil-based chemotherapy to no adjuvant treatment in patients with resectable T3-4 rectal cancer, reported no beneficial effects of adjuvant chemotherapy if the cancer did not respond to the preoperative radiation or chemoradiation therapy<sup>[16]</sup>.

The role of postoperative radiation has recently been limited to inadvertent tumor perforations intraoperatively or involved resection margins if irradiation treatment was not given preoperatively. Intraoperative radiation therapy (IORT) can be given in cancers locally invading the pelvic side walls<sup>[17]</sup>. The definite role of postoperative chemotherapy for rectal cancer remains unclear<sup>[10]</sup>.

However, the situation is even more complicated. Current discussion is not just about which is the best treatment, but also which patients should receive such treatment. Generally accepted international treatment guidelines are yet to be developed. Some countries recommend preoperative radiation or chemoradiation to almost all rectal cancer patients<sup>[11,18]</sup>, whereas others recommend neoadjuvant chemotherapy to all patients with stage II and III rectal cancer<sup>[19]</sup>. Finally, others argue for a more selective neoadjuvant treatment policy offering it only to patients with preoperative MRI showing threatened CRM (nearest tumor tissue < 3 mm from predicted CRM) or for tumors in the lower half of the rectum<sup>[3,20-22]</sup>.

## SURGICAL TREATMENT OF RECTAL CANCER

Surgery is the only method to offer cure for rectal cancer. Rectal cancer surgery performed either as a minimally invasive or as an open procedure has four goals<sup>[23]</sup>: (1) To cure the patients and give long term survival; (2) To give local control and avoid local recurrence; (3) To preserve normal defecation-, bladder- and sexual functions when possible; (4) To maintain or improve the patients quality of life.

The best way to achieve goal number 1-cure and long term survival; and goal number 2-local control and avoidance of local recurrence, is by means of major surgery. However, this has its price and considerable efforts have been made to reduce the negative impact of rectal resections upon goals number 3-to preserve normal defecation-, bladder- and sexual functions and goal number 4-to maintain or improve the patients quality of life.

Functional disturbances such as impotency, retrograde ejaculation, urinary retention or disturbed urinary bladder function as well as defecational problems or formation of a stoma have negative impact on quality of life after surgical treatment. One of the main steps during the dissection of the mesorectum is to identify and preserve the hypogastric and parasympatic pelvic nerves and thereby preserve functions. Functional disturbances are still a problem after rectal cancer surgery in about 20% of the patients<sup>[24]</sup>. Table 1 shows a summary of abbreviations that are commonly used in the surgical treatment of rectal cancer.

### Local resections

Local resections are performed transanally using both specially developed instruments and sutures to expose the rectal mucosa (transanal excision, TAE), or the operation might be performed endoscopically using a microscope to improve visualization through a specially designed proctoscope to secure access and instrumentation of the tumor (transanal endoscopic microsurgery, TEM). Local resections would be the operation of choice if only goals 3 and 4 were to be considered. Early rectal cancers treated with local resections have been reported to be associated with unacceptably high local recurrence rates of up to 40%<sup>[25]</sup>, and should only be offered to carefully selected patients, or to those who otherwise would need a permanent end stoma<sup>[26]</sup>. For patients with severe comorbidities or with extremely high risk from anesthesia and abdominal surgery, a local resection procedure can be the optimal solution despite its limitations regarding local recurrences. Studies are underway in which the results of combining chemoradiation therapy and TEM will be determined<sup>[27]</sup>.

Studies of the mesorectum in rectal cancer have shown that 10% of early rectal cancer (T1) has micrometastasis in mesorectal lymph nodes, and close to 20% have local lymph node metastasis in T2 cases<sup>[28]</sup>. Performing local resections that leave metastatic lymph nodes is undoubtedly likely to increase local recurrence rates, although the exact risk has yet to be evaluated and

Table 1 Vocabulary for rectal cancer treatment

	Treatment
Anterior resection	Resection of rectum with an anastomosis above the pelvic peritoneal reflection.
Low anterior resection	Resection of rectum with an anastomosis below the pelvic peritoneal reflection.
TME	Total mesorectal excision. The fatty tissue which contains the draining lymph nodes surrounding the lateral and posterior part of the rectal tube, are dissected down to the pelvic floor and resected. The hypogastric nerves are preserved.
PME	Partial mesorectal excision. The mesorectum is divided 5 cm below the cancer and rectum transected. PME is performed for cancers located in the upper rectum and rectosigmoid junction.
TEM	Transanal endoscopic microsurgery. A specially constructed proctoscope with an attached microscope permits local resection of premalignant lesions and selected cases of early rectal cancer up to 20 cm from the anal verge.
TAE	Transanal excision. Lesions in the lower third of rectum can be resected transanally.
APR	Abdominoperineal resection. Low rectal cancers that cannot be resected with a sphincter-saving procedure are resected with perianal tissue and the anal channel en block with the whole rectum and mesorectum.
Adjuvant	Additional treatment (chemotherapy, radiation therapy or chemoradiation) given after surgical resection.
Neoadjuvant CRT	Preoperative treatment. Chemoradiation treatment. Chemotherapeutic drugs, typically 5'-fluorouracil and/or leucovorin are given in order to increase cancer cells sensitivity to the radiation. CRT is frequently offered to patients preoperatively (neoadjuvant) in order to reduce the chances for local recurrence and improve survival.
Intersphincteric resection	The upper part of the internal anal sphincter muscle is resected continuously with the lower rectum in order to preserve anal function and avoid colostomy in cases of ultralow rectal cancer.
CRM	Circumferential resection margin is the distance in mm from the mesorectal fascia (the resection plane) to the nearest tumor growth.
DRM	Distal resection margin.

the risk is likely dependent on the exact individual tumor stage biology.

### Total mesorectal excision (TME)

Heald and coworkers standardized the approach to rectal cancer by performing a TME with sharp dissection in the avascular plane surrounding the mesorectum with preservation of the hypogastric and parasympathetic pelvic nerves<sup>[29]</sup>. They reported a 5-year recurrence rate of 5%-7% or lower, depending on the cancer stage, without the use of neoadjuvant treatment, showing the importance of adequate surgical quality upon local recurrence. By contrast, traditional rectal cancer surgery with blunt dissection and ignoring the importance of an intact mesorectum with adequate tumor resection margins,

has yielded local recurrence rates of 30% or higher<sup>[30]</sup>. The benefits of the mesorectal dissection technique have been confirmed in several European countries after introduction of training programs and national consensus of TME as the standard operation method for rectal cancer<sup>[11,18,31]</sup>. It has been documented that cancers located in the upper rectum do not need to be removed along with all the fatty tissue surrounding the rectum (mesorectum) down to the pelvic floor<sup>[32]</sup>. They do need a TME-like radial margin, but can be resected with a 5-cm distal margin to the cancer, ie a partially mesorectal excision (PME), without compromising the oncological result. This helps minimize some of the functional disturbances seen after a coloanal anastomosis.

The development of suturing devices with stapled circular anastomosis has also made the formation of anastomoses in the lower pelvis feasible, reducing the need for permanent stomas. However, the reported rates of anastomotic complications still vary considerably between surgeons<sup>[33]</sup>. It is common practice to protect the lowest anastomosis, especially after radiation treatment, with a temporary diverting loop ileostomy. The ileostomy is normally closed after 8 to 12 wk.

The low anterior syndrome describes the functional disturbances that may be seen after rectal cancer surgery. Improved defecation function can be achieved by anastomosing a colon J pouch to the top of the anal channel or to the top of a short rectal remnant<sup>[34]</sup>.

### **Laparoscopic mesorectal excision**

Laparoscopic resection of the rectum has not gained the same international acceptance as laparoscopic colon surgery. However, it has proven to be technically feasible and safe with no more or perhaps fewer complications than after open rectal surgery<sup>[35,36]</sup>. Low anterior resection (LAR) technically performed as laparoscopic TME or PME has the same oncological outcome when compared to traditional open rectal surgery<sup>[37-40]</sup>. For patients, laparoscopic surgery gives benefits regarding reduced postoperative pain, shortened postoperative ileus with faster bowel recovery after surgery, improved abdominal cosmesis, fewer wound infections, less postoperative small bowel obstruction and ventral hernias<sup>[41,42]</sup>. For the health care providers the benefits are shorter hospital stay and reduced overall costs<sup>[43]</sup> and thereby more effective use of health care resources.

Because of the technical challenges of laparoscopic pelvic surgery a standardization of the technique is important to reduce the rate of conversion and improve the operating team performance. The learning curve for laparoscopic mesorectal resection is higher than commonly stated for other laparoscopic procedures<sup>[44]</sup>. This has probably contributed to the centralization of laparoscopic rectal resections to high volume hospitals with trained and experienced surgeons.

### **Abdominoperineal resection (APR)**

About one third of rectal cancers are located in the distal third of the rectum. Traditionally this tumor location has led to an APR and a permanent colostomy. A frequency

of 30% or more of APR has therefore been reported in many series<sup>[45]</sup>. However, improved surgical technique and neoadjuvant CRT have made it possible to perform low resections and stapled or handsewn coloanal anastomosis<sup>[46]</sup>. For the ultralow rectal cancers, intersphincteric resection and a handsewn colonic J-pouch anastomosis can be performed with good oncological results<sup>[47]</sup>. Increased focus on sphincter saving surgery has reduced the frequency of APR to around 10% or less in some hands. Some authors even regard the frequency of APR as a surrogate marker of the surgical quality in rectal cancer treatment<sup>[48]</sup>.

### **Hartmann's procedure**

The Hartmann's procedure is a rectosigmoid resection where the bowel continuity is not restored by an anastomosis. Instead the proximal colon is diverted as an end colostomy and the distal rectum, or sometimes just the anal canal, is left behind as a pouch (Hartman's pouch). This procedure is performed in selected rectal cancer patients, such as those with preexisting fecal incontinence, or unacceptably high risk after an anastomotic complication.

### **Loop ileostomy**

A loop ileostomy can be performed to divert the flow of stool until the anastomosis has healed. The ileostomy does not reduce the rate of anastomotic leakage but it will limit the infectious consequences and mortality of the leakage<sup>[49]</sup>. In cases with obstructive symptoms from the cancer, a loop ileostomy can relieve symptoms before preoperative chemoradiation therapy is initiated, as well as reducing the risk of complications associated with emergency surgery by converting emergency cases into later elective surgery.

## **ENHANCED RECOVERY PROGRAMS**

The development of fast track surgery or enhanced recovery programs has dramatically reduced the recovery time and length of hospital stay after colorectal surgery<sup>[50,51]</sup>. By combining laparoscopic rectal surgery and enhanced recovery programs, hospital stay of 4 d or less can be expected for 90% of the patients<sup>[52]</sup>. Fast track pathways may include avoidance of preoperative mechanical bowel preparation, drinking of a carbohydrate enriched solution 2 h prior to surgery, use of total intravenous anaesthesia, early postoperative mobilization, avoidance of nasogastric tubes and abdominal drains, early postoperative intake of liquids and solid food, minimizing opiates for pain control and use of bowel stimulating drugs. Effective pain control can be achieved by patient controlled analgesia (PCA) pumps in most cases. Intravenous and urinary catheters are removed on postoperative day one. Using these strategies as a combined pathway leads to early recovery, with low risk for readmission within 30 d<sup>[53]</sup>.

## **SURGICAL QUALITY**

The aim to cure and improve survival as well as number



of lymph nodes in the specimen the surgical quality can be evaluated within a few days. Similarly, many of the important outcomes of early recovery after surgery can only be achieved in patients having high quality surgery. Tumor biology and stage are important prognostic factors, but so is the performance of the surgeon. The importance of the surgical quality can easily be obscured by focusing on short term and long term over all survival, cancer specific survival, long term and short term local recurrence rates, different radiation regimens with or without pre- or post operative chemotherapy, local versus major resections, or laparoscopic versus open technique. Overall local recurrence rates > 10% should lead to concerns about the surgical quality. However, it is rather late to change the technique, when the rates of local recurrence are commonly calculated 3-5 years after surgery.

By using the recommended pathological description<sup>[54]</sup>, TME grading, CRM, distal mesorectal and mural margins as well as number of lymph nodes in the specimen can be evaluated within a few days of surgery. If preoperative MRI showed more than 2 mm distance from tumor to the lateral resection margin, the CRM measured in the specimen should be at least 2 mm. Because of distal spread of tumor cells in mesorectum, a 5-cm distal resection margin is advocated in cases of PME. When performing a TME all the mesorectal fatty tissue is removed, and the surgeon can focus on achieving a safe distal rectal wall resection margin which is shown to be 1 cm or even less in cases with preoperative chemoradiation<sup>[55]</sup>.

If the surgeon repeatedly has tumor involvement in the CRM, too short distal mesorectal resection margins, or involved distal rectal wall resection margin, then his patients will suffer unnecessary local recurrence and shortened survival. Few, if any national colorectal associations have considered the consequences of this and started a certification program for colorectal surgeons who operate on rectal cancer. Development of centers of excellence could also help improve the quality of all aspects of rectal cancer treatment.

The complexity of individualized multimodal treatment plans and the challenges and technical difficulties of open or laparoscopic pelvic surgery, have centralized rectal cancer treatment to high volume institutions, hopefully to the benefit of the patients.

Additionally, there has been no broad discussion in the literature of possible overtreatment by giving neoadjuvant chemoradiation to all rectal cancer patients, since less than 10% of all rectal cancer patients will have local recurrence after optimal surgery alone.

## COMPLICATIONS AFTER SURGICAL TREATMENT

The narrow pelvic cavity and the close relations of the rectum to functionally important organs and structures as the hypogastric and parasympathetic nerves, the urinary tract including ureters, bladder and urethra, the seminal vesicles and prostate gland in males, uterus and

posterior vaginal wall in females, pelvic and sacral vessels, make rectal surgery technical challenging and risky. Impotency and sexual dysfunctions, bladder dysfunctions, defecational problems including evacuation difficulties, fecal incontinence and urgency significantly add to the mental stress of a recent cancer diagnosis. Stoma problems with fear of malodorous leakage can be socially crippling. An increased focus on quality of life has included preservation of normal defecation-, bladder- and sexual functions and maintaining or improvement of the patient's quality of life as main goals of the surgical therapy for rectal cancer. Still, up to 20% of the patients will experience one or more of the above-mentioned side effects of the surgical treatment<sup>[56]</sup>.

## POSTOPERATIVE SURVEILLANCE

The medical endoscopist frequently meets rectal cancer patients when they are coming in for colonoscopy, commonly at 6 mo and at 4 years in their postoperative surveillance program. The clinical benefit of a postoperative surveillance program is disputed<sup>[57]</sup>, but there are several considerations. One is to discover signs of cancer local recurrence or metastatic disease. Another is to educate the patient to recognize signs and symptoms of recurrent disease as well as to encourage the patients to cope with the sequelae of treatment. Thirdly, it is an important way to monitor the results and quality of the rectal cancer treatment.

Details of recent development of weight loss despite normal appetite, increased fatigue, changes in bowel habits and vague abdominal discomfort should be questioned at every postoperative consultation. Physical examination, including palpation of the abdomen for any possible mass, surgical scars, the lower edge of the liver and palpation around stomas will be performed. The presence of ventral or parastomal hernias should be recorded, but any suggestions about surgical treatment should be balanced against symptoms, impact on quality of life or other possible benefits and risks. The left supraclavicular fossa (Virchows lymph node) and the groins should be palpated for enlarged lymph nodes. The perineal region should be inspected and palpated and a DRE performed in all cases with a residual anal canal. During DRE the anastomosis should be palpated if within reach and any pelvic mass recognized.

As mentioned earlier, postoperative CEA level should return to normal if elevated preoperatively. In these patients elevated CEA levels can be indicative of local recurrence or metastatic disease.

Unsuspected findings should be verified by CT or MRI scans. PET scan is the most accurate method to rule out presence and the extent of local or metastatic disease<sup>[58]</sup>.

## LOCAL RECURRENCE

If a local recurrence is verified, surgical resection must be considered either with curative intent or as a palliative effort. However, the side effects and complications of

any surgery for recurrent disease must not be underestimated. The plan for the investigation is to determine resectability and to assess risks to the patient. Second line chemotherapy is an option, however it is non-curative and with considerable side effects. Most cases of recurrent disease will be discovered between the surveillance controls, and two thirds within two years after surgery.

## LIVER METASTASIS

The attitude towards liver metastasis from colorectal cancer has also changed during recent decades. An aggressive approach has been shown to prolong survival and increase chances for cure<sup>[59]</sup>. Even patients with multiple liver metastases should be considered for liver surgery because combination of surgical resection and ablation (radiofrequency ablation or cryo ablation) after downstaging chemotherapy can be a valuable option for the patient unless there is evidence of systemic cancer disease. Selective hepatic intraarterial chemotherapy and segmental liver embolization are also treatment options in selected cases.

## LUNG METASTASIS

Rectal cancer does also spread to the lungs. In an otherwise fit patient with no other signs of metastatic disease, an aggressive surgical approach will prolong survival. Segmental pulmonary resection or lobectomy is advocated for selected patients<sup>[60]</sup>. Multilobular and bilateral location is a sign of systemic disease and is a contraindication for surgical treatment.

## PALLIATIVE SURGERY FOR ADVANCED AND INOPERABLE RECTAL CANCER

Preoperative chemoradiation therapy might downstage a fixed and inoperable cancer to become resectable and even curable. All efforts should be made to resect a rectal cancer in order to avoid the painful and devastating conditions associated with an uncontrollable cancer growth inside the pelvic cavity. Stoma, intestinal bypass, stent, fulguration (burning down the cancer with diathermy) or laser evaporation can give temporary relief from an obstructing rectal cancer or its metastasis.

Large procedures as hemipelvectomy or anterior or total pelvic exenteration with or without combination with intraoperative radiation (IORT) have been performed in order to achieve a R0 resection (all cancer tissue removed) and thereby reduce the chances of local recurrence. Obviously, this has side effects for the patients.

## CONCLUSION

The medical endoscopist is not commonly involved in the multidisciplinary teams deciding the treatment plans for patients with rectal cancer. However, the endoscopist frequently is the first health care provider to meet the patient with suspected rectal cancer in the setting of

endoscopy for colorectal symptoms or screening, and is frequently the person that performs the postoperative endoscopic surveillance. By having knowledge about the complex investigation plans and treatment options available, the endoscopist can provide important information in order to help the patient to prepare for the coming meeting with the surgeon.

## REFERENCES

- 1 **Jemal A**, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. *CA Cancer J Clin* 2007; **57**: 43-66
- 2 **Wu JS**, Fazio VW. Management of rectal cancer. *J Gastrointest Surg* 2004; **8**: 139-149
- 3 **Daniels IR**, Fisher SE, Heald RJ, Moran BJ. Accurate staging, selective preoperative therapy and optimal surgery improves outcome in rectal cancer: a review of the recent evidence. *Colorectal Dis* 2007; **9**: 290-301
- 4 **Kaminski MF**, Regula J. Colorectal cancer screening by colonoscopy--current issues. *Digestion* 2007; **76**: 20-25
- 5 **Muthusamy VR**, Chang KJ. Optimal methods for staging rectal cancer. *Clin Cancer Res* 2007; **13**: 6877s-6884s
- 6 **Hall NR**, Finan PJ, Stephenson BM, Purves DA, Cooper EH. The role of CA-242 and CEA in surveillance following curative resection for colorectal cancer. *Br J Cancer* 1994; **70**: 549-553
- 7 **Korner H**, Soreide K, Stokkeland PJ, Soreide JA. Diagnostic accuracy of serum-carcinoembryonic antigen in recurrent colorectal cancer: a receiver operating characteristic curve analysis. *Ann Surg Oncol* 2007; **14**: 417-423
- 8 **Kapiteijn E**, Marijnen CA, Nagtegaal ID, Putter H, Steup WH, Wiggers T, Rutten HJ, Pahlman L, Glimelius B, van Krieken JH, Leer JW, van de Velde CJ. Preoperative radiotherapy combined with total mesorectal excision for resectable rectal cancer. *N Engl J Med* 2001; **345**: 638-646
- 9 **Sauer R**, Becker H, Hohenberger W, Rodel C, Wittekind C, Fietkau R, Martus P, Tschmelitsch J, Hager E, Hess CF, Karstens JH, Liersch T, Schmidberger H, Raab R. Preoperative versus postoperative chemoradiotherapy for rectal cancer. *N Engl J Med* 2004; **351**: 1731-1740
- 10 **Bosset JF**, Collette L, Calais G, Mineur L, Maingon P, Radosevic-Jelic L, Daban A, Bardet E, Beny A, Ollier JC. Chemotherapy with preoperative radiotherapy in rectal cancer. *N Engl J Med* 2006; **355**: 1114-1123
- 11 **Peeters KC**, Marijnen CA, Nagtegaal ID, Kranenbarg EK, Putter H, Wiggers T, Rutten H, Pahlman L, Glimelius B, Leer JW, van de Velde CJ. The TME trial after a median follow-up of 6 years: increased local control but no survival benefit in irradiated patients with resectable rectal carcinoma. *Ann Surg* 2007; **246**: 693-701
- 12 **Arnoletti JP**, Bland KI. Neoadjuvant and adjuvant therapy for rectal cancer. *Surg Oncol Clin N Am* 2006; **15**: 147-157
- 13 **Habr-Gama A**, Perez RO, Kiss DR, Rawet V, Scanavini A, Santinho PM, Nadalin W. Preoperative chemoradiation therapy for low rectal cancer. Impact on downstaging and sphincter-saving operations. *Hepatogastroenterology* 2004; **51**: 1703-1707
- 14 **Glynn-Jones R**, Mawdsley S, Novell JR. The clinical significance of the circumferential resection margin following preoperative pelvic chemo-radiotherapy in rectal cancer: why we need a common language. *Colorectal Dis* 2006; **8**: 800-807
- 15 **den Dulk M**, Collette L, van de Velde CJ, Marijnen CA, Calais G, Mineur L, Maingon P, Radosevic-Jelic L, Daban A, Bosset JF. Quality of surgery in T3-4 rectal cancer: involvement of circumferential resection margin not influenced by preoperative treatment. Results from EORTC trial 22921. *Eur J Cancer* 2007; **43**: 1821-1828
- 16 **Collette L**, Bosset JF, den Dulk M, Nguyen F, Mineur L, Maingon P, Radosevic-Jelic L, Pierart M, Calais G.

- Patients with curative resection of cT3-4 rectal cancer after preoperative radiotherapy or radiochemotherapy: does anybody benefit from adjuvant fluorouracil-based chemotherapy? A trial of the European Organisation for Research and Treatment of Cancer Radiation Oncology Group. *J Clin Oncol* 2007; **25**: 4379-4386
- 17 **Williams CP**, Reynolds HL, Delaney CP, Champagne B, Obias V, Joh YG, Merlino J, Kinsella TJ. Clinical results of intraoperative radiation therapy for patients with locally recurrent and advanced tumors having colorectal involvement. *Am J Surg* 2008; **195**: 405-409
  - 18 **Pahlman L**, Bohe M, Cedermark B, Dahlberg M, Lindmark G, Sjobahl R, Ojerskog B, Damber L, Johansson R. The Swedish rectal cancer registry. *Br J Surg* 2007; **94**: 1285-1292
  - 19 **Benson AB 3rd**, Choti MA, Cohen AM, Doroshow JH, Fuchs C, Kiel K, Martin EW Jr, McGinn C, Petrelli NJ, Posey JA, Skibber JM, Venook A, Yeatman TJ. NCCN Practice Guidelines for Colorectal Cancer. *Oncology* (Williston Park) 2000; **14**: 203-212
  - 20 **Strassburg J**, Lewin A, Ludwig K, Kilian L, Linke J, Loy V, Knuth P, Puttcher O, Ruehl U, Stockmann F, Hackenthal M, Hopfenmuller W, Huppertz A. Optimised surgery (so-called TME surgery) and high-resolution MRI in the planning of treatment of rectal carcinoma. *Langenbecks Arch Surg* 2007; **392**: 179-188
  - 21 **Simunovic M**, Sexton R, Rempel E, Moran BJ, Heald RJ. Optimal preoperative assessment and surgery for rectal cancer may greatly limit the need for radiotherapy. *Br J Surg* 2003; **90**: 999-1003
  - 22 **Eriksen MT**, Wibe A, Haffner J, Wiig JN. Prognostic groups in 1,676 patients with T3 rectal cancer treated without preoperative radiotherapy. *Dis Colon Rectum* 2007; **50**: 156-167
  - 23 **Balch GC**, De Meo A, Guillem JG. Modern management of rectal cancer: a 2006 update. *World J Gastroenterol* 2006; **12**: 3186-3195
  - 24 **Moriya Y**. Function preservation in rectal cancer surgery. *Int J Clin Oncol* 2006; **11**: 339-343
  - 25 **Madbouly KM**, Remzi FH, Erkek BA, Senagore AJ, Baeslach CM, Khandwala F, Fazio VW, Lavery IC. Recurrence after transanal excision of T1 rectal cancer: should we be concerned? *Dis Colon Rectum* 2005; **48**: 711-719; discussion 719-721
  - 26 **Bentrem DJ**, Okabe S, Wong WD, Guillem JG, Weiser MR, Temple LK, Ben-Porat LS, Minsky BD, Cohen AM, Paty PB. T1 adenocarcinoma of the rectum: transanal excision or radical surgery? *Ann Surg* 2005; **242**: 472-477; discussion 477-479
  - 27 **Ota DM**, Nelson H. Local excision of rectal cancer revisited: ACOSOG protocol Z6041. *Ann Surg Oncol* 2007; **14**: 271
  - 28 **Fang WL**, Chang SC, Lin JK, Wang HS, Yang SH, Jiang JK, Chen WC, Lin TC. Metastatic potential in T1 and T2 colorectal cancer. *Hepatogastroenterology* 2005; **52**: 1688-1691
  - 29 **MacFarlane JK**, Ryall RD, Heald RJ. Mesorectal excision for rectal cancer. *Lancet* 1993; **341**: 457-460
  - 30 **Martling A**, Holm T, Rutqvist LE, Johansson H, Moran BJ, Heald RJ, Cedermark B. Impact of a surgical training programme on rectal cancer outcomes in Stockholm. *Br J Surg* 2005; **92**: 225-229
  - 31 **Wibe A**, Moller B, Norstein J, Carlsen E, Wiig JN, Heald RJ, Langmark F, Myrvold HE, Soreide O. A national strategic change in treatment policy for rectal cancer--implementation of total mesorectal excision as routine treatment in Norway. A national audit. *Dis Colon Rectum* 2002; **45**: 857-866
  - 32 **Law WL**, Chu KW. Anterior resection for rectal cancer with mesorectal excision: a prospective evaluation of 622 patients. *Ann Surg* 2004; **240**: 260-268
  - 33 **Kong AP**, Kim J, Holt A, Konyalian V, Huynh R, Udani SM, Stamos MJ, Kumar RR. Selective treatment of rectal cancer with single-stage coloanal or ultralow colorectal anastomosis does not adversely affect morbidity and mortality. *Int J Colorectal Dis* 2007; **22**: 897-901
  - 34 **Fazio VW**, Zutshi M, Remzi FH, Parc Y, Ruppert R, Furst A, Celebrezze J Jr, Galanduk S, Orangio G, Hyman N, Bokey L, Tietz E, Kirchdorfer B, Medich D, Tietze M, Hull T, Hammel J. A randomized multicenter trial to compare long-term functional outcome, quality of life, and complications of surgical procedures for low rectal cancers. *Ann Surg* 2007; **246**: 481-488; discussion 488-490
  - 35 **Leroy J**, Jamali F, Forbes L, Smith M, Rubino F, Mutter D, Marescaux J. Laparoscopic total mesorectal excision (TME) for rectal cancer surgery: long-term outcomes. *Surg Endosc* 2004; **18**: 281-289
  - 36 **Kim SH**, Park IJ, Joh YG, Hahn KY. Laparoscopic resection for rectal cancer: a prospective analysis of thirty-month follow-up outcomes in 312 patients. *Surg Endosc* 2006; **20**: 1197-1202
  - 37 **Jayne DG**, Guillou PJ, Thorpe H, Quirke P, Copeland J, Smith AM, Heath RM, Brown JM. Randomized trial of laparoscopic-assisted resection of colorectal carcinoma: 3-year results of the UK MRC CLASICC Trial Group. *J Clin Oncol* 2007; **25**: 3061-3068
  - 38 **Leung KL**, Kwok SP, Lam SC, Lee JF, Yiu RY, Ng SS, Lai PB, Lau WY. Laparoscopic resection of rectosigmoid carcinoma: prospective randomised trial. *Lancet* 2004; **363**: 1187-1192
  - 39 **Bianchi PP**, Rosati R, Bona S, Rottoli M, Elmore U, Ceriani C, Malesci A, Montorsi M. Laparoscopic surgery in rectal cancer: a prospective analysis of patient survival and outcomes. *Dis Colon Rectum* 2007; **50**: 2047-2053
  - 40 **Aziz O**, Constantinides V, Tekkis PP, Athanasiou T, Purkayastha S, Paraskeva P, Darzi AW, Heriot AG. Laparoscopic versus open surgery for rectal cancer: a meta-analysis. *Ann Surg Oncol* 2006; **13**: 413-424
  - 41 **Breukink S**, Pierie J, Wiggers T. Laparoscopic versus open total mesorectal excision for rectal cancer. *Cochrane Database Syst Rev* 2006; CD005200
  - 42 **Duepre HJ**, Senagore AJ, Delaney CP, Fazio VW. Does means of access affect the incidence of small bowel obstruction and ventral hernia after bowel resection? Laparoscopy versus laparotomy. *J Am Coll Surg* 2003; **197**: 177-181
  - 43 **Senagore AJ**, Brannigan A, Kiran RP, Brady K, Delaney CP. Diagnosis-related group assignment in laparoscopic and open colectomy: financial implications for payer and provider. *Dis Colon Rectum* 2005; **48**: 1016-1020
  - 44 **Park JS**, Kang SB, Kim SW, Cheon GN. Economics and the laparoscopic surgery learning curve: comparison with open surgery for rectosigmoid cancer. *World J Surg* 2007; **31**: 1827-1834
  - 45 **Ptok H**, Marusch F, Kuhn R, Gastinger I, Lippert H. Influence of hospital volume on the frequency of abdominoperineal resection and long-term oncological outcomes in low rectal cancer. *Eur J Surg Oncol* 2007; **33**: 854-861
  - 46 **Crane CH**, Skibber JM, Feig BW, Vauthey JN, Thames HD, Curley SA, Rodriguez-Bigas MA, Wolff RA, Ellis LM, Delclos ME, Lin EH, Janjan NA. Response to preoperative chemoradiation increases the use of sphincter-preserving surgery in patients with locally advanced low rectal carcinoma. *Cancer* 2003; **97**: 517-524
  - 47 **Chamlou R**, Parc Y, Simon T, Bennis M, Dehni N, Parc R, Tietz E. Long-term results of intersphincteric resection for low rectal cancer. *Ann Surg* 2007; **246**: 916-921; discussion 921-922
  - 48 **Tilney HS**, Heriot AG, Purkayastha S, Antoniou A, Aylin P, Darzi AW, Tekkis PP. A national perspective on the decline of abdominoperineal resection for rectal cancer. *Ann Surg* 2008; **247**: 77-84
  - 49 **Matthiessen P**, Hallbook O, Rutegard J, Simert G, Sjobahl R. Defunctioning stoma reduces symptomatic anastomotic leakage after low anterior resection of the rectum for cancer: a randomized multicenter trial. *Ann Surg* 2007; **246**: 207-214
  - 50 **Delaney CP**, Fazio VW, Senagore AJ, Robinson B, Halverson AL, Remzi FH. 'Fast track' postoperative management protocol for patients with high co-morbidity undergoing

- complex abdominal and pelvic colorectal surgery. *Br J Surg* 2001; **88**: 1533-1538
- 51 **Basse L**, Hjort Jakobsen D, Billesbolle P, Werner M, Kehlet H. A clinical pathway to accelerate recovery after colonic resection. *Ann Surg* 2000; **232**: 51-57
- 52 **Lindsetmo RO**, Champagne B, Delaney CP. Results of perioperative care protocols for laparoscopic low anterior resections. Lindsetmo RO, B Champagne, Delaney CP. *Am J Surg* 2008; In press
- 53 **Kariv Y**, Wang W, Senagore AJ, Hammel JP, Fazio VW, Delaney CP. Multivariable analysis of factors associated with hospital readmission after intestinal surgery. *Am J Surg* 2006; **191**: 364-371
- 54 **Quirke P**, Dixon MF. The prediction of local recurrence in rectal adenocarcinoma by histopathological examination. *Int J Colorectal Dis* 1988; **3**: 127-131
- 55 **Guillem JG**, Chessin DB, Shia J, Suriawinata A, Riedel E, Moore HG, Minsky BD, Wong WD. A prospective pathologic analysis using whole-mount sections of rectal cancer following preoperative combined modality therapy: implications for sphincter preservation. *Ann Surg* 2007; **245**: 88-93
- 56 **Pocard M**, Zinzindohoue F, Haab F, Caplin S, Parc R, Tiret E. A prospective study of sexual and urinary function before and after total mesorectal excision with autonomic nerve preservation for rectal cancer. *Surgery* 2002; **131**: 368-372
- 57 **Abir F**, Alva S, Longo WE, Audiso R, Virgo KS, Johnson FE. The postoperative surveillance of patients with colon cancer and rectal cancer. *Am J Surg* 2006; **192**: 100-108
- 58 **Watson AJ**, Lolohea S, Robertson GM, Frizelle FA. The role of positron emission tomography in the management of recurrent colorectal cancer: a review. *Dis Colon Rectum* 2007; **50**: 102-114
- 59 **Hirai I**, Kimura W, Fuse A, Isobe H, Hachiya O, Moriya T, Suto K, Mizutani M. Surgical management for metastatic liver tumors. *Hepatogastroenterology* 2006; **53**: 757-763
- 60 **Pfannschmidt J**, Dienemann H, Hoffmann H. Surgical resection of pulmonary metastases from colorectal cancer: a systematic review of published series. *Ann Thorac Surg* 2007; **84**: 324-338

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## EDITORIAL

# Autoimmune liver diseases

Pietro Invernizzi, Ian R Mackay

Pietro Invernizzi, Division of Internal Medicine and Liver Unit, San Paolo Hospital School of Medicine, University of Milan, Milan 20142, Italy

Pietro Invernizzi, Division of Rheumatology, Allergy, and Clinical Immunology, University of California at Davis, Davis, CA, United States

Ian R Mackay, Department of Biochemistry & Molecular Biology, Monash University, Clayton, Victoria 3800, Australia

Correspondence to: Pietro Invernizzi, Division of Internal Medicine and Liver Unit, San Paolo Hospital School of Medicine, University of Milan, Milan 20142,

Italy. [pietro.invernizzi@unimi.it](mailto:pietro.invernizzi@unimi.it)

Telephone: +39-2-50323088 Fax: +39-2-50323089

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## Abstract

The liver was one of the earliest recognized sites among autoimmune diseases yet autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, and their overlap forms, are still problematic in diagnosis and causation. The contributions herein comprise 'pairs of articles' on clinical characteristics, and concepts of etiopathogenesis, for each of the above diseases, together with childhood autoimmune liver disease, overlaps, interpretations of diagnostic serology, and liver transplantation. This issue is timely, since we are witnessing an ever increasing applicability of immunology to a wide variety of chronic diseases, hepatic and non-hepatic, in both developed and developing countries. The 11 invited expert review articles capture the changing features over recent years of the autoimmune liver diseases, the underlying immunomolecular mechanisms of development, the potent albeit still unexplained genetic influences, the expanding repertoire of immunoserological diagnostic markers, and the increasingly effective therapeutic possibilities.

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The liver is one of the earliest recognized sites among those affected by autoimmune diseases. Such diseases became recognized during the 1950s as novel pathogenetic entities in humans and, later, in laboratory animals. Today there are 80 different disorders attributable to autoimmunity. Over the past five decades, clinical awareness of autoimmune liver disease has been greatly enhanced, knowledge of pathogenesis has become more refined, laboratory diagnosis far more precise, and therapy more effective. These advances are authoritatively described by the expert contributors to this dedicated issue of the *World Journal of Gastroenterology*.

The health burden of autoimmune liver diseases, numerically not of the same magnitude resulting from liver diseases due to alcohol abuse, hepatitis virus infection or steatosis-related pathology, is still very substantial. Thus autoimmune liver diseases can affect individuals in childhood, at highly productive stages of adult life, as well as in later years; and these diseases are life long, and have degrading effects on *joie de vivre* due to distressing symptoms and complications, or side-effects of therapy. Moreover, there is an impression sometimes given by doctors that medical knowledge has not yet fully explained the exact nature or cause of autoimmune disorders. This might not be surprising because autoimmune diseases, including those affecting the liver, result from intricate derangements of immunological functions, and the idea that "immunity" can work against the well-being of the individual is counter-intuitive. Furthermore, immunology as a discipline of science is still not well accommodated in the curricula for students of medicine; it is a fast-evolving and strongly laboratory-based discipline with its own arcane terminology; and it may be disadvantaged by a still incomplete severance from microbiology in many university departments. Expectedly, clinicians may not readily engage with the theoretical pillars of modern immunology, nor fully appreciate the intimate applicability of immunology to a wide variety of chronic diseases, whether in developed or developing countries.

The autoimmune liver diseases considered among these reviews are autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC), and primary (autoimmune) sclerosing cholangitis (PSC). AIH and PBC are very well proved in terms of an autoimmune background whereas PSC, as readers will discern, fits less readily into this category. Nevertheless, the evidence in PSC for some forms of immune derangement is quite impressive, and we can reasonably attribute "guilt by association" with, for example, ulcerative colitis, as pointed out by our authors

on this topic.

We have made special provisions in this series of articles on the etiopathogenesis for each of the three autoimmune liver diseases. We often read that “such-and-such” a disease is “an autoimmune disorder of unknown etiology”. We would contend that autoimmunity, synonymous with tolerance deficit, should be regarded as aetiology in its own right, meaning that failure of natural immune tolerance itself can be pathogenic even in the absence of any overt environmental provocation. The regular and predictable occurrence of autoimmunity in certain tolerance-deficient inbred and genetically tilted strains of mice (NOD, NZB) is an ample witness to this. Among the autoimmune liver diseases, an environmental provocation is seldom discernible for AIH except for some “transient” examples after exposure to particular sensitizing medications, such as minocycline, whereas for PBC, there are a great variety of agents or processes claimed to act as “initiators”. As for PSC, the co-morbidity with ulcerative colitis leads to an indictment of pathogenetic immune hyper-responsiveness to the normally tolerated microbial flora of the colon. Tolerance deficits in humans, as in animals, will be largely genetically based, and deciphering the nature of these errors is an urgent and exciting challenge for the future studies.

Each of the three reviews dealing with etiopathogenesis of an autoimmune liver disease has a partner article on clinical features and management, and readers will note that clinical presentations have changed compared with earlier days. For example, AIH is currently presenting more as an indolent disease with an onset in later life, in contrast to its major impact on young women in the past years. PBC occurs more often at minimally symptomatic earlier stages, with cases frequently ascertained as a result of automated laboratory screening, sometimes for unrelated purposes. It is encouraging for clinicians that both AIH and PBC are gratifyingly responsive to the current standardized treatment regimens, even though, for ursodeoxycholic acid in PBC, the undoubted therapeutic benefits are not readily explicable on theoretical grounds. Finally, some patients will still require liver transplantation for eventual end-stage disease, but readers will be glad to

learn that AIH and PBC provide liver transplantation with more satisfying results in terms of post-transplantation morbidity and mortality. And, speaking of transplantation, the inescapable complications of allograft rejection or graft-versus-host disease do exist, but are now eminently manageable. Of much interest, there are credible examples of recurrence of autoimmune liver disease, or even *de novo* autoimmune hepatitis, in an allografted liver. This puzzling immunological scenario is engaged in one of our articles. Finally, a review article focuses on the particular aspects of autoimmune liver diseases occurring during childhood.

We have included an authoritative review on diagnostic serology in autoimmune liver disease. Previously diagnostic serological tests were usually provided by “academic” laboratories in either universities or major teaching hospitals. Currently commercial sectors are providing (increasingly more efficiently) the source materials and/or assay kits for private laboratories to perform autoimmune serologic assays. However, there is still a pressing need for the standardization of the assay procedures worldwide, and for a ready availability of calibrated anti-sera with which the laboratories can evaluate and quantitate their results. Clinicians should also be fully informed about the interpretation of the assay data rather than entirely rely on the printed results from the computer. For example, the hepatologist will be confronted from time to time with what is called an “overlap syndrome”. This is a topic that has attracted the attention of authors of several recent review articles, given that the partner diseases, AIH and PBC, or AIH and PSC require different regimens of therapy. The theme is reviewed in these articles. The diagnostic serological laboratory can often help the clinicians to identify the dominant partner to which therapy should primarily be directed.

We recommend to readers this series of reviews as a timely and “state-of-the-art” outline of autoimmunity and liver, by research centres esteemed for their contributions to the science and practice of hepatimmunology. Finally, we would express our deep appreciation to the invited authors for their painstaking preparation of the highly informative articles in this issue.

S- Editor Li DL L- Editor Ma JY E- Editor Ma WH



## TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, Series Editors

# Historical reflections on autoimmune hepatitis

Ian R Mackay

Ian R Mackay, Department of Biochemistry & Molecular Biology, Monash University, Clayton Victoria 3800, Australia  
Correspondence to: Ian R Mackay, MD, Department of Biochemistry & Molecular Biology, Monash University, Clayton Victoria 3800, Australia. [ian.mackay@med.monash.edu.au](mailto:ian.mackay@med.monash.edu.au)  
Telephone: +61-3-99051437 Fax: +61-3-96822751  
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## Abstract

Autoimmune hepatitis (AIH), initially known as chronic active or active chronic hepatitis (and by various other names), first came under clinical notice in the late 1940s. However, quite likely, chronic active hepatitis (CAH) had been observed prior to this and was attributed to a persistently destructive virus infection of the liver. An earlier (and controversial) designation in 1956 as lupoid hepatitis was derived from associated L.E. cell test positivity and emphasized accompanying multisystem features and immunological aberrations. Young women featured prominently in early descriptions of CAH. AIH was first applied in 1965 as a descriptive term. Disease-characteristic autoantibodies were defined from the early 1960s, notably antinuclear antibody (ANA), smooth muscle antibody (SMA) and liver-kidney microsomal (LKM) antibody. These are still widely used diagnostically but their relationship to pathogenesis is still not evident. A liver and disease specific autoantigen has long been searched for but unsuccessfully. Prolonged immunosuppressive therapy with prednisolone and azathioprine in the 1960s proved beneficial and remains standard therapy today. AIH like many other autoimmune diseases is associated with particular HLA alleles especially with the "ancestral" B8, DR3 haplotype, and also with DR4. Looking forwards, AIH is one of the several enigmatic autoimmune diseases that, despite being (relatively) organ specific, are marked by autoimmune reactivities with non-organ-specific autoantigens. New paradigms are needed to explain the occurrence, expressions and pathogenesis of such diseases.

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**Key words:** Medical history; Autoimmune hepatitis; Lupoid hepatitis; Liver disease autoantibodies; Immunosuppressive therapy; HLA-disease associations

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## EARLY DAYS: 1940s-1950s

The recognition in the 1950s of the disease now known as autoimmune hepatitis (AIH) with its various guises and appellations is a richly interesting story, as hitherto recounted<sup>[1,2]</sup>. The history was recently embellished by Reuben in one of his memorable "Landmarks" published in *Hepatology*<sup>[3]</sup> (Figure 1). AIH, known in earlier days as chronic active hepatitis (CAH), was generally regarded as a "new" disease when reported in 1950 by Waldenström<sup>[4]</sup> in an out-of-the-way Conference Proceedings on nutrition. However, since it is hardly believable that any disease of autoimmune nature would have arisen then *de novo*, it is likely that it did exist but was unrecognized, for two reasons: first, autoimmunity in the 1940s had scarcely entered the medical mind and, second, most of the necessary diagnostic laboratory procedures (liver biopsy, serum aminotransferases, serum autoantibodies) were not then routinely available. The exception was the hallmark feature of hyperglobulinaemia, recognized by Waldenström<sup>[4]</sup>, and also by others writing at around the same time (see below). Indeed increased levels of serum gamma globulin, first ascertained by moving boundary electrophoresis, are well illustrated in cases of cirrhosis of the liver reported in the early 1940s<sup>[5]</sup>. In fact, early descriptions that would fit AIH are discernible under the non-committal names of subacute hepatitis or subacute hepatic necrosis: such cases were ascribed to non-healing infectious hepatitis. As one example, Himsworth<sup>[6]</sup> in his 1947 monograph alluded to:

"the form of subacute hepatitis which appears to arise as such, without the patient ever having had acute illness suggestive of liver disease... jaundice being absent or so faint as to unnoticed... the conditions affects women more often than men... the patient may date her present illness from an acute infection, such as cystitis or bronchitis, some 1 or 2 years previously... since then she has never felt really well ... rheumatic pains, without evidence of articular damage are often noticed... or she may delay attending until the condition has passed into

the next stage of post necrotic scarring... a particular problem is why do nearly all cases of sub-acute massive necrosis inevitably progress...?"

Similarly, Zimmerman *et al*<sup>[7]</sup> in 1951 described a case of subacute hepatic necrosis in a 36-year-old man with slowly developing jaundice and extreme hyperglobulinaemia (87 g/L) and in fact reached for an immunological explanation, in commenting that:

"the initial injury causes an alteration in liver protein, which stimulates the formation of anti-liver antibodies. These newly formed antibodies produce more liver injury, thus releasing more altered liver protein, which again contributes to the vicious circle of continuing necrosis."

The general interpretation in the 1940s of subacute or chronic hepatitis was that it was a sequel to, and a consequence of, an unresolved acute infectious hepatitis. For example Neefe<sup>[8]</sup>, writing from a long experience of viral hepatitis, comments in his review in 1946 on the occurrence of chronic hepatitis as a sequel of viral hepatitis. The stage was actually set for the later nomenclature of CAH in 1945 by Barker, Capps and Allen<sup>[9]</sup> who followed the course of viral hepatitis in military personnel in the Mediterranean theatre. They defined "non-recovery" as persistence of symptoms after 4 mo and applied the term chronic hepatitis "without any implications regarding the nature of the pathologic process or the eventual prognosis". Further, their cases of "chronic hepatitis" were divided into active if symptoms were present, or inactive if there were only laboratory defined abnormalities<sup>[10]</sup>. Thereafter CAH became the standard descriptor of later years, albeit for a disease quite different from that exhibited by the soldiers. Even so, there still remained the strong impression that the precursor of CAH was hepatitis virus infection, according to articles by Wood and colleagues<sup>[11]</sup> in Melbourne in 1948, Kunkel and Labby<sup>[12]</sup> in New York in 1950, and Liebowitz<sup>[13]</sup> in New York in 1950 who followed up 68 patients with presumed acute viral hepatitis and reported that seven (11%) developed CAH. Waldenstrom<sup>[4]</sup> in his 1950 report had been more circumspect in specifying that the cause of the disease was unknown, *wie wir alle wissen*, although he also speculated on persisting viral infection (see below).

In the event, the disease seen during 1948-1950 that fulfils the modern description of AIH is illustrated by key phrases reproduced from the 1950 report of Waldenstrom<sup>[4]</sup>, and by a 1951 Meeting Abstract of Kunkel and colleagues<sup>[14]</sup>. According to Waldenstrom:

"Dazu kommt bei fast allen Fällen das Auftreten von sog. Sternchen (Naevi aranei) und bei den Mädchen auch eine besondere Tendenz zur schweren Akneeruption. Eine langdauernde Amenorrhöe ist charakteristisch, die wahrscheinlich anovulatorisch ist... Es ist damit möglich, dass in der Zukunft eine Anzahl von diesen Fällen mit ACTH verbessert werden können... Die zweite Frage, ob die nachgewiesene Erhöhung des Gammaglobulins als Zeichen eines chronischen Immunisierungsprozesses aufzufassen ist, verdient meines Erachtens grösste Auf-



**Figure 1** Montage and legend as prepared by Dr. Adrian Reuben for "Landmarks in Hepatology"<sup>[3]</sup>. **A:** Jan Gösta Waldenström (right) with Dr Göran Bauer, President of the Swedish College of Physicians, Stockholm, c.1989 (courtesy of Dr. Frank Wollheim); **B:** Henry George Kunkel, Paris, 1979, being informed of his naming for the Lita Annenberg Hazxen Award (courtesy of Dr. Eng M Tan); **C:** Ian Reay Mackay, on the occasion of his Retirement Symposium in 1987 organized by the Walter and Eliza Hall Institute (courtesy of the subject). Reproduced by courtesy of Dr. Reuben and Wiley and Sons Inc., publishers of *Hepatology*).

merksamkeit... Die Ätiologie dieser chronischen Leberleiden ist-wie wir alle wissen-immer noch unbekannt. Es werden toxische, infektiöse und Nahrungsfaktoren als Ursache angenommen... Es scheint sehr wohl möglich, dass die Gammaglobulinvermehrung als Symptom einer Immunisierung gegen das im Körper verbleibende Virus aufzufassen ist". Kunkel and colleagues<sup>[14]</sup> used the following phrases:

"Total proteins ranged from 9-13 per cent...this rise was due entirely to gamma globulin increase... eleven of these twelve patients were females... the maximum age was 32...onset of disease was insidious... course was prolonged and either stationary or downhill... frequently marked by periods of high fever, arthralgia, and arthritis ... remarkable degree of plasma cell infiltration in the liver... which diminished during the course of disease... the etiology of the syndrome remains unknown."

This apparently new liver disease came under particular scrutiny in Melbourne following the report of Wood *et al*<sup>[11]</sup> referred to above, and particularly from the standpoint of clinical and histological correlations, described by Saint *et al*<sup>[15]</sup> in 1953, who noted:

"fairly well defined clinical features, a highly characteristic pattern of biochemical tests including hypergammaglobulinaemia, and a histological picture which seems to indicate active chronic inflammatory changes... in many cases an initial history of contact or



even a history of typical acute infectious hepatitis was lacking... but little doubt existed that the liver disease was due to infection with a virus”.

This latter article followed the earlier nomenclature of Capps<sup>[10]</sup> in 1948 of active and inactive chronic hepatitis but used histological rather than clinical criteria to draw the distinction. Accordingly Saint *et al* introduced the term active chronic hepatitis<sup>[15]</sup> that for a while coexisted with CAH, although the latter eventually prevailed. The dire outcome was recorded as follows:

“the course was progressively downhill... jaundice became a permanent feature... bleeding episodes became more frequent... alternatively these patients became permanently bedridden owing to their dropsical enfeebled state, and finally lapsed into coma... the time or presentation until death has varied between six months and two years”.

Among the cases assembled by Saint *et al*<sup>[15]</sup>, one stands out. This was a 36-year-old woman, described further in 1955 in a case study by Joske and King<sup>[16]</sup> as active chronic viral hepatitis with positivity for the lupus erythematosus (L.E.) cell test. Their report includes the following:

“liver biopsy showed the typical picture of an active chronic viral hepatitis... cellular infiltration with lymphocytes and plasma cells and fibroblastic activity... fairly numerous L.E. cells present... cortisone produced a dramatic improvement in her arthralgia... we may recall that Leonie (1954) found L. E. cells in ascitic fluid from a patient with hepatic cirrhosis... we suggest that the L.E. cell and related phenomena might be based either: (1) on an abnormality of the antibody-producing mechanism... or (2) on changes in red or white cells or their constituents... which modify their characteristic “self markers”.

The latter 3-4 lines can be reasonably attributed to the pen of FM Burnet who, at this time in 1955 had already turned his mind to immunological aberrations in disease states. Also noteworthy in the report are comments on the plasma cellular content of the inflammatory infiltration into the liver, and the improvement conferred by treatment with cortisone.

The L.E. (lupus erythematosus) cell effect requires a few lines here. L.E. cells had been discovered incidentally in the 1940s by Hargraves<sup>[17]</sup> in bone marrow preparations from patients with “collagen diseases” of the lupus erythematosus type, and were modestly reported after a delay of some years in 1948 in the Mayo Clinic Proceedings. Hargraves himself was quite surprised by the interest that his report aroused<sup>[18]</sup>. This interest intensified further with the discovery that the L.E. cell effect depended on a serum factor, of gamma globulin nature<sup>[19]</sup>, which only a few years thereafter became recognized by various groups as an antinuclear autoantibody. An item of interest, unreported and transmitted as a personal comment from Hargraves to this author, was that among the initial patients in whom L.E. cells were detected was a young girl (PC) suffering from chronic hepatitis! The L.E. cell test soon became

a surrogate marker for an autoimmune basis for a given disease and, as such, was deployed in Melbourne in the early 1950s as the single available routine laboratory indicator for multisystem autoimmunity-hence its application to cases of CAH.

## LUPOID TO AIH

After my return to Melbourne from abroad in 1955, it took only a little time to identify several instances of CAH in young women with hypergammaglobulinaemia and a lymphoplasmacytic infiltration in the liver and, in each, a positive test for the presence of L.E. cells in blood. Then the case for an immunological derangement as the cause of the disease became even stronger because at the time, DC Gajdusek who was a visiting scientist to the Hall Institute was attempting to develop a diagnostic serological test for viral hepatitis based on a complement fixation (CF) reaction, using as antigen liver tissue obtained at autopsy from a patient with fatal acute viral hepatitis. However sera from cases of acute hepatitis were at most only weakly positive using this CF reaction, whereas sera from cases of CAH (inserted as disease controls) tested positive, not only using as antigen the virally infected liver tissue but also using normal liver as well<sup>[20]</sup>. We can recall that during the development of the Wassermann serological test for syphilis infection, *Spirochaeta*-infected tissue was used initially, but normal tissue was found to serve equally well to elicit a positive reaction.

The several patients with CAH and a positive L.E. cell test appeared to represent a unique disease entity since most had, additionally, extrahepatic disease expressions including arthralgia, rash, haemolytic anaemia and others, typically seen in cases of systemic lupus erythematosus (SLE) wherein sera also gave a positive autoimmune CF test. In a report to *Lancet* in 1957<sup>[21]</sup>, the concept was developed thus:

“linking certain types of active chronic hepatitis and lupus erythematosus... possibly through the common factor of disturbed immunological response... this group of cases has been provisionally designated as ‘lupoid hepatitis’ since ‘lupus’ has now acquired a far broader significance than the original term suggests... we consider that immunological destruction of the host’s liver cells best explains the perpetuation of the hepatitis and progression to cirrhosis. If this is so, it would be rational to use therapeutic measures (e.g. cortisone therapy) designed to modify this process, and our experience suggests that cortisone is of benefit in this autoimmune hepatitis.”

The global response to this report was remarkable. There were journal reports of patients with lupoid hepatitis from far and wide and, as well, detailed case studies, by Bearn *et al*<sup>[22]</sup>, Reynolds *et al*<sup>[23]</sup> and others<sup>[1,2]</sup>. Thereafter there arose controversy, still not completely resolved<sup>[3,24]</sup>, as to whether “lupoid hepatitis” was intended to specify a particular form of CAH and thus a disease in the realm of hepatology, or a component of a multisystem syndrome in the realm of rheumatology.

Admittedly, this dilemma was not helped by our earlier writings which attempted to distinguish cases of “lupoid hepatitis” from those of “CAH” in which L.E. cell positivity was not demonstrable<sup>[25]</sup>. The development of the immunofluorescence test for antinuclear antibody (ANA), and its application to patients with CAH<sup>[26]</sup> soon revealed that cases of “lupoid” and “ordinary” CAH were indistinguishable on important criteria such as biochemical indices of liver dysfunction, histological abnormalities in the liver, and responsiveness to immunosuppressive therapy. Also, by the early 1960s, it was clearly evident that the disease in all its guises was best accounted for by an autoimmune reaction in the liver and thus, in 1965, we suggested that the disease be named “autoimmune hepatitis”<sup>[27]</sup>, although this appellation did not become formally endorsed until 1993<sup>[28]</sup>. Meanwhile, lupoid hepatitis lived on for quite some years, having heuristic appeal in some jurisdictions and causing semantic grievance in others, to the extent that one publication was directed to establishing that lupoid hepatitis was a “non-entity” within the disease group known as CAH<sup>[29]</sup>. Actually our Clinic had already discarded a possible association between lupoid hepatitis and SLE by showing in 1959 that liver lesions in classic instances of SLE were relatively trivial and nondescript<sup>[30]</sup>, and a serological distinction was forthcoming a few years later (see below).

## THERAPIES FOR CHRONIC ACTIVE/AIH

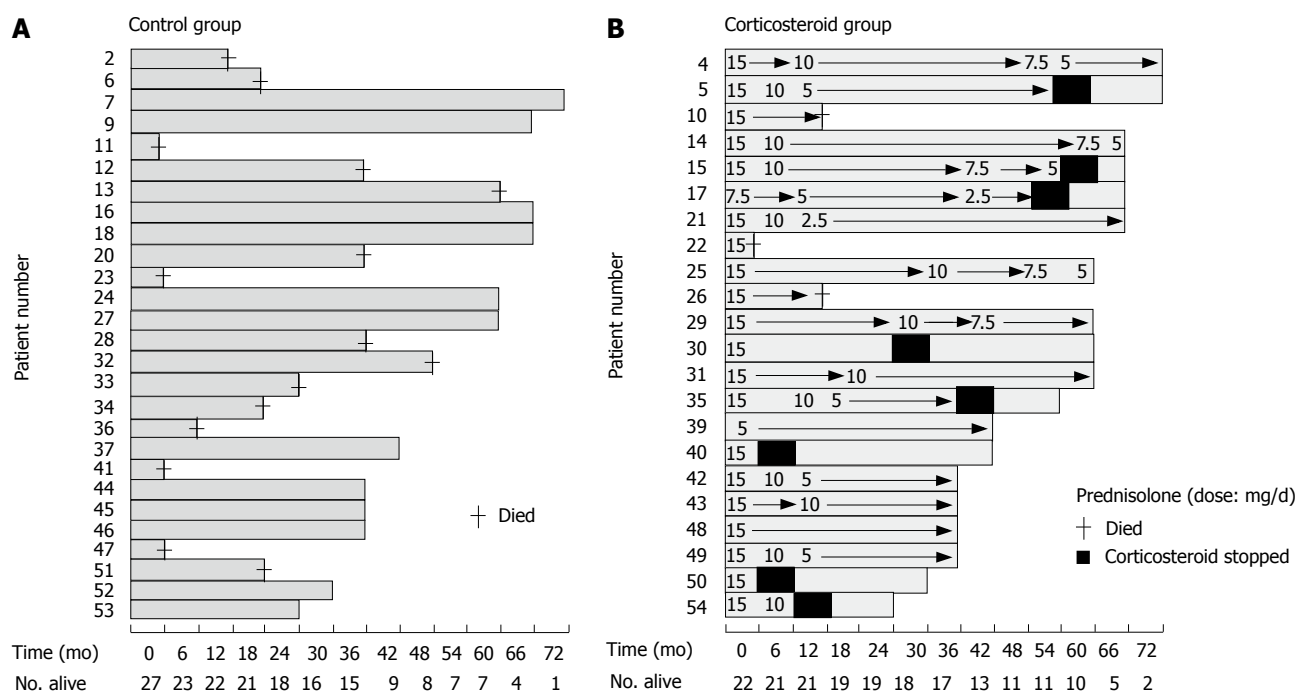
In our 1957 publication on lupoid hepatitis<sup>[21]</sup>, the notion of immunological destruction of host liver cells was seen to provide a rationale for the use of “anti-immune” therapies to modify the process. Corticosteroid therapy in fact had been used in cases of CAH in Melbourne from as early as 1953, and, although initial results were equivocal<sup>[15]</sup>, later experience proved more encouraging<sup>[31]</sup>. Also, Bearn *et al*<sup>[21]</sup> in their 1956 article that formalised observations made in the 1951 Meeting Abstract<sup>[14]</sup>, reported that cortisone induced improvement in symptoms, and in physical and biochemical expressions of disease, and that withdrawal of cortisone from two patients resulted in prompt relapse. However they did retain the proviso that “there was no conclusive evidence that cortisone modified the disease process or that it will alter the eventual outcome”. A telling observation in Melbourne, reported in 1958<sup>[32]</sup>, was that by serial daily monitoring of levels of serum aspartate transaminase after treatment was started, prompt falls in highly raised levels occurred, often within hours, and other indices of impaired liver function improved in turn. Moreover, since relapse tended to occur when cortisone was withdrawn, the need for long term maintenance therapy became evident. Hence co-therapy with a “steroid-sparing” agent was sought and, on theoretical grounds, there was chosen azathioprine, a derivative of the immunosuppressive drug 6-mercaptopurine, and this conferred added benefit<sup>[33]</sup>. However there was scepticism whether our routine prednisolone-azathioprine regimen, albeit

symptomatically beneficial, actually altered the natural history of the disease. The view in Melbourne was that a trial that included randomly allocated placebo control patients was ethically problematic. Our decision was that all trial patients would receive active therapy after a 4-8 wk non-treatment run-up with close monitoring of multiple liver functional indices, and comparison would be made of mean levels for such indices for a group of 15 patients pre- and post -therapy with prednisolone, or prednisolone plus azathioprine. The outcome, published in 1968<sup>[34]</sup>, led to the recommendation for long term (2-3 years) maintenance therapy with either prednisolone monotherapy, or combined prednisolone-azathioprine. However, conventional trials with randomly allocated non-treated controls were still called for, and these convincingly indicated survival benefits for the treated patients. The study of Cook *et al*<sup>[35]</sup> is exemplary, albeit with a substantial burden of mortality among the control group (Figure 2). Detailed studies on immunosuppressive therapy by the Mayo Clinic through the 1970s-1980s, with substantial case numbers, have shown sustained efficacy according to various criteria, survival, biochemical and histological<sup>[36]</sup>.

## PATHOLOGICAL FEATURES OF CHRONIC ACTIVE/AIH

The increasing use of percutaneous biopsy of the liver in the 1950s greatly contributed to better knowledge of the nature of chronic hepatitis, well illustrated by the description in 1958 by Klatskin<sup>[37]</sup> of nine cases bearing the pre-1950s diagnoses of subacute hepatic necrosis/post-necrotic cirrhosis, and attributed to “anicteric infections with the hepatitis virus” because histopathological appearances met existing criteria for subacute hepatic necrosis of viral origin. However scrutiny of the clinical, biochemical and histological features strongly suggests that the illness in some, perhaps most, of the nine cases was actually AIH: there was female preponderance, hyperglobulinaemia, and corticosteroid responsiveness. However the particular feature of Klatskin’s study was the introduction of, or emphasis on, detailed histological features characteristic of those associated with autoimmune (as well as with viral) hepatitis. These included extensive “bridging” hepatocellular necrosis, ballooning of hepatocytes, regenerating hepatocytes with arrangement of cells in the form of “rosettes”, intense mononuclear inflammatory reaction; regenerative nodules, and acidophilic bodies (Councilman bodies) which are rounded intensely eosinophilic homogeneous cytoplasmic masses derived from hepatic cells undergoing coagulation necrosis. These latter appearances of course represent apoptosis of hepatocytes, recognized by Kerr<sup>[38]</sup> some 30-40 years before but not widely appreciated until interest developed in the 1980s in the apoptosis process. A detailed analysis of apoptosis in the context of chronic hepatitis and liver cell degeneration is given by Searle *et al*<sup>[39]</sup>.

There were two morphological features additional



**Figure 2** Combined Figures 1 and 2 from a publication<sup>[35]</sup> on long-term prospective controlled trial of corticosteroid treatment of patients with active chronic (autoimmune) hepatitis. The graphs show duration of treatment and survival for (A), left, non-treated control group and (B), right, corticosteroid treated group, and length of time (months) that the individual patients in either group had been in the investigation at the time of assessment. Numbers indicate the sequence of entry to the trial; +, time of death. Survival was 19/22 for the treated group and 12/27 for the control group. Reproduced with permission of Oxford University Press, publishers of Quarterly Journal of Medicine.

to those described by Klatskin that deserve comment. One was the concentration of inflammatory activity and necrosis in the junctional region between the portal tract and liver lobule, initially described by Popper *et al*<sup>[40]</sup> as piecemeal necrosis, and currently referred to as interface hepatitis. The other feature was the striking accumulation among the inflammatory infiltrates of plasma cells, noted in most pathological descriptions, although not all<sup>[41]</sup>, and earlier on leading to the disease being designated as “plasma cell hepatitis”<sup>[42]</sup>. This feature of CAH, i.e. prominence of plasma cells in liver, and bone marrow as well, aligns with the characteristic hypergammaglobulinaemia, although the antigenic specificity of these plasma cells and their secreted immunoglobulins has not yet been ascertained.

Finally we can note the protracted debate on whether histological features, irrespective of clinical diagnosis, would distinguish cases of chronic hepatitis with a progressive course and cirrhotic potential-CAH-from those without such potential-chronic persistent hepatitis<sup>[43,44]</sup>. After much elaboration in the 1970s these terms slowly disappeared from the lexicon.

## HETEROGENEITY OF CAH

The term CAH used in 1950s and 1960s was generic, for what tended to be regarded as a single disease entity, with the prototypic cases being those that would fulfil criteria that presently define the autoimmune type of disease. However, impressions developed from the 1960s that CAH was not a homogeneous disease entity. Thus a collaborative study with colleagues in

Singapore ascertained that CAH seen among Caucasians in Melbourne and Chinese in Singapore differed substantially, clinically, histologically and serologically<sup>[45]</sup>. Similarly, in unpublished observations in Melbourne, features of cases of chronic hepatitis and cirrhosis among recent Southern European immigrants differed from those among Australian-born individuals, such that we spoke of “Mediterranean cirrhosis”. An explanation was soon forthcoming. This depended on the fortuitous discovery by Blumberg and colleagues of an antigenic particle in serum initially called “Australia antigen” (Au), because it was first detected in serum from an Australian aboriginal serum donor<sup>[46]</sup>. Au was later found to be a surface protein of a virus identified with as the transmissible agent responsible for infectious (serum) hepatitis type B. The particle became known as hepatitis B surface antigen-HBsAg<sup>[47]</sup>.

It became evident, as reported from several centres that cases of “CAH” segregated into those that were autoantibody positive and HBsAg negative, or vice versa and, in fact, prototypic (autoimmune) CAH became referred to as HBsAg-negative CAH<sup>[36]</sup>. Moreover, certain histological features of CAH, chiefly interface hepatitis, could be recognized in various other causally different cases chronic liver disease, whether due to viral infection, ethanol abuse, Wilson’s disease or other causes. An invitation was extended to me in 1972 to write an editorial on the “Prognosis (I changed this to Prognoses) of CAH” which led to a proposal that CAH was indeed heterogeneous in terms of aetiology, histological appearances, immunogenetic background, therapy requirements and outcome<sup>[48]</sup>. Moreover it seemed

reasonable to assume that aetiological proportions among all cases of CAH would differ according to geographic region, and this proved to be the case, as judged by differences ascertained for cases of CAH in Australia and Singapore (see above) or Yugoslavia wherein proportions of cases due to chronic hepatitis B were substantially higher<sup>[49]</sup>. Finally, there were cases regarded as cryptogenic CAH, with some perhaps resulting from infection with non-A, non-B hepatitis virus; these later became attributable to the subsequently discovered hepatitis C virus (HCV). Currently, worldwide, the proportion of cases of chronic hepatitis B, followed by that of hepatitis C, far outnumber that of all other types.

All this led to the perception of a need for a clearer definition of CAH. This was met by the convening of a widely representative expert panel of hepatologists, the International AIH Group whose consensus deliberations led to reports in 1993 and 1999 on generally acceptable diagnostic criteria<sup>[28,50]</sup>.

## GENETIC FEATURES OF CHRONIC ACTIVE/AIH

The wide regional differences in prevalence of different types of chronic hepatitis depend on differing environmental exposures and genetic composition of the particular population groups. Among the former, the endemicity of hepatitis virus infections is one important element.

The first genetic factor of interest in AIH was female predisposition, common to most autoimmune diseases, and for which the basis is still not well understood. Then, recognized in 1972, were genes that encoded for human leucocyte antigens, HLA. Cases of “classical” CAH, selected for typing for the then testable HLA alleles encoded at the HLA A and B loci, showed a significantly increased frequency of HLA-B8<sup>[51]</sup>, an allele already implicated among Caucasian subjects with certain other autoimmune diseases. This association was soon confirmed in other centres, and was followed by the finding of an increased frequency in CAH of the D-related (DR) locus allele, DR3; a family study showed the combined inheritance of HLA A1, B8 and DR3 *en bloc* (a haplotype) from one or other parent, or both<sup>[52]</sup>. Possession of HLA DR3, particularly in those homozygous for these alleles, was predictive of a severe course and lesser responsiveness to immunosuppressive therapy<sup>[53]</sup>. The culprit allele is now styled as *HLA-DRB1\*0301*. Later an additional HLA type, DR4 (*HLA-DRB1\*0401*), not evident in our earlier studies, was identified<sup>[54]</sup>. The 6-7 fold risk for disease conferred by HLA DR3/4 is substantial but not highly potent meaning that, like all other “complex” autoimmune diseases, there must exist multiple other polymorphisms in “tolerance/ autoimmunity” genes that contribute to susceptibility: these are mostly undiscerned pending application of population genetics by genome wide screening.

## IMMUNOSEROLOGICAL AND T-CELL STUDIES IN CHRONIC ACTIVE/AIH

The reactivities that initially (in the 1950s) were indicative of autoimmunity in CAH, the L.E. cell test and the AICF reaction, were soon superseded by more discriminatory and simpler laboratory assays. These are described in detail in other articles in this issue and in contemporary reviews<sup>[55-57]</sup>.

### Nuclear antigen(s)

Detection of ANA by indirect immunofluorescence (IIF) was introduced in the early 1960s<sup>[26]</sup> and remains the standard diagnostic screening procedure<sup>[57]</sup>. Superficially at least, the nuclear reactant(s) is the same as that responsible for the ANA reactivity observed in SLE i.e. the nucleosome (chromatin), although anti-DNA is much less frequent<sup>[56]</sup>. The idea that patients with AIH and SLE share one or more of the gene loci that determine ANA reactivity may be revealed by future population genome studies.

### Smooth muscle antigen(s)

In 1963 there was observed a novel reactivity with smooth muscle of rodent gastric mucosa<sup>[58]</sup>. Detection of this smooth muscle antibody (SMA) to high titre proved to have high specificity for the diagnosis of CAH and notably, in “conventional” cases of SLE in which inflammatory destruction of liver cells is not evident, the test proved negative<sup>[59]</sup>. Further observations showed that some SMA+ve sera reacted by IIF with the mesangium of renal glomeruli, indicative of a wider distribution of the antigenic reactant than merely gastric smooth muscle tissue<sup>[60]</sup>. A subsequent observation was that some positive sera gave reactivity only with blood vessel walls (SMAv), and others reacted as well with renal glomeruli and renal tubular cells (SMAgt)<sup>[61]</sup>. The recognition that SMAv pointed to non-specific reactivity, and SMAvgt to reactivity specifically associated with AIH has led laboratory serologists to retain the designations SMAv and SMAgt in their diagnostic reporting.

The first indication of the identity of a reactant for SMA+ve sera was that reactivity could be absorbed from serum by exposure to the cytoskeletal protein F-actin<sup>[62]</sup>. Further studies using IIF on cultured tissue cells revealed that SMA+ve sera stained cytoskeletal microfilaments (actin “cables”), representing polymeric F-actin, whereas SMA+ve sera from cases other than CAH stained intermediate filaments representing vimentin, desmin or others<sup>[63]</sup>. After much developmental work, there are now commercially available ELISA formats based on highly purified F-actin that have good specificity and sensitivity for the diagnosis of AIH<sup>[56]</sup>. The need at present is for better knowledge on the basis of anti-F-actin reactivity, including the significance (if any) for the pathogenesis of AIH, the epitope specificity of the antibodies, the relationship of epitopes to binding sites for the numerous F-actin binding proteins in the cell, and functional effects of anti-F-actin on cell motility<sup>[64]</sup>.



### LKM-1 antigen

In 1973, yet another serum reactant in AIH was discovered by IIF, to an antigen that was enriched in cytoplasm of liver and kidney proximal tubular cells<sup>[65]</sup>. This so-called liver-kidney “microsomal” (LKM) antigen, later designated LKM-1 because other LKM antigens became demonstrable<sup>[56]</sup>, is actually located in the endoplasmic reticulum of liver and kidney proximal tubular cells<sup>[66]</sup>. A notable feature of anti-LKM-1-positive-AIH, versus ANA/SMA positive AIH, is that the serologically defined reactivities appear mutually exclusive, providing grounds for distinguishing these serological variants of AIH as type 2 and type 1<sup>[67]</sup>; this distinction has been retained by hepatologists even though few other differences exist. The point of interest heuristically on the serological distinction is that the respective autoantibody responses (ANA/SMA, anti-LKM-1) cannot be ascribed simply to (hepato) cellular injury, an explanation often levelled for the appearance of at least for some types of autoantibody. Although cases of anti-LKM-1-positive AIH are numerically far less than the traditional type, the ratios being about 1:10 in adults and 1:4 in children, type 2 AIH has proven far more amenable to investigation, since the LKM-1 antigen has been molecularly identified by screening a gene expression library as the cytochrome P450 isoform 2D6, enabling epitopes to be mapped<sup>[68]</sup>; there is demonstrated a CD4+ T-cell responsiveness to peptide antigens of CYP450 2D6<sup>[69]</sup>; and an experimentally credible model in mice has been developed<sup>[70]</sup>.

### Soluble liver/pancreas antigen

A soluble cytoplasmic antigen was independently discovered by CF<sup>[71]</sup>, and ELISA<sup>[72]</sup>, using pancreas or liver cell extracts respectively, and the reactant was found to be identical; it is generally known as “soluble liver/pancreas antigen (SLA). Sero-positivity, initially thought to identify a type 3 AIH, occurs in cases of AIH that are negative for other reactivities, but also in sero-positive cases of type 1 AIH. SLA has been cloned, identified and purified<sup>[56]</sup>, and commercially available ELISA kits are diagnostically reliable. The pathogenetic significance of SLA is uncertain.

### A liver-specific and disease specific antigen

This has been long searched for, since AIH behaves very much like an organ-specific autoimmune disease. In earlier times much effort was put into the characterisation of a preparation called liver-specific lipoprotein (LSP)<sup>[73]</sup>, and assessment of the autoantigenic potential of this claimed liver-specific molecule<sup>[74]</sup>. Stemming from this was the recognition of the liver-specific membrane antigen, the asialoglycoprotein receptor<sup>[75]</sup>, but this has not quite fulfilled the earlier hopes<sup>[76]</sup>. The liver cell membrane has been repeatedly studied, mostly by immunoblotting using AIH sera, for a molecular signal corresponding to a specific autoantigenic moiety, with the consistent finding being that of multiple reactive components of mw ranging from 20 to > 100 kDa<sup>[77,78]</sup>, but with none of these

reaching candidate status as a liver-specific or disease-specific autoantigen.

## CONCLUSION

Despite weighty circumstantial evidence for autoimmunity as one proximal cause of chronic hepatitis, and the existence of diagnostic serological reactants of high sensitivity and specificity, there is a disconcerting lack of mechanistic immunological explanation for AIH, and particularly the more prevalent type 1. There are high expectations for “the way we hope to be”<sup>[79]</sup> but, for these to be realised, new paradigms will be needed to explain AIH as well as other examples of organ-specific autoimmunity with non-organ-specific immune-dependent accompaniments.

## REFERENCES

- 1 Mackay IR. Chronic active hepatitis. *Front Gastrointest Res* 1975; **1**: 142-187
- 2 Mackay IR, Tait BD. The history of autoimmune hepatitis. In: Autoimmune Hepatitis, Nishioka M, Toda G, Zeniya M Eds. Amsterdam: Elsevier, 1994: 3-23
- 3 Reuben A. A sheep in wolf's clothing. *Hepatology* 2003; **38**: 1596-1601
- 4 Waldenstrom J. Leber, Blutproteine und Nahrungseiweiss. *Dtsch Ges Verdau Stoffwechselkr* 1950; **15**: 113-119
- 5 Stern KG, Reiner M. Electrophoresis in medicine. *Yale J Biol Med* 1946; **19**: 67-99
- 6 Himsworth HP. Lectures on the Liver and its Diseases. Oxford: Blackwell, 1947: 158-161
- 7 Zimmerman HJ, Heller P, Hill RP. Extreme hyperglobulinemia in subacute hepatic necrosis. *N Engl J Med* 1951; **244**: 245-249
- 8 Neefe JM. Recent advances in the knowledge of ‘virus hepatitis’. *Med Clin N Amer* 1946; **39**: 1407-1443
- 9 Barker MH, Capps RB, Allen, FW. Chronic hepatitis in the Mediterranean theatre. *JAMA* 1945; **129**: 653-659
- 10 Capps RB. Clinical aspects of the sequelae of acute hepatitis. *Gastroenterology* 1948; **11**: 680-690
- 11 Wood IJ, King WE, Parsons PJ, Perry JW, Freeman M, Lim-Brick L. Non-suppurative hepatitis: a study of acute and chronic forms with special reference to biochemical and histological changes. *Med J Austr* 1948; **1**: 249-261
- 12 Kunkel HG, Labby DH. Chronic liver disease following infectious hepatitis. II. Cirrhosis of the liver. *Ann Intern Med* 1950; **32**: 433-450
- 13 Leibowitz S. Virus hepatitis implications of its chronic stages. *J Insur Med* 1950; **5**: 10-18
- 14 Kunkel HG, Ahrens EH, Eisenmenger WJ, Bongiovanni AM, Slater RJ. Extreme hypergammaglobulinemia in young women with liver disease. *J Clin Invest* 1951; **30**: 654
- 15 Saint EG, King WE, Joske RA, Finckh ES. The course of infectious hepatitis with special reference to prognosis and the chronic stage. *Australas Ann Med* 1953; **2**: 113-127
- 16 Joske RA, King WE. The L.E.-cell phenomenon in active chronic viral hepatitis. *Lancet* 1955; **269**: 477-480
- 17 Hargraves MM, Richmond H, Morton R. Presentation of two bone marrow elements: the “tart” cell and the “L.E.” cell. *Proc Staff Meet Mayo Clin* 1948; **23**: 25-28
- 18 Hargraves MM. Discovery of the LE cell and its morphology. *Mayo Clin Proc* 1969; **44**: 579-599
- 19 Haserick JR, Lewis LA, Bortz DW. Blood factor in acute disseminated lupus erythematosus; determination of gamma globulin as specific plasma fraction. *Am J Med Sci* 1950; **219**: 660-663
- 20 Mackay IR, Gajdusek DC. An autoimmune reaction against

- human tissue antigens in certain acute and chronic diseases. II. Clinical correlations. *AMA Arch Intern Med* 1958; **101**: 30-46
- 21 **Mackay IR**, Taft LI, Cowling DC. Lupoid hepatitis. *Lancet* 1956; **271**: 1323-1326
  - 22 **Bearn AG**, Kunkel HG, Slater RJ. The problem of chronic liver disease in young women. *Am J Med* 1956; **21**: 3-15
  - 23 **Reynolds TB**, Edmondson HA, Peters RL, Redeker A. Lupoid hepatitis. *Ann Intern Med* 1964; **61**: 650-666
  - 24 **Mackay IR** **Hepatic disease and systemic lupus erythematosus: coincidence or convergence**. In: Lahita RG, editor. *Systemic Lupus Erythematosus*. 4ed. San Diego: Elsevier, 2004: 993-1017
  - 25 **Mackay IR**. The problem of persisting destructive disease of the liver. *Gastroenterology* 1961; **40**: 617-626
  - 26 **Holborow EJ**, Asherson GL, Johnson GD, Barnes RD, Carmichael DS. Antinuclear factor and other antibodies in blood and liver diseases. *Br Med J* 1963; **1**: 656-658
  - 27 **Mackay IR**, Weiden S, Hasker J. Autoimmune hepatitis. *Ann N Y Acad Sci* 1965; **124**: 767-780
  - 28 **Johnson PJ**, McFarlane IG. Meeting report: International Autoimmune Hepatitis Group. *Hepatology* 1993; **18**: 998-1005
  - 29 **Soloway RD**, Summerskill WH, Baggenstoss AH, Schoenfield LJ. "Lupoid" hepatitis, a nonentity in the spectrum of chronic active liver disease. *Gastroenterology* 1972; **63**: 458-465
  - 30 **Mackay IR**, Taft LI, Cowling DC. Lupoid hepatitis and the hepatic lesions of systemic lupus erythematosus. *Lancet* 1959; **1**: 65-69
  - 31 **Last PM**. The treatment of active chronic infectious hepatitis with ACTH (corticotrophin) and cortisone. *Med J Aust* 1957; **44**: 672-676
  - 32 **O'Brien EN**, Goble AJ, Mackay IR. Plasma-transaminase activity as an index of the effectiveness of cortisone in chronic hepatitis. *Lancet* 1958; **1**: 1245-1249
  - 33 **Mackay IR**, Weiden S, Ungar B. Treatment of active chronic hepatitis and lupoid hepatitis with 6-mercaptopurine and azathioprine. *Lancet* 1964; **1**: 899-902
  - 34 **Mackay IR**. Chronic hepatitis: effect of prolonged suppressive treatment and comparison of azathioprine with prednisolone. *Q J Med* 1968; **37**: 379-392
  - 35 **Cook GC**, Mulligan R, Sherlock S. Controlled prospective trial of corticosteroid therapy in active chronic hepatitis. *Q J Med* 1971; **40**: 159-185
  - 36 **Czaja AJ**, Davis GL, Ludwig J, Taswell HF. Complete resolution of inflammatory activity following corticosteroid treatment of HBsAg-negative chronic active hepatitis. *Hepatology* 1984; **4**: 622-627
  - 37 **Klatskin G**. Subacute hepatic necrosis and postnecrotic cirrhosis due to anicteric infections with the hepatitis virus. *Am J Med* 1958; **25**: 333-358
  - 38 **Kerr JF**. Shrinkage necrosis: a distinct mode of cellular death. *J Pathol* 1971; **105**: 13-20
  - 39 **Searle J**, Harmon BV, Bishop CJ, and Kerr JFR. Significance of single cell death by apoptosis in hepatobiliary disease. *J Gastroenterol Hepatol* 1987; **2**: 77-96
  - 40 **Popper H**, Paronetto F, Schaffner F. Immune processes in the pathogenesis of liver disease. *Ann N Y Acad Sci* 1965; **124**: 781-799
  - 41 **Dienes HP**. Viral and autoimmune hepatitis. Stuttgart: Fischer Verlag, 1989: 47-51
  - 42 **Page AR**, Good RA. Plasma-cell hepatitis, with special attention to steroid therapy. *AMA J Dis Child* 1960; **99**: 288-314
  - 43 **De Groote J**, Desmet VJ, Gedigk P, Korb G, Popper H, Poulsen H, Scheuer PJ, Schmid M, Thaler H, Uehlinger E. A classification of chronic hepatitis. *Lancet* 1968; **2**: 626-628
  - 44 **Acute and chronic hepatitis revisited**. Review by an international group. *Lancet* 1977; **2**: 914-919
  - 45 **Whittingham S**, Mackay IR, Thanabalasundrum RS, Chuttani HK, Manjuran R, Seah CS, Yu M, Viranuvatti V. Chronic liver disease: differences in autoimmune serological reactions between Australians and Asians. *Br Med J* 1973; **4**: 517-519
  - 46 **Blumberg BS**, Gerstley BJ, Hungerford DA, London WT, Sutnick AI. A serum antigen (Australia antigen) in Down's syndrome, leukemia, and hepatitis. *Ann Intern Med* 1967; **66**: 924-931
  - 47 **Prince AM**. An antigen detected in the blood during the incubation period of serum hepatitis. *Proc Natl Acad Sci USA* 1968; **60**: 814-821
  - 48 **Mackay IR**. The prognoses of chronic hepatitis. *Ann Intern Med* 1972; **77**: 649-651
  - 49 **Pedersen JS**, Toh BH, Mackay IR, Tait BD, Gust ID, Kastelan A, Hadzic N. Segregation of autoantibody to cytoskeletal filaments, actin and intermediate filaments with two types of chronic active hepatitis. *Clin Exp Immunol* 1982; **48**: 527-532
  - 50 **Alvarez F**, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, Chapman RW, Cooksley WG, Czaja AJ, Desmet VJ, Donaldson PT, Eddleston AL, Fainboim L, Heathcote J, Homberg JC, Hoofnagle JH, Kakumu S, Krawitt EL, Mackay IR, MacSween RN, Maddrey WC, Manns MP, McFarlane IG, Meyer zum Beschenfelde KH, Zeniya M. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; **31**: 929-938
  - 51 **Mackay IR**, Morris PJ. Association of autoimmune active chronic hepatitis with HL-A1.8. *Lancet* 1972; **2**: 793-795
  - 52 **Mackay IR**, Tait BD. HLA associations with autoimmune-type chronic active hepatitis: identification of B8-DRw3 haplotype by family studies. *Gastroenterology* 1980; **79**: 95-98
  - 53 **Czaja AJ**, Donaldson PT. Genetic susceptibilities for immune expression and liver cell injury in autoimmune hepatitis. *Immunol Rev* 2000; **174**: 250-259
  - 54 **Donaldson PT**, Doherty DG, Hayllar KM, McFarlane IG, Johnson PJ, Williams R. Susceptibility to autoimmune chronic active hepatitis: human leukocyte antigens DR4 and A1-B8-DR3 are independent risk factors. *Hepatology* 1991; **13**: 701-706
  - 55 **Zachou K**, Rigopoulou E, Dalekos GN. Autoantibodies and autoantigens in autoimmune hepatitis: important tools in clinical practice and to study pathogenesis of the disease. *J Autoimmune Dis* 2004; **1**: 2
  - 56 **Mackay IR**. Chronic hepatitis. In: Rose NR, Mackay IR, editors. *The Autoimmune Diseases*. 4th ed. San Diego: Academic Press, 2006: 729-747
  - 57 **Vergani D**, Alvarez F, Bianchi FB, Cancado EL, Mackay IR, Manns MP, Nishioka M, Penner E. Liver autoimmune serology: a consensus statement from the committee for autoimmune serology of the International Autoimmune Hepatitis Group. *J Hepatol* 2004; **41**: 677-683
  - 58 **Johnson GD**, Holborow EJ, Glynn LE. Antibody to smooth muscle in patients with liver disease. *Lancet* 1965; **2**: 878-879
  - 59 **Whittingham S**, Irwin J, Mackay IR, Smalley M. Smooth muscle autoantibody in "autoimmune" hepatitis. *Gastroenterology* 1966; **51**: 499-505
  - 60 **Whittingham S**, Mackay IR, Irwin J. Autoimmune hepatitis. Immunofluorescence reactions with cytoplasm of smooth muscle and renal glomerular cells. *Lancet* 1966; **1**: 1333-1335
  - 61 **Bottazzo GF**, Florin-Christensen A, Fairfax A, Swana G, Doniach D, Groeschel-Stewart U. Classification of smooth muscle autoantibodies detected by immunofluorescence. *J Clin Pathol* 1976; **29**: 403-410
  - 62 **Gabbiani G**, Ryan GB, Lamelin JP, Vassalli P, Majno G, Bouvier CA, Cruchaud A, Lescher EF. Human smooth muscle autoantibody. Its identification as antiactin antibody and a study of its binding to "nonmuscular" cells. *Am J Pathol* 1973; **72**: 473-488
  - 63 **Toh BH**. Smooth muscle autoantibodies and autoantigens. *Clin Exp Immunol* 1979; **38**: 621-628
  - 64 **Mackay IR**, Martinez-Neira R, Whittingham S, Nicolau D, Toh B-H. Autoantigenicity of Actin In: dos Remedios C, Chhabra D Eds, *Actin, Actin-binding Proteins and Disease*, New York: Springer, 2008: 50-64

- 65 **Rizzetto M**, Swana G, Doniach D. Microsomal antibodies in active chronic hepatitis and other disorders. *Clin Exp Immunol* 1973; **15**: 331-344
- 66 **Alvarez F**, Bernard O, Homberg JC, Kreibich G. Anti-liver-kidney microsome antibody recognizes a 50,000 molecular weight protein of the endoplasmic reticulum. *J Exp Med* 1985; **161**: 1231-1236
- 67 **Homberg JC**, Abuaf N, Bernard O, Islam S, Alvarez F, Khalil SH, Poupon R, Darnis F, Levy VG, Gripon P. Chronic active hepatitis associated with antiliver/kidney microsome antibody type 1: a second type of "autoimmune" hepatitis. *Hepatology* 1987; **7**: 1333-1339
- 68 **Manns MP**, Johnson EF, Griffin KJ, Tan EM, Sullivan KF. Major antigen of liver kidney microsomal autoantibodies in idiopathic autoimmune hepatitis is cytochrome P450db1. *J Clin Invest* 1989; **83**: 1066-1072
- 69 **Ma Y**, Bogdanos DP, Hussain MJ, Underhill J, Bansal S, Longhi MS, Cheeseman P, Mieli-Vergani G, Vergani D. Polyclonal T-cell responses to cytochrome P450IID6 are associated with disease activity in autoimmune hepatitis type 2. *Gastroenterology* 2006; **130**: 868-882
- 70 **Lapierre P**, Djilali-Saiah I, Vitozzi S, Alvarez F. A murine model of type 2 autoimmune hepatitis: Xenoinmunization with human antigens. *Hepatology* 2004; **39**: 1066-1074
- 71 **Berg PA**, Stechemesser E, Strienz J. Hypergammaglobulinämische chronisch aktive Hepatitis mit Nachweis von leber-pankreas-spezifischen komplemtbindenden Autoantikörpern. *Verh Dtsch Ges Inn Med* 1981; **87**: 921-927
- 72 **Manns M**, Gerken G, Kyriatsoulis A, Staritz M, and Meyer zum Büschenfelde KH. Characterization of a new subgroup of autoimmune chronic active hepatitis by autoantibodies against a soluble liver antigen. *Lancet* 1987; **1**: 292-294
- 73 **Meyer zum Buschenfelde KH**, Hutteroth TH, Manns M, Moller B. The role of liver membrane antigens as targets in autoimmune type liver disease. *Springer Semin Immunopathol* 1980; **3**: 297-315
- 74 **Chisari FV**. Liver-specific protein in perspective. *Gastroenterology* 1980; **78**: 168-170
- 75 **McFarlane IG**, McFarlane BM, Major GN, Tolley P, Williams R. Identification of the hepatic asialo-glycoprotein receptor (hepatic lectin) as a component of liver specific membrane lipoprotein (LSP). *Clin Exp Immunol* 1984; **55**: 347-354
- 76 **Treichel U**, McFarlane BM, Seki T, Krawitt EL, Alessi N, Stickel F, McFarlane IG, Kiyosawa K, Furuta S, Freni MA. Demographics of anti-asialoglycoprotein receptor autoantibodies in autoimmune hepatitis. *Gastroenterology* 1994; **107**: 799-804
- 77 **Swanson NR**, Reed WD, Yarred LJ, Shilkin KB, Joske RA. Autoantibodies to isolated human hepatocyte plasma membranes in chronic active hepatitis. II. Specificity of antibodies. *Hepatology* 1990; **11**: 613-621
- 78 **Matsuo I**, Ikuno N, Omagari K, Kinoshita H, Oka M, Yamaguchi H, Kohno S, Mackay IR. Autoimmune reactivity of sera to hepatocyte plasma membrane in type 1 autoimmune hepatitis. *J Gastroenterol* 2000; **35**: 226-234
- 79 **Mackay IR**, Toh BH. Autoimmune hepatitis: the way we were, the way we are today and the way we hope to be. *Autoimmunity* 2002; **35**: 293-305

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Pietro Invernizzi, MD; Ian R Mackay, MD, Series Editors

## Clinical features and management of autoimmune hepatitis

Edward L Krawitt

Edward L Krawitt, Departments of Medicine, University of Vermont, Burlington, Vermont, and Dartmouth College, Hanover, New Hampshire, Vermont 05405-0068, United States  
Correspondence to: Edward L Krawitt, MD, Departments of Medicine, University of Vermont, Given C-246 Burlington, Vermont 05405-0068, United States. [edward.krawitt@uvm.edu](mailto:edward.krawitt@uvm.edu)  
Telephone: +1-802-6564290 Fax: +1-802-6560168  
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### Abstract

Autoimmune hepatitis (AIH) is a chronic hepatitis of unknown etiology which can progress to cirrhosis. Its clinical manifestations are highly variable and sometimes follow a fluctuating course. Diagnosis is based on characteristic histologic, clinical, biochemical and serological findings. Anti-inflammatory/immunosuppressive treatment frequently induces remission but long-term maintenance therapy is often required. Liver transplantation is generally successful in patients with decompensated cirrhosis unresponsive to or intolerant of medical therapy.

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### INTRODUCTION

Autoimmune hepatitis (AIH) is chronic hepatitis of unknown etiology, which is thought to occur as a result of escape from normal suppression of self-reactivity. It occurs worldwide in children and adults. Clinical manifestations are highly variable and sometimes follow a fluctuating course. Diagnosis is based on characteristic histologic, clinical, biochemical and serological findings. Anti-inflammatory/immunosuppressive treatment induces remission but long-term maintenance therapy is often required. Liver transplantation is generally success-

ful in patients with decompensated cirrhosis unresponsive to or intolerant of medical therapy.

### HISTOLOGY

The histologic appearance of AIH is that of chronic hepatitis, and, although certain changes are characteristic, there are no findings specific to the disease. The histologic differential diagnosis of chronic hepatitis is shown in Table 1. Based on the advances in virologic studies and refinements of cholangiographic methods, exclusion of other entities has become easier, although co-existence of chronic viral hepatitis and AIH may make the diagnosis difficult.

The inflammatory component is characterized by a mononuclear cell infiltrate, which invades the sharply demarcated hepatocyte boundary (limiting plate) surrounding the portal triad and permeates the surrounding parenchyma (periportal infiltrate; piecemeal necrosis; interface hepatitis) and beyond (lobular hepatitis). It may include an abundance of plasma cells and/or eosinophils, but the portal lesion generally spares the biliary tree. In all but the mildest forms of AIH, fibrosis is present. In advanced disease, fibrosis is extensive (bridging fibrosis) and, with distortion of the hepatic lobule and appearance of regenerative nodules, cirrhosis occurs. On occasion, centrilobular disease may be present.

The histologic findings differ somewhat comparing patients with acute onset AIH to those with an insidious presentation. Patients presenting with fulminant hepatic failure have more interface and lobular hepatitis, lobular disarray, hepatocyte necrosis, central necrosis and submassive necrosis, but less fibrosis and cirrhosis compared to patients presenting with a more chronic course<sup>[1,2]</sup>. Steatosis occurs in a minority of patients, although, given the increasing prevalence of diabetes, dyslipidemia and obesity in many parts of the world, non-alcoholic fatty liver disease may be seen more often accompanying AIH. Whether the co-morbidity of steatosis and/or steatohepatitis accelerate progression of disease in AIH is unknown. The prevalence of cirrhosis in patients  $\geq 60$  years at presentation was found to be higher than that in patients  $\leq 30$  years; when comparing groups of patients  $\geq 60$  years with those  $< 60$  years, however, no differences were found<sup>[2,3]</sup>. In patients with a spontaneous or pharmacologically-induced remission, histologic findings may revert to normal; inflammation



Table 1 Histologic differential diagnosis of chronic hepatitis

Histologic differential diagnosis
Autoimmune liver disease
Autoimmune hepatitis
Primary biliary cirrhosis
Primary sclerosing cholangitis
Variant syndromes
Chronic viral hepatitis
Chronic hepatitis B
Chronic hepatitis C
Chronic hepatitis delta
Chronic hepatitis due to other viruses
Chronic drug-induced hepatitis
Alpha <sub>1</sub> -antitrypsin deficiency
Wilson's disease
Granulomatous hepatitis
Systemic lupus erythematosus
Graft-versus-host disease
Alcoholic steatohepatitis
Nonalcoholic steatohepatitis

may be confined to portal areas; cirrhosis may become inactive; and fibrosis may regress or disappear<sup>[4]</sup>.

## CLASSIFICATION

The most commonly accepted classification of AIH is based on patterns of circulating antibodies, although there is little evidence to support a role for these antibodies in pathogenesis (Table 2).

Type 1 AIH is most frequently characterized by antinuclear antibody (ANA), smooth muscle antibody (SMA) and anti-actin antibody (AAA). Titers of significance vary depending on the autoantibody in question and assays employed<sup>[5]</sup>. Anti-actin (IgG anti F actin) antibodies measured by ELISA appear to be more sensitive than SMA measured by immunofluorescence<sup>[6,7]</sup>.

The identification of other circulating autoantibodies, in particular anti-soluble liver antigen/liver-pancreas antigen (anti-SLA/LP) and atypical perinuclear anticytoplasmic antibody (pANCA) are sometimes helpful in diagnosing type 1 disease. Anti-SLA/LP is the most specific autoantibody detected in type 1 AIH but is found in only 10%-30% of type 1 AIH. Atypical pANCA is non-specific, but commonly present. Antimitochondrial antibodies (AMA) occur infrequently in type 1 AIH. At times AMA may be the sole antibody present and identify an entity sometimes referred to as AMA-positive AIH or the overlap syndrome<sup>[8]</sup>.

Anti-liver/kidney microsome -1 (ALKM-1) and anti-liver cytosol-1 (ALC-1) antibodies occurring alone or together characterize type 2 AIH. Anti-liver cytosol-1 generally occurs in conjunction with anti-liver/kidney microsome-1, but may be the sole autoantibody<sup>[9]</sup>.

Type 1 AIH in Caucasians is associated with the *HLA-DR3* serotype, which is found in linkage disequilibrium with *HLA-B8* and *HLA-A1* and in *HLA-DR3*-negative patients with *HLA-DR4*. *HLA-DR3*-associated disease is more commonly found in patients  $\leq 40$  years at presentation<sup>[2]</sup>. In Japan, where *HLA-DR3* is rare, the primary association is with *HLA-DR4*. Polymerase chain reaction

Table 2 Classification of autoimmune hepatitis

Disorder	Characteristic autoantibodies
Type 1	ANA (antinuclear antibody) SMA (smooth muscle antibody) AAA (anti-actin antibody) Anti SLA/LP (anti-soluble liver antigen/liver-pancreas antigen) pANCA (atypical perinuclear antineutrophil cytoplasmic antibody) AMA (antimitochondrial antibody) <sup>1</sup>
Type 2	ALKM-1 (anti-liver/kidney microsome-1) ALC-1 (anti-liver cytosol-1)

<sup>1</sup>Occurs infrequently in association with other characteristic autoantibodies. It may be the sole antibody present in AMA-negative autoimmune hepatitis, also referred to as the overlap syndrome.

studies genotyping for *HLA-DRB*, *DQA* and *DQB* have confirmed the serologic findings. In children, type 1 AIH is commonly associated with the *HLA-DRB1\*03* and *HLA-DRB1\*13* alleles. Type 2 AIH has been associated with *HLA-DRB1* as well as *HLA-DQB1* alleles<sup>[10]</sup>.

## CLINICAL FEATURES

Although there is a female predominance, AIH occurs in children and adults of both sexes in diverse ethnic groups worldwide. Type 2 disease, which is seen predominantly in children and young women, is rare in North America<sup>[9,10]</sup>. Although AIH was thought previously to be primarily a disease of the young or middle aged, it is now clear that it also occurs in the elderly (generally defined as  $\geq 60$  years of age)<sup>[2,3,11]</sup>.

The heterogeneous, sometimes fluctuating nature of AIH, leads to marked variability in clinical manifestations. Presentation may be asymptomatic or insidious, with mild non-specific symptoms only or may mimic acute viral hepatitis. Rarely, AIH presents as fulminant hepatic failure<sup>[11,12]</sup>. Patients with occult disease may have undetected cirrhosis and present only when decompensation occurs. The group of patients now labeled as cryptogenic cirrhosis, includes some patients with seronegative AIH, underscoring the possibility of the absence of circulating autoantibodies in AIH<sup>[13]</sup>.

Many patients with an acute presentation have histological evidence of chronic disease in the liver biopsy, indicating that they have had antecedent subclinical disease, although the duration of the subclinical anicteric course is generally difficult to ascertain. In retrospect, a fluctuating course, which had been thought to reflect some other diseases, can be identified occasionally. Long periods of subclinical disease may also ensue after presentation. Recent surveys of pregnancy in AIH have indicated that the initial presentations of AIH may occur not only during pregnancy but in the early post-partum period<sup>[14,15]</sup>. AIH may occur in conjunction with a variety of autoimmune disorders, including celiac disease<sup>[16,17]</sup>. Arthralgia involving small joints is common, and inflammatory arthritis may be particularly troublesome.

One presentation of AIH is in the setting of medications, or herbal agents, used for other diseases. It is not

clear if they unmask and/or induce AIH or simply result in a drug-induced hepatitis with histological findings that mimic AIH. Minocycline and, more recently, statins<sup>[18]</sup>, both of which induce other autoimmune syndromes, have been considered as drugs capable of “triggering” AIH.

Complications of AIH are those seen in any progressive liver disease and primary hepatocellular carcinoma is an expected, although uncommon, consequence<sup>[2,19,20]</sup>. There are no established guidelines for hepatocellular carcinoma screening in cirrhosis associated with AIH. A reasonable approach would be surveillance with an ultrasound and alpha feta-protein every 6-12 mo.

## DIAGNOSIS

In the presence of a compatible histologic picture, the diagnosis of AIH is based on characteristic clinical and biochemical findings, circulating autoantibodies and abnormalities of serum globulins. A scoring system, proposed and subsequently revised by the International AIH Group for experimental purposes to standardize diagnosis for clinical trials and population studies, has been adopted by clinicians, but found to be problematic when applied to individual patients, especially children. Thus attempts were undertaken by the International AIH Group to devise a less complicated and more accurate system for wider application in clinical practice. A scoring system, using autoantibodies, gamma globulins, absence of viral hepatitis and histologic findings from patients form a wide geographic distribution, has been proposed as a sufficiently sensitive and specific scoring system<sup>[21]</sup>.

## TREATMENT OF ADULTS WITH AIH

Appropriate management of AIH can mitigate inflammation, slow progression of disease, prolong survival, improve quality of life and delay or avoid liver transplantation. However, depending on a variety of definitions of response, success rates only range from 65% to 80%, which leaves a significant number of patients in need of other than standard treatment. Considerable challenges still exist in the areas of initial and maintenance regimens, management of relapse, non-response, drug toxicity and intolerance, and non-compliance<sup>[8,22]</sup>.

Standard medications for initial and maintenance regimens are still considered to be prednisone (or prednisolone) alone or in combination with azathioprine (or 6-mercaptopurine) (Table 3). A recent retrospective analysis of corticosteroid treatment in AIH patients with severe and fulminant AIH, suggested that steroids did not obviate the need for transplantation and may have promoted septic complications<sup>[23]</sup>.

One issue of treatment of particular concern is toxicity and/or intolerance to 6-mercaptopurine and its pro-drug azathioprine. The methylation of 6-mercaptopurine and 6-thioguanosine 5'-monophosphate is catalyzed by thiopurine methyltransferase (TPMT). The genes encoding thiopurine methyltransferase are highly polymorphic.

**Table 3** Drugs used in standard treatment of autoimmune hepatitis in adults

Regimen	Single-drug therapy	Combination therapy
Initial	Prednisone 20-60 mg/d	Prednisone 15-30 mg/d and azathioprine 50-100 mg/d
Maintenance	Prednisone 5-15 mg/d or azathioprine 50-200 mg/d	Prednisone 5-10 mg/d and azathioprine 50-150 mg/d

Homozygosity and heterozygosity for mutations in TPMT genes occur in Caucasian and other populations, and these patients may accumulate high levels of thio-guanine nucleotides in bone marrow cells. Patients who are homozygous for a mutation of TPMT are at high risk for severe toxicity, including death. Patients, who are heterozygous for the TPMT mutation, probably have an intermediate risk of toxicity. These findings have led to the suggestion that prior to placing patients on azathioprine or 6-mercaptopurine, TPMT genotyping may be appropriate. Despite reliable methods for TPMT genotyping and measurement of levels of 6-mercaptopurine metabolites, their assessment in the clinical management of AIH is not established, and must be evaluated in the context of severity of disease, as well, as advanced fibrosis has been shown to predict azathioprine toxicity<sup>[24-26]</sup>.

Although some patients will remain in remission when drug treatment is withdrawn, the majority requires long-term maintenance therapy. In general, the response is better with milder disease. Adults with cirrhosis at the time of initial biopsy and children, particularly those with type 2 disease, rarely stay in remission when treatment is withdrawn and will almost require life-long maintenance therapy.

No firm guidelines exist for decisions regarding withdrawal of medications because histologic changes may lag biochemical responses and a quiescent histologic appearance and normal biochemical findings while patients are still receiving therapy, are not necessarily predictive of continued remission once therapy is withdrawn. Although, in the past, aminotransferase levels  $\leq 2 \times$  normal were proposed as a guideline to reducing medications, relapse has been shown to be less likely in patients who achieve normal transaminase and gamma globulin (or IgG) levels<sup>[27]</sup>.

Progress in non-standard treatment for patients with inadequate responses or intolerance to therapy with glucocorticosteroids alone or in combination with azathioprine or 6-mercaptopurine (including mycophenolate mofetil, methotrexate, cyclophosphamide, tacrolimus, budesonide and ursodeoxycholic acid) has been slow. In view of the paucity of trials with non-standard forms of therapy most decisions must be based on data obtained from case reports and series of small numbers of patients. Cyclosporine, which has been used successfully in children to induce remission<sup>[28]</sup>, and tacrolimus are used occasionally to treat adults<sup>[29,30]</sup>. Off-label use of mycophenolate mofetil has become more frequently employed in intolerant or non-responsive patients<sup>[31,32]</sup>. The roles of cyclosporine, tacrolimus, mycophenolate mofetil,

methotrexate, cyclophosphamide, ursodeoxycholic acid, budesonide<sup>[33,34]</sup> and other immunosuppressive medications have not been established.

AIH patients who develop decompensated cirrhosis may require liver transplantation. Five-year patient and graft survivals range from 80% to 90%. As in other autoimmune liver diseases, recurrence and cirrhosis may occur after transplantation<sup>[35,36]</sup> and mandate modifications of the post-transplantation therapeutic regimens. So-called *de novo* AIH, also referred to as post-transplantation immune hepatitis or graft dysfunction mimicking AIH, occurs after liver transplantation for diseases other than AIH in adults and children, and may require changes in post-transplantation therapy as well<sup>[37,38]</sup>.

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## REFERENCES

- 1 Kessler WR, Cummings OW, Eckert G, Chalasani N, Lumeng L, Kwo PY. Fulminant hepatic failure as the initial presentation of acute autoimmune hepatitis. *Clin Gastroenterol Hepatol* 2004; **2**: 625-631
- 2 Al-Chalabi T, Boccatto S, Portmann BC, McFarlane IG, Heneghan MA. Autoimmune hepatitis (AIH) in the elderly: a systematic retrospective analysis of a large group of consecutive patients with definite AIH followed at a tertiary referral centre. *J Hepatol* 2006; **45**: 575-583
- 3 Czaja AJ, Carpenter HA. Distinctive clinical phenotype and treatment outcome of type 1 autoimmune hepatitis in the elderly. *Hepatology* 2006; **43**: 532-538
- 4 Czaja AJ, Carpenter HA. Decreased fibrosis during corticosteroid therapy of autoimmune hepatitis. *J Hepatol* 2004; **40**: 646-652
- 5 Vergani D, Alvarez F, Bianchi FB, Cancado EL, Mackay IR, Manns MP, Nishioka M, Penner E. Liver autoimmune serology: a consensus statement from the committee for autoimmune serology of the International Autoimmune Hepatitis Group. *J Hepatol* 2004; **41**: 677-683
- 6 Frenzel C, Herkel J, Luth S, Galle PR, Schramm C, Lohse AW. Evaluation of F-actin ELISA for the diagnosis of autoimmune hepatitis. *Am J Gastroenterol* 2006; **101**: 2731-2736
- 7 Granito A, Muratori L, Muratori P, Pappas G, Guidi M, Cassani F, Volta U, Ferri A, Lenzi M, Bianchi FB. Antibodies to filamentous actin (F-actin) in type 1 autoimmune hepatitis. *J Clin Pathol* 2006; **59**: 280-284
- 8 Krawitt EL. Autoimmune hepatitis. *N Engl J Med* 2006; **354**: 54-66
- 9 Bridoux-Henno L, Maggiore G, Johanet C, Fabre M, Vajro P, Dommergues JP, Reinert P, Bernard O. Features and outcome of autoimmune hepatitis type 2 presenting with isolated positivity for anti-liver cytosol antibody. *Clin Gastroenterol Hepatol* 2004; **2**: 825-830
- 10 Djilali-Saiah I, Renous R, Caillat-Zucman S, Debray D, Alvarez F. Linkage disequilibrium between HLA class II region and autoimmune hepatitis in pediatric patients. *J Hepatol* 2004; **40**: 904-909
- 11 Strassburg CP, Manns MP. Autoimmune hepatitis in the elderly: what is the difference? *J Hepatol* 2006; **45**: 480-482
- 12 Abe M, Onji M, Kawai-Ninomiya K, Michitaka K, Matsuura B, Hiasa Y, Horiike N. Clinicopathologic features of the severe form of acute type 1 autoimmune hepatitis. *Clin Gastroenterol Hepatol* 2007; **5**: 255-258
- 13 Gassert DJ, Garcia H, Tanaka K, Reinus JF. Corticosteroid-responsive cryptogenic chronic hepatitis: evidence for seronegative autoimmune hepatitis. *Dig Dis Sci* 2007; **52**: 2433-2437
- 14 Samuel D, Riordan S, Strasser S, Kurtovic J, Singh-Grewel I, Koorey D. Severe autoimmune hepatitis first presenting in the early post partum period. *Clin Gastroenterol Hepatol* 2004; **2**: 622-624
- 15 Schramm C, Herkel J, Beuers U, Kanzler S, Galle PR, Lohse AW. Pregnancy in autoimmune hepatitis: outcome and risk factors. *Am J Gastroenterol* 2006; **101**: 556-560
- 16 Abdo A, Meddings J, Swain M. Liver abnormalities in celiac disease. *Clin Gastroenterol Hepatol* 2004; **2**: 107-112
- 17 Villalta D, Girolami D, Bidoli E, Bizzaro N, Tampona M, Liguori M, Pradella M, Tonutti E, Tozzoli R. High prevalence of celiac disease in autoimmune hepatitis detected by anti-tissue transglutaminase autoantibodies. *J Clin Lab Anal* 2005; **19**: 6-10
- 18 Alla V, Abraham J, Siddiqui J, Raina D, Wu GY, Chalasani NP, Bonkovsky HL. Autoimmune hepatitis triggered by statins. *J Clin Gastroenterol* 2006; **40**: 757-761
- 19 Miyake Y, Iwasaki Y, Terada R, Okamoto R, Ikeda H, Makino Y, Kobashi H, Takaguchi K, Sakaguchi K, Shiratori Y. Persistent elevation of serum alanine aminotransferase levels leads to poor survival and hepatocellular carcinoma development in type 1 autoimmune hepatitis. *Aliment Pharmacol Ther* 2006; **24**: 1197-1205
- 20 Meza-Junco J, Montano-Loza AJ, Martinez-Benitez B, Kimura-Hayama E. Hepatocellular carcinoma in patients with autoimmune liver diseases: two case reports and literature review. *Ann Hepatol* 2007; **6**: 122-126
- 21 Hennes EM, Zeniya M, Czaja AJ, Parés A, Dalekos GN, Krawitt EL, Bittencourt PL, Porta G, Boberg KM, Hofer H, Bianchi FB, Shibata M, Schramm C, de Torres BE, Galle PR, McFarlane I, Dienes HP, Lohse AW. Simplified criteria for the diagnosis of autoimmune hepatitis. *Hepatology* 2008; **9999** (999A): Epub ahead of print
- 22 Czaja AJ, Bianchi FB, Carpenter HA, Krawitt EL, Lohse AW, Manns MP, McFarlane IG, Mieli-Vergani G, Toda G, Vergani D, Vierling J, Zeniya M. Treatment challenges and investigational opportunities in autoimmune hepatitis. *Hepatology* 2005; **41**: 207-215
- 23 Ichai P, Duclos-Vallee JC, Guettier C, Hamida SB, Antonini T, Delvart V, Saliba F, Azoulay D, Castaing D, Samuel D. Usefulness of corticosteroids for the treatment of severe and fulminant forms of autoimmune hepatitis. *Liver Transpl* 2007; **13**: 996-1003
- 24 Heneghan MA, Allan ML, Bornstein JD, Muir AJ, Tendler DA. Utility of thiopurine methyltransferase genotyping and phenotyping, and measurement of azathioprine metabolites in the management of patients with autoimmune hepatitis. *J Hepatol* 2006; **45**: 584-591
- 25 Czaja AJ, Carpenter HA. Thiopurine methyltransferase deficiency and azathioprine intolerance in autoimmune hepatitis. *Dig Dis Sci* 2006; **51**: 968-975
- 26 Tamori A, Shinzaki M, Kosaka S, Hayashi T, Iwai S, Enomoto M, Habu D, Sakaguchi H, Kawada N, Hino M, Shiomi S, Nishiguchi S. Thiopurine S-methyltransferase gene polymorphism in Japanese patients with autoimmune liver diseases. *Liver Int* 2007; **27**: 95-100
- 27 Montano-Loza AJ, Carpenter HA, Czaja AJ. Improving the end point of corticosteroid therapy in type 1 autoimmune hepatitis to reduce the frequency of relapse. *Am J Gastroenterol* 2007; **102**: 1005-1012
- 28 Cuarterolo M, Ciocca M, Velasco CC, Ramonet M, Gonzalez T, Lopez S, Garsd A, Alvarez F. Follow-up of children with autoimmune hepatitis treated with cyclosporine. *J Pediatr Gastroenterol Nutr* 2006; **43**: 635-639
- 29 Larsen FS, Vainer B, Eefsen M, Bjerring PN, Adel Hansen B. Low-dose tacrolimus ameliorates liver inflammation and fibrosis in steroid refractory autoimmune hepatitis. *World J Gastroenterol* 2007; **13**: 3232-3236
- 30 Aqel BA, Machicao V, Rosser B, Satyanarayana R, Harnois DM, Dickson RC. Efficacy of tacrolimus in the treatment of

- steroid refractory autoimmune hepatitis. *J Clin Gastroenterol* 2004; **38**: 805-809
- 31 **Inductivo-Yu I**, Adams A, Gish RG, Wakil A, Bzowej NH, Frederick RT, Bonacini M. Mycophenolate mofetil in autoimmune hepatitis patients not responsive or intolerant to standard immunosuppressive therapy. *Clin Gastroenterol Hepatol* 2007; **5**: 799-802
- 32 **Devlin SM**, Swain MG, Urbanski SJ, Burak KW. Mycophenolate mofetil for the treatment of autoimmune hepatitis in patients refractory to standard therapy. *Can J Gastroenterol* 2004; **18**: 321-326
- 33 **Csepregi A**, Rocken C, Treiber G, Malfertheiner P. Budesonide induces complete remission in autoimmune hepatitis. *World J Gastroenterol* 2006; **12**: 1362-1366
- 34 **Wiegand J**, Schuler A, Kanzler S, Lohse A, Beuers U, Kreisel W, Spengler U, Koletzko S, Jansen PL, Hochhaus G, Mollmann HW, Prols M, Manns MP. Budesonide in previously untreated autoimmune hepatitis. *Liver Int* 2005; **25**: 927-934
- 35 **Gautam M**, Cheruvattath R, Balan V. Recurrence of autoimmune liver disease after liver transplantation: a systematic review. *Liver Transpl* 2006; **12**: 1813-1824
- 36 **Seyam M**, Neuberger JM, Gunson BK, Hubscher SG. Cirrhosis after orthotopic liver transplantation in the absence of primary disease recurrence. *Liver Transpl* 2007; **13**: 966-974
- 37 **Evans HM**, Kelly DA, McKiernan PJ, Hubscher S. Progressive histological damage in liver allografts following pediatric liver transplantation. *Hepatology* 2006; **43**: 1109-1117
- 38 **Riva S**, Sonzogni A, Bravi M, Bertani A, Alessio MG, Candusso M, Stroppa P, Melzi ML, Spada M, Gridelli B, Colledan M, Torre G. Late graft dysfunction and autoantibodies after liver transplantation in children: preliminary results of an Italian experience. *Liver Transpl* 2006; **12**: 573-577

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## TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, Series Editors

# Aetiopathogenesis of autoimmune hepatitis

Diego Vergani, Giorgina Mieli-Vergani

Diego Vergani, Giorgina Mieli-Vergani, Institute of Liver Studies, King's College London School of Medicine at King's College Hospital, London SE5 9RS, United Kingdom  
Correspondence to: Diego Vergani, Professor, Institute of Liver Studies, King's College Hospital, Denmark Hill, London SE5 9RS, United Kingdom. [diego.vergani@kcl.ac.uk](mailto:diego.vergani@kcl.ac.uk)  
Telephone: +44-20-32993305 Fax: +44-20-32993700  
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## Abstract

The histological hallmark of autoimmune hepatitis (AIH) is a dense portal mononuclear cell infiltrate that invades the surrounding parenchyma and comprises T and B lymphocytes, macrophages, and plasma cells. An unknown but powerful stimulus must be promoting the formation of this massive inflammatory cellular reaction that is likely to initiate and perpetuate liver damage. An autoimmune attack can follow different pathways to inflict damage on hepatocytes. Liver damage is likely to be orchestrated by CD4<sup>+</sup> T lymphocytes recognizing an autoantigenic liver peptide. To trigger an autoimmune response, the peptide must be embraced by an HLA class II molecule and presented to naïve CD4<sup>+</sup> T helper (Th0) cells by professional antigen presenting cells, with the co-stimulation of ligand-ligand fostering interaction between the two cells. Th0 cells become activated, differentiate into functional phenotypes according to the cytokines prevailing in the microenvironment and the nature of the antigen, and initiate a cascade of immune reactions determined by the cytokines produced by the activated T cells. Th1 cells, arising in the presence of the macrophage-derived interleukin (IL) -12, secrete mainly IL-2 and interferon-gamma (IFN- $\gamma$ ), which activate macrophages, enhance expression of HLA class I (increasing liver cell vulnerability to a CD8<sup>+</sup> T cell cytotoxic attack), and induce expression of HLA class II molecules on hepatocytes. Th2 cells, which differentiate from Th0 if the microenvironment is rich in IL-4, produce mainly IL-4, IL-10, and IL-13 which favour autoantibody production by B lymphocytes. Physiologically, Th1 and Th2 antagonize each other. Th17 cells, a recently described population, arise in the presence of transforming growth factor beta (TGF- $\beta$ ) and IL-6 and appear to have an important effector role in inflammation and autoimmunity. The

process of autoantigen recognition is strictly controlled by regulatory mechanisms, such as those exerted by CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells, which derive from Th0 in the presence of TGF- $\beta$ , but in the absence of IL-6. If regulatory mechanisms fail, the autoimmune attack is perpetuated. Over the past three decades different aspects of the above pathogenic scenario have been investigated. In particular, a defect in immunoregulation affecting CD4<sup>+</sup>CD25<sup>+</sup> regulatory T cells (T-regs) has been demonstrated in AIH, particularly at diagnosis or during relapse. Advances in the study of autoreactive T cells have occurred mostly in AIH type 2, since the knowledge that CYP2D6 is the main autoantigen has enabled the characterization of both CD4 and CD8 T cells targeting this cytochrome. CD4 T cells from patients with type 2 AIH positive for the predisposing HLA allele *DRB1\*0701* recognize seven regions of CYP2D6, five of which are also recognized by CD8 T cells. High numbers of IFN- $\gamma$  producing CD4 T cells and CD8 T cells are associated with biochemical evidence of liver damage, suggesting a combined cellular immune attack.

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**Key words:** Autoimmune hepatitis; Aetiopathogenesis; Lymphocyte; Cellular immune attack; Histocompatibility lymphocyte antigen

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## INTRODUCTION

Autoimmune hepatitis (AIH) is an inflammatory liver disease, affecting mainly females, characterized by elevated serum transaminase activity, positive organ and non-organ specific autoantibodies, elevated IgG, and a histological picture of interface hepatitis. There are two types of AIH according to their serology: type 1 is characterized by anti-nuclear (ANA) and/or anti-smooth muscle (SMA) antibodies; type 2 by anti-liver kidney microsomal type 1 (anti-LKM-1) antibody. The aetiology of AIH is unknown, though both genetic and environmental factors are involved in its expression.

Immune reactions against host liver antigens are believed to be the major pathogenic mechanism.

## GENETICS

AIH is a “complex trait” disease, i.e. a condition not inherited in a Mendelian autosomal dominant, autosomal recessive, or sex-linked fashion. The mode of inheritance of a complex trait disorder is unknown and involves one or more genes, operating alone or in concert, to increase or reduce the risk of the trait, and interacting with environmental factors.

Susceptibility to AIH is imparted by genes within the major histocompatibility complex (MHC) - the human leukocyte antigen (HLA) region - on the short arm of chromosome 6, especially genes encoding HLA *DRB1* alleles. Since the role of class II MHC molecules is to present peptide antigens to CD4 T cells, HLA class II antigen presentation with ensuing T cell activation is likely to be involved in the pathogenesis of AIH.

In Europe and North America, susceptibility to AIH type 1 is conferred by the presence of HLA *DR3* (*DRB1\*0301*) and *DR4* (*DRB1\*0401*), both heterodimers containing a lysine residue at position 71 of the *DRB1* polypeptide and the hexameric amino acid sequence LLEQKR at positions 67-72<sup>[1,2]</sup>. In Japan, Argentina, and Mexico, susceptibility is linked to *DRB1\*0405* and *DRB1\*0404*, alleles encoding arginine rather than lysine at position 71, but sharing the motif LLEQ-R with *DRB1\*0401* and *DRB1\*0301*<sup>[3]</sup>. Thus, K or R at position 71 in the context of LLEQ-R may be critical for susceptibility to AIH, favouring the binding of autoantigenic peptides, complementary to this hexameric sequence. However, an alternative model based on valine/glycine dimorphism at position 86 of the DR- $\beta$  polypeptide has been proposed, better representing the key HLA associations in patients from Argentina and Brazil<sup>[1,2]</sup>. In a study from Japan, patients with AIH type 1 were found to have *DRB1* alleles which encode histidine at position 13<sup>[1,2]</sup>. There appears therefore to be at least three different models, suggesting that different genetic associations are present in different populations and that the peptides presented by HLA class II molecules to the T cell receptors are different and may be derived from different antigens. Thus, these HLA associations may be the molecular footprints of the prevailing environmental triggers that precipitate AIH type 1 in different environments, though at the effector level the same autoantigenic target would be recognized. In this context, it is of interest that in South America presence of the HLA *DRB1\*1301* allele, which predisposes to paediatric AIH type 1 in that population, is also associated with persistent infection with the endemic hepatitis A virus.

The lysine-71 and other models for AIH type 1 cannot explain completely the disease, since for example in European and North American patients presence of lysine-71 is associated with a severe, mainly juvenile, disease in those *DRB1\*0301* positive, but to a mild, late onset, disease in those *DRB1\*0401* positive. Other genes

within or/and without the MHC are, therefore, likely to be involved in determining the phenotype. Possible candidates are the MHC encoded complement and tumour necrosis factor  $\alpha$  genes, that are located in the class III MHC region, and the MHC class I chain-related (MICA) A and B genes.

Susceptibility to AIH type 2 is conferred by HLA *DR7* (*DRB1\*0701*) and *DR3* (*DRB1\*0301*); patients positive for *DRB1\*0701* have a more aggressive disease and worse outcome<sup>[4]</sup>.

In an attempt to define additional susceptibility genes, a genome-wide approach was applied to a Japanese cohort of patients with AIH type 1<sup>[5]</sup>. This study found that 2 microsatellite markers (on chromosomes 11 and 18) out of 400 studied are associated with AIH type 1, though no protein of clear relevance to the disease is encoded in proximity of these two markers. The use of a larger number of microsatellites may prove more informative.

A form of AIH resembling AIH type 2 affects some 20% of patients with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), a condition also known as autoimmune polyendocrine syndrome 1. APECED is a monogenic autosomal recessive disorder caused by homozygous mutations in the *AIRE1* gene and characterized by a variety of organ-specific autoimmune diseases, the most common of which are hypoparathyroidism and primary adrenocortical failure, accompanied by chronic mucocutaneous candidiasis<sup>[6,7]</sup>. The *AIRE1* gene sequence consists of 14 exons containing 45 different mutations, with a 13 bp deletion at nucleotide 964 in exon 8 accounting for more than 70% of APECED alleles in the UK<sup>[6]</sup>. The protein predicted to be encoded by *AIRE1* is a transcription factor. *AIRE1* is highly expressed in thymic medullary epithelial cells and thymic stromal cells involved in clonal deletion of self-reactive T cells. Studies in a murine model indicate that the gene inhibits organ specific autoimmunity by inducing thymic expression of peripheral antigens in the medulla leading to central deletion of autoreactive T cells. Interestingly, APECED has a high level of variability in symptoms, especially between populations. Since various gene mutations have the same effect on thymic transcription of ectopic genes in animal models, it is likely that the clinical variability across human populations relates to environmental or genetic modifiers. Of the various genetic modifiers, perhaps the most likely to synergize with *AIRE* mutations are polymorphisms in the HLA region. HLA molecules are not only highly variable and strongly associated with multiple autoimmune diseases, but are also able to affect thymic repertoire selection of autoreactive T cell clones. Carriers of a single *AIRE* mutation do not develop APECED. However, although the inheritance pattern of APECED indicates a strictly recessive disorder, there are anecdotal data of mutations in a single copy of *AIRE* being associated with human autoimmunity of a less severe form than classically defined APECED<sup>[6,7]</sup>. A role of the heterozygote state for mutant *AIRE1* in the development of AIH remains

to be established. *AIRE1* mutations have been reported in 3 children with severe AIH type 2 and extrahepatic autoimmune manifestations<sup>[8]</sup>.

## IMMUNE MECHANISMS

The liver is regarded as a lymphoid organ with unique immunological properties<sup>[9]</sup>. Because of its location and function, the liver is continuously exposed to a large antigenic load that includes pathogens, toxins, tumour cells, dietary, and self-antigens. The liver contains large numbers of phagocytic cells, antigen presenting cells (APC) and lymphocytes and is a site for the abundant production of cytokines, complement components and acute phase proteins. The intrinsic lymphocyte population mainly resides in the portal tracts but is also scattered throughout the parenchyma, consisting of both cells of the innate (natural killer T cells, natural killer cells, and macrophages) and the adaptive (T and B cells) arms of the immune system. The blood entering the liver from the gut is rich in bacterial and dietary antigens that intermingle with lymphocytes. Immunoregulatory mechanisms are required to determine whether an antigen encounter will result in immunological unresponsiveness (tolerance) or reactivity. Liver autoimmunity implies loss of self-tolerance. Programmed cell death - apoptosis - which is responsible for the normal turnover of hepatocytes and the elimination of liver cells and unwanted lymphocytes in inflammatory pathologies is also relevant to the breakdown and/or maintenance of liver tolerance. First, death by apoptosis allows for non-inflammatory elimination of cell components in contrast to necrosis, which is pro-inflammatory and potentially autoantigenic. Second, apoptosis is the mechanism whereby the immune system is "cleansed" of autoreactive T and B lymphocytes as illustrated by the process of "activation induced cell death".

Various mechanisms have been proposed to account for the onset of an autoimmune liver response with no single initiating event being able to explain all instances of autoimmunity. Two general conditions, however, should prevail: self reactive B and T lymphocytes must exist in the immunological repertoire and autoantigens must be presented in conjunction with MHC class II molecules by APC.

### Humoral autoimmunity

Titres of antibodies to liver specific protein (LSP), a macromolecular complex present on the hepatocyte membrane<sup>[10]</sup>, and to its well characterized components asialoglycoprotein receptor (ASGPR)<sup>[11]</sup> and alcohol dehydrogenase (ADH)<sup>[12]</sup> correlate with the biochemical and histological severity of AIH. Immunofluorescence studies on monodispersed suspensions of liver cells obtained from patients with AIH show that these cells are coated *in vivo* with antibodies reacting with antigens on the liver cell membrane<sup>[13]</sup>. A pathogenic role for these autoantibodies has been indicated by cytotoxicity assays demonstrating that autoantibody-coated hepato-

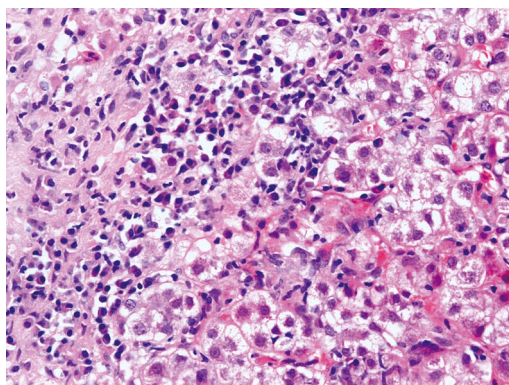
cytes from patients with AIH are killed when incubated with autologous or allogeneic lymphocytes<sup>[13]</sup>. The effector cell was identified as an Fc receptor positive mononuclear cell, presumably a natural killer (NK) cell.

In AIH type 2 the target of the disease-defining antibody, anti-LKM-1, is CYP2D6, a member of the hepatic P450 cytochrome family. Since CYP2D6 is expressed on the membrane of the hepatocytes and readily "accessible"<sup>[14]</sup>, anti-LKM-1 antibodies might well have a pathogenic effect. In AIH type 2 anti-LKM-1 antibodies recognize linear regions (autoepitopes) of CYP2D6 in a hierarchical manner. Thus, the principal linear B-cell epitope, CYP2D6<sub>193-212</sub> is recognized by 93% of patients, CYP2D6<sub>257-269</sub> by 85%, CYP2D6<sub>321-351</sub> by 53%, and two additional minor epitopes CYP2D6<sub>373-389</sub> and CYP2D6<sub>410-429</sub> are recognized by 7% and 13% respectively<sup>[15]</sup>. Intriguingly, anti-LKM-1 antibodies are also found in some 5% of patients with hepatitis C virus (HCV) infection, among whom they appear to correlate with increased disease severity and adverse reactions to interferon  $\alpha$  treatment<sup>[16]</sup>. The major CYP2D6 epitope recognized by patients with AIH type 2, CYP2D6<sub>193-212</sub>, is also recognized by 50% of patients with anti-LKM-1 positive HCV infection<sup>[15]</sup>. Interestingly, these patients have antibodies that cross-react with homologous regions of HCV (NS5B HCV<sub>2985-2990</sub>) and CYP2D6 (CYP2D6<sub>204-209</sub>), and also of cytomegalovirus (exon CMV<sub>130-135</sub>)<sup>[15]</sup>. Cross-reactive mechanisms to explain the emergence of CYP2D6 specific autoimmunity have also been suggested for other sequences of CYP2D6 which share homologies with HCV and herpes simplex virus (HSV), such as the sequence spanning aa 310-324 of E1 HCV and aa 156-170 of IE175 HSV1, which share homology with the CYP2D6 region comprising aa 254-271. As anti-LKM-1 antibodies cross-react with homologous regions of CYP2D6, HCV, HSV, and CMV, a "multi-hit" mechanism for the generation of these antibodies and possibly of AIH type 2 may be envisaged. In this model, multiple exposures to CMV or HSV, common viral pathogens, may establish permissive immunological conditions, by priming a cross-reactive subset of T cells, in a genetically predisposed host. Depending on the degree of immunological priming, i.e. level of exposure and the degree of genetic susceptibility (particularly at the HLA locus and coding regions for "innate" components of immunity), a minority of recurrently infected individuals may progress to autoimmune disease. It is therefore conceivable that an as yet unsuspected virus infection may be part of the origin of the autoimmune attack in AIH; this is to some degree in agreement with the concept expressed by Rolf Zinkernagel that an autoimmune disease is a viral disease in which the virus is unknown<sup>[17]</sup>.

### Molecular mimicry

The central function of the adaptive immune system is to generate T and B lymphocytes that can specifically recognize a potentially infinite number of non-self antigens without any prior information as to their structure. This is achieved by randomly generating a





**Figure 1** The portal and periportal inflammatory infiltrate characteristic of autoimmune hepatitis is composed by lymphocytes and plasma cells (interface hepatitis) (HE,  $\times 40$ , provided by Dr. Alberto Quaglia).

large number of T and B cell specificities (*via* their respective antigen receptors-the T cell receptor and the antibody receptor) that are then able to clonally expand and recruit effector mechanisms on recognition of their cognate antigen or epitope. It is however, becoming clear that even this versatile system cannot cope with the extent of non-self antigenic diversity, and in the past decade convincing evidence has emerged for cross reactivity as an inherent property of immune ontogeny<sup>[18]</sup>. This has been studied primarily in the context of T lymphocytes, where it is clear that altered peptide ligands (APLs) - peptides similar in structure to the peptide antigen which are initially encountered - are able to induce both stimulatory and inhibitory T cell responses and, indeed, endogenous APLs operate in selecting the T cell repertoire in the thymus. This implies that a single T cell, rather than responding to a single antigen specificity, is able to cross-reactively respond to a number of antigens, thus expanding the antigenic specificities of the immune system to a level that reflects the antigenic diversity of the external environment<sup>[19]</sup>.

This inherent potential for cross-reactivity, whilst allowing efficient responses to a vast array of pathogens also provides the immune system with the potential to cross-react with self, leading to autoimmunity. This process has been termed “molecular mimicry” as described above, whereby immune responses to external pathogens become directed towards structurally similar self components. Molecular mimicry has been shown to participate to the pathogenesis of autoimmune disease both in experimental models and in the human setting at the level of both T and B cells<sup>[18]</sup>.

### Cellular autoimmunity

The histological picture of interface hepatitis (Figure 1), with its striking infiltrate of lymphocytes, plasma cells, and macrophages was the first to suggest an autoaggressive cellular immune attack in the pathogenesis of AIH. Whatever is the initial trigger, this massive recruitment of activated inflammatory cells is likely to cause damage. Immunohistochemical studies have identified a predominance of T lymphocytes mounting the  $\alpha/\beta$  T cell receptor<sup>[20]</sup>. Amongst the

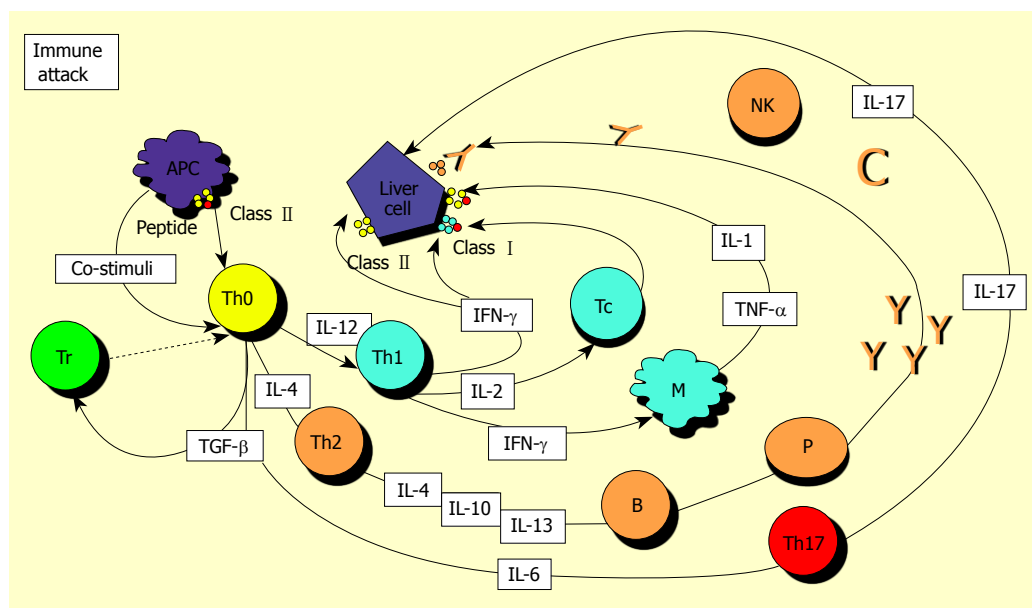
T cells, a majority are positive for the CD4 helper/inducer phenotype, and a sizeable minority for the CD8 cytotoxic phenotype. Lymphocytes of non-T cell lineage are fewer and include (in decreasing order of frequency) natural killer cells (CD16/CD56 positive), macrophages, B cells, and plasma cells. The involvement of natural killer T cells is the focus of ongoing studies.

There are different possible pathways that an immune attack can follow to inflict damage on hepatocytes (Figure 2) as discussed below.

### Impairment of T regulatory cells

An impairment of immunoregulatory mechanisms, which would enable the autoimmune response to develop, has been repeatedly reported in the setting of both human and experimental autoimmunity. Thus, in early studies it was shown that patients with AIH have low levels of circulating T cells expressing the CD8 marker<sup>[21]</sup>, and impaired suppressor cell function which segregates with the possession of the disease-predisposing HLA haplotype B8/DR3<sup>[22]</sup> and is correctable by therapeutic doses of corticosteroids<sup>[23]</sup>. Furthermore, patients with AIH have been reported to have a defect specifically in a subpopulation of T cells that control the immune response to an as yet unidentified liver-specific membrane antigen(s)<sup>[24]</sup>. Recent experimental evidence confirms an impairment of the immunoregulatory function in AIH. Thus, among recently defined T cell subsets with potential immunosuppressive function, CD4<sup>+</sup> T cells constitutively expressing the interleukin 2 receptor  $\alpha$  chain (CD25) (T-regulatory cells, T-regs) have emerged as the dominant immunoregulatory lymphocytes<sup>[25]</sup>. These cells, which in health represent 5%-10% of the total population of peripheral CD4<sup>+</sup> T cells, control the innate and the adaptive immune responses by preventing the proliferation and effector function of autoreactive T cells. Their mechanism of action involves mainly a direct contact with the target cells, and to a lesser extent the release of immunoregulatory cytokines, such as interleukin 10 and transforming growth factor  $\beta$  1 (TGF- $\beta$ ). In addition to CD25, which is also present on T cells undergoing activation, T-regs express a number of additional markers such as the glucocorticoid induced tumour necrosis factor receptor, CD62L, the cytotoxic T lymphocyte associated protein-4 (CTLA-4) and the forkhead/winged helix transcription factor FOXP3, the expression of which is closely associated with the acquisition of regulatory properties. In patients with AIH, T-regs are defective both in number and function compared to normal controls and these abnormalities relate to the stage of disease, being more evident at diagnosis than during drug-induced remission<sup>[26-28]</sup>. The percentage of T-regs inversely correlates with markers of disease severity, such as levels of antibodies to anti-soluble liver antigen<sup>[29]</sup> and anti-LKM-1 autoantibody titres, suggesting that a reduction T-regs favours the serological manifestations of autoimmune liver disease. If loss of immunoregulation was central to the pathogenesis of autoimmune liver disease, treatment should concentrate on restoring T-regs ability to expand, with consequent





**Figure 2** Autoimmune attack to the liver cell. A specific autoantigenic peptide is presented to an uncommitted T helper (Th0) lymphocyte within the HLA class II molecule of an antigen-presenting cell (APC). Th0 cells become activated and, according to the presence in the microenvironment of IL-12 or IL-4 and the nature of the antigen, differentiate into Th1 or Th2 and initiate a series of immune reactions determined by the cytokines they produce: Th2 secrete mainly IL-4, IL-10 and IL-13, and direct autoantibody production by B lymphocytes; Th1 secrete IL-2 and IFN- $\gamma$ , which stimulate T cytotoxic (Tc) lymphocytes, enhance expression of class I and induce expression of class II HLA molecules on hepatocytes and activate macrophages; activated macrophages release IL-1 and tumour necrosis factor alpha (TNF- $\alpha$ ). If regulatory T cells do not oppose, a variety of effector mechanisms are triggered: liver cell destruction could derive from the action of Tc lymphocytes; cytokines released by Th1 and recruited macrophages; complement activation or engagement of Fc receptor-bearing cells such as natural killer (NK) lymphocytes by the autoantibody bound to the hepatocyte surface. The role of the recently described Th17 cells, which arise in the presence of transforming growth factor beta (TGF- $\beta$ ) and IL-6, is under investigation.

increase in their number and function. This is at least partially achieved by standard immunosuppression, since T-reg numbers do increase during remission<sup>[26,28]</sup>.

### CD4 autoreactive T cells

To trigger an autoimmune response, a peptide must be embraced by an HLA class II molecule and presented to uncommitted T helper (Th0) cells by professional APC, with the co-stimulation of ligand-ligand (CD28 on Th0, CD80 on APC) interaction between the cells (Figure 2). Once the autoimmune response has been initiated and in the absence of effective immunosuppressive treatment, tissue damage ensues and persists. In an inflammatory milieu, hepatocytes from patients with AIH, in contrast to normal hepatocytes, express HLA class II molecules<sup>[20]</sup>, as well as class I. Although lacking the antigen processing machinery typical of APC, MHC-class II-bearing hepatocytes may present peptides through a bystander mechanism<sup>[30]</sup>. In the presence of impaired immunoregulation and inappropriate expression of HLA class II antigens on the hepatocytes, an intracellular autoantigenic peptide from intact hepatocytes could be presented to the CD4 helper/inducer T cells leading to their activation. Although no direct evidence exists as yet that an autoantigenic peptide is in fact presented by MHC-class II-bearing hepatocytes and recognized by CD4 T helper cells, activation of such cells has been documented in AIH<sup>[21]</sup>. Circulating T cells specific for liver autoantigens are found also in normal subjects, but in AIH their frequency is at least 10-fold higher<sup>[31]</sup>. This

finding suggests that the pool of liver-autoreactive T cells undergoes a significant expansion in patients with AIH and hence may be involved in the initiation and perpetuation of the immune attack to the liver.

Given that T cells recognize antigens in a precise fashion, studies in the early 1990s were conducted at a single T cell level in order to characterize antigen-specific T cell recognition. T cell clones generated from the peripheral blood were mainly CD4<sup>+</sup>  $\alpha/\beta$  T cells<sup>[32]</sup>, while a large proportion of liver-derived clones were either CD4/CD8<sup>+</sup>  $\gamma/\delta$  or CD8<sup>+</sup>  $\alpha/\beta$  T cells<sup>[31,33,34]</sup>. Both  $\alpha/\beta$  and  $\gamma/\delta$  T cell clones proliferated in the presence of a crude liver membrane preparation, liver specific protein and asialoglycoprotein receptor,  $\alpha/\beta$  being more reactive than  $\gamma/\delta$  clones. Some of the liver membrane reactive clones also proliferated in the presence of LSP and/or ASGPR, responded in an HLA class II restricted fashion and helped autologous B cells to produce immunoglobulins, and in particular autoantibodies to LSP and ASGPR<sup>[32]</sup>.

T cell ligands are best studied in AIH type 2, since the target of anti-LKM-1 has been characterized as CYP2D6. CYP2D6<sub>262-285</sub> specific T cell clones generated from liver tissue and peripheral blood express a Th1 CD4<sup>+</sup> phenotype<sup>[33,34]</sup>. In contrast to the latter study that focused on a short antigenic sequence of CYP2D6, a systematic approach based on the construction of overlapping peptides covering the whole CYP2D6 molecule was recently adopted to define the specificity of *ex vivo* CYP2D6 reactive T cells in patients with AIH type

2<sup>[4]</sup>. This study showed that T cells from patients positive for the predisposing HLA allele *DRB1\*0701* recognize in a proliferation assay seven regions of CYP2D6, four of which are also partially recognized by T cells of *DRB1\*0701* negative patients. While distinct peptides induce production of IFN- $\gamma$ , IL-4 or IL-10, peptides that induced IFN- $\gamma$  and proliferative responses overlap. There was also an overlap between sequences inducing T and B cell responses. The number of epitopes recognized and the quantity of cytokine produced by T cells are directly correlated to biochemical and histological markers of disease activity. These results indicate that the T cell response to CYP2D6 in AIH type 2 is polyclonal, involves multiple effector types targeting different epitopes, and is associated with hepatocyte damage<sup>[4]</sup>.

### CD8 autoreactive T cells

In addition to the unfolding role of CYP2D6 specific CD4 T cells in AIH type 2, there is growing evidence implicating an HLA class I restricted CD8 response in the pathogenesis of autoimmune liver damage. In the early 1990s CD8 T cell clones specific for ASGPR were described in patients with AIH<sup>[32]</sup>. Recent studies have identified CYP2D6 specific CD8 T cells capable of secreting IFN- $\gamma$  and of exerting cytotoxicity after recognition of CYP2D6 epitopic sequences in an HLA class I restricted fashion<sup>[35]</sup>.

Taken together, the data presented above suggest that a failure of immune homeostatic processes, normally keeping the response against self-antigens under control, is involved in the pathogenesis of AIH. The prime mechanism for tolerance breakdown remains to be elucidated. There is some evidence that molecular mimicry mechanisms involving viral self-mimicking and autologous sequences may be involved<sup>[36,37]</sup> and such mechanisms are the focus of ongoing studies.

### ANIMAL MODELS

Research on the pathogenesis of AIH has been hampered by the lack of animal models reproducing faithfully the human condition. The ideal model for AIH should have a well-defined initiating event followed by chronic inflammation leading to fibrosis. Recently, researchers have focused on animal models of AIH type 2, since in this condition the autoantigen is well defined. The model produced by the group of Alvarez<sup>[38]</sup> is based on immunizing every two weeks for three times C57BL/6 female mice with a plasmid containing cDNA for the antigenic region of human CYP2D6, which is the target of anti-LKM-1, and formimino-transferase cyclodeaminase, which is the target of anti-liver cytosol-1 and an additional marker for AIH type 2<sup>[39]</sup>, together with the end of the terminal region of murine CTLA-4. The latter was added to facilitate antigen uptake by antigen presenting cells. In a parallel set of experiments a plasmid containing the cDNA encoding IL-12, a Th1 skewing pro-inflammatory cytokine, was also used. When autoantigens and IL-12 were used to

break tolerance, antigen specific autoantibodies were produced, a relatively modest elevation of transaminase levels at 4 and 7 mo was observed, and a portal and periportal inflammatory infiltrate composed of CD4 and CD8 T cells and, to a lesser extent, B cells was demonstrated 8-10 mo after the third immunization. When the same immunization protocol was used in different mouse strains, either a mild hepatitis or no inflammatory changes were observed indicating the importance of a specific genetic background. Another model of AIH type 2 uses CYP2D6 transgenic mice and aims at breaking tolerance with an adenovirus-CYP2D6 vector<sup>[40]</sup>. While focal hepatocyte necrosis was seen in both mice treated with the adenovirus-CYP2D6 vector and control mice treated with adenovirus alone, only the former developed chronic histological changes, including fibrosis, reminiscent of AIH. The hepatic lesion was associated to a specific immune response to an immunodominant region of CYP2D6 and a cytotoxic T cell response to adenovirus-CYP2D6 vector infected target cells. Though these two experimental approaches provide useful information on the possible pathogenic mechanisms leading to human AIH type 2, a model that closely reproduces human AIH type 1 is still lacking, hampering the elucidation of pathogenic mechanisms in this form of AIH.

### REFERENCES

- 1 Donaldson PT. Genetics in autoimmune hepatitis. *Semin Liver Dis* 2002; **22**: 353-364
- 2 Donaldson PT. Genetics of autoimmune and viral liver diseases; understanding the issues. *J Hepatol* 2004; **41**: 327-332
- 3 Czaja AJ, Donaldson PT. Genetic susceptibilities for immune expression and liver cell injury in autoimmune hepatitis. *Immunol Rev* 2000; **174**: 250-259
- 4 Ma Y, Bogdanos DP, Hussain MJ, Underhill J, Bansal S, Longhi MS, Cheeseman P, Mieli-Vergani G, Vergani D. Polyclonal T-cell responses to cytochrome P450IID6 are associated with disease activity in autoimmune hepatitis type 2. *Gastroenterology* 2006; **130**: 868-882
- 5 Yokosawa S, Yoshizawa K, Ota M, Katsuyama Y, Kawa S, Ichijo T, Umemura T, Tanaka E, Kiyosawa K. A genomewide DNA microsatellite association study of Japanese patients with autoimmune hepatitis type 1. *Hepatology* 2007; **45**: 384-390
- 6 Simmonds MJ, Gough SC. Genetic insights into disease mechanisms of autoimmunity. *Br Med Bull* 2004; **71**: 93-113
- 7 Liston A, Lesage S, Gray DH, Boyd RL, Goodnow CC. Genetic lesions in T-cell tolerance and thresholds for autoimmunity. *Immunol Rev* 2005; **204**: 87-101
- 8 Lankisch TO, Strassburg CP, Debray D, Manns MP, Jacquemin E. Detection of autoimmune regulator gene mutations in children with type 2 autoimmune hepatitis and extrahepatic immune-mediated diseases. *J Pediatr* 2005; **146**: 839-842
- 9 Mackay IR. Hepatoimmunology: a perspective. *Immunol Cell Biol* 2002; **80**: 36-44
- 10 Jensen DM, McFarlane IG, Portmann BS, Eddleston AL, Williams R. Detection of antibodies directed against a liver-specific membrane lipoprotein in patients with acute and chronic active hepatitis. *N Engl J Med* 1978; **299**: 1-7
- 11 McFarlane BM, McSorley CG, Vergani D, McFarlane IG, Williams R. Serum autoantibodies reacting with the hepatic asialoglycoprotein receptor protein (hepatic lectin) in acute and chronic liver disorders. *J Hepatol* 1986; **3**: 196-205

- 12 **Ma Y**, Gaken J, McFarlane BM, Foss Y, Farzaneh F, McFarlane IG, Mieli-Vergani G, Vergani D. Alcohol dehydrogenase: a target of humoral autoimmune response in liver disease. *Gastroenterology* 1997; **112**: 483-492
- 13 **Vergani D**, Mieli-Vergani G, Mondelli M, Portmann B, Eddleston AL. Immunoglobulin on the surface of isolated hepatocytes is associated with antibody-dependent cell-mediated cytotoxicity and liver damage. *Liver* 1987; **7**: 307-315
- 14 **Muratori L**, Parola M, Ripalti A, Robino G, Muratori P, Bellomo G, Carini R, Lenzi M, Landini MP, Albano E, Bianchi FB. Liver/kidney microsomal antibody type 1 targets CYP2D6 on hepatocyte plasma membrane. *Gut* 2000; **46**: 553-561
- 15 **Kerkar N**, Choudhuri K, Ma Y, Mahmoud A, Bogdanos DP, Muratori L, Bianchi F, Williams R, Mieli-Vergani G, Vergani D. Cytochrome P4502D6(193-212): a new immunodominant epitope and target of virus/self cross-reactivity in liver kidney microsomal autoantibody type 1-positive liver disease. *J Immunol* 2003; **170**: 1481-1489
- 16 **Muratori L**, Lenzi M, Cataleta M, Giostra F, Cassani F, Ballardini G, Zauli D, Bianchi FB. Interferon therapy in liver/kidney microsomal antibody type 1-positive patients with chronic hepatitis C. *J Hepatol* 1994; **21**: 199-203
- 17 **Aichele P**, Bachmann MF, Hengartner H, Zinkernagel RM. Immunopathology or organ-specific autoimmunity as a consequence of virus infection. *Immunol Rev* 1996; **152**: 21-45
- 18 **Vergani D**, Choudhuri K, Bogdanos DP, Mieli-Vergani G. Pathogenesis of autoimmune hepatitis. *Clin Liver Dis* 2002; **6**: 727-737
- 19 **Bogdanos DP**, Choudhuri K, Vergani D. Molecular mimicry and autoimmune liver disease: virtuous intentions, malign consequences. *Liver* 2001; **21**: 225-232
- 20 **Senaldi G**, Portmann B, Mowat AP, Mieli-Vergani G, Vergani D. Immunohistochemical features of the portal tract mononuclear cell infiltrate in chronic aggressive hepatitis. *Arch Dis Child* 1992; **67**: 1447-1453
- 21 **Lobo-Yeo A**, Alvirgi L, Mieli-Vergani G, Portmann B, Mowat AP, Vergani D. Preferential activation of helper/inducer T lymphocytes in autoimmune chronic active hepatitis. *Clin Exp Immunol* 1987; **67**: 95-104
- 22 **Nouri-Aria KT**, Donaldson PT, Hegarty JE, Eddleston AL, Williams R. HLA A1-B8-DR3 and suppressor cell function in first-degree relatives of patients with autoimmune chronic active hepatitis. *J Hepatol* 1985; **1**: 235-241
- 23 **Nouri-Aria KT**, Hegarty JE, Alexander GJ, Eddleston AL, Williams R. Effect of corticosteroids on suppressor-cell activity in "autoimmune" and viral chronic active hepatitis. *N Engl J Med* 1982; **307**: 1301-1304
- 24 **Vento S**, Hegarty JE, Bottazzo G, Macchia E, Williams R, Eddleston AL. Antigen specific suppressor cell function in autoimmune chronic active hepatitis. *Lancet* 1984; **1**: 1200-1204
- 25 **Shevach EM**, Piccirillo CA, Thornton AM, McHugh RS. Control of T cell activation by CD4+CD25+ suppressor T cells. *Novartis Found Symp* 2003; **252**: 24-36; discussion 36-44, 106-114
- 26 **Longhi MS**, Ma Y, Mitry RR, Bogdanos DP, Heneghan M, Cheeseman P, Mieli-Vergani G, Vergani D. Effect of CD4+CD25+ regulatory T-cells on CD8 T-cell function in patients with autoimmune hepatitis. *J Autoimmun* 2005; **25**: 63-71
- 27 **Longhi MS**, Hussain MJ, Mitry RR, Arora SK, Mieli-Vergani G, Vergani D, Ma Y. Functional study of CD4+CD25+ regulatory T cells in health and autoimmune hepatitis. *J Immunol* 2006; **176**: 4484-4491
- 28 **Longhi MS**, Ma Y, Mitry RR, Bogdanos DP, Heneghan M, Cheeseman P, Mieli-Vergani G, Vergani D. Effect of CD4+CD25+ regulatory T-cells on CD8 T-cell function in patients with autoimmune hepatitis. *J Autoimmun* 2005; **25**: 63-71
- 29 **Ma Y**, Okamoto M, Thomas MG, Bogdanos DP, Lopes AR, Portmann B, Underhill J, Dürr R, Mieli-Vergani G, Vergani D. Antibodies to conformational epitopes of soluble liver antigen define a severe form of autoimmune liver disease. *Hepatology* 2002; **35**: 658-664
- 30 **Chen M**, Shirai M, Liu Z, Arichi T, Takahashi H, Nishioka M. Efficient class II major histocompatibility complex presentation of endogenously synthesized hepatitis C virus core protein by Epstein-Barr virus-transformed B-lymphoblastoid cell lines to CD4(+) T cells. *J Virol* 1998; **72**: 8301-8308
- 31 **Wen L**, Ma Y, Bogdanos DP, Wong FS, Demaine A, Mieli-Vergani G, Vergani D. Pediatric autoimmune liver diseases: the molecular basis of humoral and cellular immunity. *Curr Mol Med* 2001; **1**: 379-389
- 32 **Wen L**, Peakman M, Lobo-Yeo A, McFarlane BM, Mowat AP, Mieli-Vergani G, Vergani D. T-cell-directed hepatocyte damage in autoimmune chronic active hepatitis. *Lancet* 1990; **336**: 1527-1530
- 33 **Lohr H**, Manns M, Kyriatsoulis A, Lohse AW, Trautwein C, Meyer zum Buschenfelde KH, Fleischer B. Clonal analysis of liver-infiltrating T cells in patients with LKM-1 antibody-positive autoimmune chronic active hepatitis. *Clin Exp Immunol* 1991; **84**: 297-302
- 34 **Lohr H**, Treichel U, Poralla T, Manns M, Meyer zum Buschenfelde KH. Liver-infiltrating T helper cells in autoimmune chronic active hepatitis stimulate the production of autoantibodies against the human asialoglycoprotein receptor in vitro. *Clin Exp Immunol* 1992; **88**: 45-49
- 35 **Longhi MS**, Hussain MJ, Bogdanos DP, Quaglia A, Mieli-Vergani G, Ma Y, Vergani D. Cytochrome P450IID6-specific CD8 T cell immune responses mirror disease activity in autoimmune hepatitis type 2. *Hepatology* 2007; **46**: 472-484
- 36 **Mackie FD**, Peakman M, Yun M, Sallie R, Smith H, Davies ET, Mieli-Vergani G, Vergani D. Primary and secondary liver/kidney microsomal autoantibody response following infection with hepatitis C virus. *Gastroenterology* 1994; **106**: 1672-1675
- 37 **Bogdanos DP**, Lenzi M, Okamoto M, Rigopoulou EI, Muratori P, Ma Y, Muratori L, Tsantoulas D, Mieli-Vergani G, Bianchi FB, Vergani D. Multiple viral/self immunological cross-reactivity in liver kidney microsomal antibody positive hepatitis C virus infected patients is associated with the possession of HLA B51. *Int J Immunopathol Pharmacol* 2004; **17**: 83-92
- 38 **Lapierre P**, Djilali-Saiah I, Vitozzi S, Alvarez F. A murine model of type 2 autoimmune hepatitis: Xenoimmunization with human antigens. *Hepatology* 2004; **39**: 1066-1074
- 39 **Lapierre P**, Hajoui O, Homberg JC, Alvarez F. Formimino-transferase cyclodeaminase is an organ-specific autoantigen recognized by sera of patients with autoimmune hepatitis. *Gastroenterology* 1999; **116**: 643-649
- 40 **Holdener M**, Hintermann E, Bayer M, Rhode A, Rodrigo E, Hintereder G, Johnson EF, Gonzalez FJ, Pfeilschifter J, Manns MP, Herrath MV, Christen U. Breaking tolerance to the natural human liver autoantigen cytochrome P450 2D6 by virus infection. *J Exp Med* 2008; **205**: 1409-1422

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Pietro Invernizzi, MD; Ian R Mackay, MD, Series Editors

## Clinical features and management of primary biliary cirrhosis

Andrea Crosignani, Pier Maria Battezzati, Pietro Invernizzi, Carlo Selmi, Elena Prina, Mauro Podda

Andrea Crosignani, Pier Maria Battezzati, Pietro Invernizzi, Carlo Selmi, Elena Prina, Mauro Podda, Division of Internal Medicine and Liver Unit, San Paolo Hospital School of Medicine, University of Milan, Milano 20142, Italy

Pietro Invernizzi, Carlo Selmi, Division of Rheumatology, Allergy, and Clinical Immunology, University of California at Davis, Davis, CA 95616, United States

Correspondence to: Andrea Crosignani, MD, Dipartimento di Medicina Interna, Polo Universitario, Ospedale San Paolo, Via di Rudini 8, Milano 20142, Italy. [crosihsp@unimi.it](mailto:crosihsp@unimi.it)

Telephone: +39-2-50323088 Fax: +39-2-50323089

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### Abstract

Primary biliary cirrhosis (PBC), which is characterized by progressive destruction of intrahepatic bile ducts, is not a rare disease since both prevalence and incidence are increasing during the last years mainly due to the improvement of case finding strategies. The prognosis of the disease has improved due to both the recognition of earlier and indolent cases, and to the wide use of ursodeoxycholic acid (UDCA). New indicators of prognosis are available that will be useful especially for the growing number of patients with less severe disease. Most patients are asymptomatic at presentation. Pruritus may represent the most distressing symptom and, when UDCA is ineffective, cholestyramine represents the mainstay of treatment. Complications of long-standing cholestasis may be clinically relevant only in very advanced stages. Available data on the effects of UDCA on clinically relevant end points clearly indicate that the drug is able to slow but not to halt the progression of the disease while, in advanced stages, the only therapeutic option remains liver transplantation.

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**Key words:** Primary biliary cirrhosis; Epidemiology; Clinical course; Natural history; Treatment

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### INTRODUCTION

Primary biliary cirrhosis (PBC) is an autoimmune liver disease characterized by progressive destruction of intrahepatic bile ducts with cholestasis, portal inflammation, and fibrosis which may lead to cirrhosis, to its complications, and eventually to liver transplantation or death. Thus, primary biliary cirrhosis is indeed a historically-based misnomer, since currently a substantial proportion of patients may not develop cirrhosis as the final event. The disease predominantly affects women who are usually diagnosed in their fifties mainly in an asymptomatic stage. The loss of bile ducts leads to the retention within the liver of detergent bile acids which contribute to parenchymal damage through interaction with cell membranes and cellular organelles. The derangement of the entero-hepatic circulation of bile acids may also induce important pathophysiological changes which may determine, if untreated, some of the extra-hepatic alterations characteristic of established disease. It is well known that both clinical features and natural history vary greatly among individual patients ranging from asymptomatic and stable or only slowly progressive to symptomatic and rapidly progressive disease. The clinical presentation has progressively changed from one characterized by a serious outcome to that of a slowly evolving disease since natural history and outcome have improved, during the last few decades, due to the recognition of earlier more indolent cases and, likely, to widespread use of ursodeoxycholic acid (UDCA).

Since aetiology and immunological aspects are reviewed separately in this series, the aim here is to review the evidence on epidemiology, diagnosis, clinical features, and treatment. Both management of the consequences of long-standing cholestasis and specific therapy for PBC will be discussed.

### EPIDEMIOLOGY

Descriptive epidemiology of a particular disease is important in order to establish the magnitude of the problem and to find clues for aetiopathogenesis. There are a number of epidemiologic studies reported among patients affected by PBC<sup>[1]</sup>. The key issue involving all these studies is that they rely upon the number of diagnoses recorded in a defined location rather than on the screening of the entire population at risk. Obviously, this latter approach



**Table 1** Epidemiology of primary biliary cirrhosis: Results from the most relevant studies<sup>[2-9]</sup>

Area	Patients (No.)	Prevalence (per million)	Incidence (per million/yr)	Age (yr)	Gender (M:F)
Europe (1984)	569	23	54	54	1:10
Northern Sweden (1990)	111	151 <sup>1</sup>	13.3	55	1:6
North East England (1990)	347	129 <sup>1</sup>	19	58	1:9
Ontario, Canada (1990)	225	22	3.3	59	1:13
Victoria, Australia (1995)	84	19	-	-	1:11
Newcastle, England (1997)	160	240 <sup>1</sup>	22	66	1:10
Olmsted County, MN (2000)	46	402 <sup>1</sup>	27	-	1:8
Victoria, Australia (2004)	249	51 <sup>1</sup>	-	61	1:9

<sup>1</sup>Data include survey of laboratories for antimitochondrial antibodies.

would be particularly expensive in view of the relatively low prevalence of the disease thus requiring large populations to be screened. At present, we must consider the prevalence indicated by case-finding studies as underestimates, to a degree inversely related to the accuracy of the methodology employed to identify the potential diagnoses made in the area under consideration. In Table 1, relevant data from the available epidemiologic studies are reported in chronological order<sup>[2-9]</sup>.

Several difficulties however exist when attempting to compare results of these studies among each other, and over time. Heterogeneity in the methodology of case finding and, to a lesser extent, the criteria used for the diagnosis represent the most problematic issue. In particular, only a few studies used multiple strategies to reduce selection bias by capturing the entire spectrum of illness associated with PBC, especially cases at the preclinical stage<sup>[10]</sup>. Ascertainment from laboratory determination of anti-mitochondrial antibodies (AMA), which are highly sensitive and specific markers of the disease, has been a valuable approach. Differences in estimates of incidence and prevalence of PBC among populations, coming from the earlier studies<sup>[2,11-15]</sup>, may be due to differences in diagnostic criteria and study design, as well as to the different disease awareness among physicians, and to the differing degrees of access to health care systems. Similarly the same limits may explain the lack of confirmation of preliminary observations of associations between the occurrence of PBC and environmental factors<sup>[12,16]</sup>.

The methodological quality of reported investigations has improved over time which allows some capacity to compare incidence and prevalence rates by geographic areas. Initial studies published between 1974 and 1986 described annual incidence rate of PBC ranging from 0.6 to 13.7 cases per million<sup>[2,11,13-15]</sup>. Prevalence rates from these studies varied between 23 and 128 cases per million<sup>[2,11,13-15]</sup>. The majority of data originated from the United Kingdom and Sweden. Since 1989 a larger number of studies have been reported, mainly performed in Europe but also coming from Asia, North

America, and Australia<sup>[3-10,16-31]</sup>. From these more recent studies, both the annual incidence rates and prevalence of PBC have increased<sup>[3-10,16-31]</sup>. In particular, from the United Kingdom the annual incidence rates increased from 5.8 to 20.5 cases per million between 1980 and 1999 among residents of Sheffield<sup>[12,28]</sup> and from 11 to 32 cases in Newcastle-upon-Tyne between 1976 and 1994<sup>[4,7,27]</sup>. A parallel increase of the prevalence rate occurred reaching the number of more than 200 cases per million in the middle-late nineties<sup>[4,7,27]</sup>. A similar picture has been reported by very recent studies coming from Europe<sup>[30,31]</sup>. These data may be explained by the progressively higher proportion of asymptomatic cases with early-stage disease, resulting in growing prevalence rates, and the increased use of biochemical and serologic testing leading to the increasing diagnosis of new cases per year. Interestingly the mean age at diagnosis did not change from initial to more recent studies (Table 1), thus indicating that the increasing prevalence and incidence reported by the literature is more related to wider rather than to earlier diagnoses.

Only recently, several epidemiological data are available also from the USA in full indicating an annual incidence rate of 27 cases per million with prevalence rates ranging between 160 and 402 cases per million, thus leading to an estimate of 3500 new cases each year with 47 000 prevalent cases among the white population<sup>[8]</sup>. However, these data come from specific regions and difficulties in obtaining more complete epidemiological evaluations are mainly due to two reasons: (1) the lack of an universal health care system; and (2) the large number of patients followed in secondary and tertiary centres. Lower prevalence and incidence have been reported in Canada and Australia<sup>[5,6,9,18,22]</sup>.

For PBC there is a well known high prevalence of female gender (F/M 9 to 1), and based on this observation several studies provided greater insight into the aetio-pathogenesis of the disease<sup>[32]</sup>. Little information is available regarding the influence of race or ethnicity on the descriptive epidemiology of PBC<sup>[1]</sup> indicating that host susceptibility plays a significant role in the development of the disease. PBC occurs more commonly among individuals with a family history of either PBC itself or other autoimmune disorders<sup>[33-35]</sup> and there is a high concordance rate (63%) *versus* that in other autoimmune diseases in monozygotic twins<sup>[36]</sup>. Taken altogether, these observations point towards the relevance of genetic factors in the occurrence of PBC. On the other hand, the recent finding of several clusters of PBC within defined spatial boundaries suggests that also environmental factors, such as pollution, may contribute to the development of the disease<sup>[37,38]</sup>. These associations are statistically extremely weak and may be flawed by quite a high number of biases of different types<sup>[39]</sup>. The role of a previous infection as the triggering factor for the development of PBC by the mechanism of molecular mimicry has been repeatedly suggested, in analogy with other autoimmune diseases, but data are inconsistent<sup>[40-42]</sup>.

In conclusion, data coming from more recent surveys of diagnoses performed in different geographical areas

indicate that PBC is not a rare disease and its prevalence and incidence are apparently increasing in recent years mainly due to easier recognition of the disease and improved case finding strategies. No firm suggestion on the aetiologic role of any specific environmental factors has come from epidemiology, whereas familial clustering indicates a major role for genetic background.

## DIAGNOSIS

The diagnosis of PBC is currently based on three criteria: the presence of AMA in serum which is highly specific for the disease, elevation of biochemical indices of cholestasis for more than 6 mo, and histological features in the liver that are indicative of the diagnosis. The presence of two of these criteria allows a probable diagnosis but for a definite diagnosis the occurrence of all criteria is needed<sup>[43]</sup>. However, alternative diagnoses of liver disease should be ruled out and particularly in the absence of detectable AMA, a nuclear magnetic resonance cholangiography is necessary to exclude a primary sclerosing cholangitis.

Determination of AMA using routine methods, however may lead to underestimation of their presence<sup>[44]</sup>. Up to 5% to 10% of patients have no detectable antimitochondrial antibodies, but their disease appears to be identical to that in AMA positive patients<sup>[45]</sup>.

Serum liver enzymes are the earliest biochemical indices to increase in serum: gamma glutamyl transpeptidase, alkaline phosphatase, and aminotransferases in descending order of sensitivity, but each lacks specificity, except, to some extent, alkaline phosphatase, if bone disease can be ruled out. On the other hand, serum bilirubin concentrations increase only in advanced stages of the disease, and accurate measure of serum bile acid concentrations requires state of the art methods, like gas chromatography-mass spectrometry (GC-MS), which are not available routinely<sup>[46]</sup>. In addition, serum bile acids are extremely sensitive but poorly specific and their detection by GC-MS is more useful to study derangement of the bile acid circulation or the effects of therapeutic bile acids<sup>[47]</sup>.

The utility of liver biopsy in the diagnosis of PBC has been questioned by several hepatologists<sup>[43]</sup> and even for staging purposes it is scarcely justified in patients who have obvious features of cirrhosis by clinical evaluation including imaging techniques.

## HISTOLOGICAL FEATURES

The pathological lesion typical for PBC is a chronic non-suppurative destructive cholangitis involving interlobular bile ducts of 40–80 µm in diameter<sup>[48]</sup>. Overall, coexistence of portal inflammatory infiltrate with bile duct paucity is needed for diagnosis. PBC is divided into four histological stages but the liver is not affected uniformly and even a single biopsy sample may demonstrate the presence of different stages of the disease. If this is the case, the most advanced stage of those present is assigned, according to convention<sup>[43]</sup>. Stage 1 is characterized by localization of

**Table 2** Modifications during time of the clinical spectrum of primary biliary cirrhosis at presentation<sup>[50–52]</sup>

	Sherlock 1973 ( <i>n</i> = 100)	James 1981 ( <i>n</i> = 93)	Nyberg 1989 ( <i>n</i> = 80)
Jaundice (%)	28	16	3
Pruritus (%)	57	14	26
Complications (%)	4	9	1
Asymptomatics (%)	11	61	70
Mean age (yr)	50	57	58

inflammation to portal triads. Stage 2 entails extension of inflammation beyond the portal triads into the lobular parenchyma and reduction in number of normal bile ducts. Stage 3 entails fibrous septa linking adjacent portal tracts. Stage 4 is the most advanced histological stage in which liver cirrhosis has occurred<sup>[49]</sup>.

## CLINICAL FEATURES

### Symptoms

**Asymptomatic disease:** PBC is now diagnosed earlier in its clinical course and most cases are only slowly progressive in comparison with the past, and the large majority of patients are asymptomatic at diagnosis (Table 2)<sup>[50–52]</sup>. It has been suggested that symptoms develop within five years in most asymptomatic patients, although one third of patients may remain symptom-free for many years<sup>[53,54]</sup>. Pruritus and fatigue are early symptoms and occur in about 20% of the patients<sup>[53,55]</sup>.

**Fatigue:** This is reported in up to 78% of PBC patients overall and is suggested to be a significant cause of disability from numerous studies<sup>[56–59]</sup>. However, a well-preserved quality of life has been recently reported in a very large cohort of patients with PBC in the USA thus arguing against the clinical relevance of fatigue in such a population<sup>[60]</sup>. Several studies have explored the pathogenesis of this symptom and indicated heterogeneous mechanisms ranging from autonomic dysfunction<sup>[59,61,62]</sup>, to excessive daytime somnolence<sup>[63]</sup>, and to altered manganese homeostasis within the central nervous system<sup>[64]</sup>, while concomitant depression could not be ruled out<sup>[65–67]</sup>. In addition, studies aimed at demonstrating the clinical relevance of fatigue in PBC are affected by significant flaws, since the correlation of inaccurate quantification of the symptom with both scores related to quality of life and clinically relevant events appears to be inappropriate, and a possible role of concomitant diseases could not be excluded<sup>[56–59]</sup>. Therefore fatigue seems a poorly specific symptom and a predominant psychogenic component is likely, as usually occurs in carriers of a chronic progressive illness who are aware of the potential impact on their future life.

**Pruritus:** This appears to be the most typical symptom of PBC. It was reported to occur in 20% to 70% of patients and occasionally is quite distressing<sup>[68]</sup>. In latter years its frequency in PBC has been decreasing because

the disease is increasingly recognized in its asymptomatic stage. The availability of therapeutic options such as UDCA which has been widely administered during the last two decades, seems to have also modified the occurrence and intensity of this symptom. The onset of pruritus generally precedes the onset of jaundice by months to years. The cause of pruritus remains unknown. However there is consensus that in the course of cholestasis biliary excretion of several compounds is impaired, thus leading to increased systemic concentrations of a putative “pruritogenic” compound. The occurrence of pruritus would result from the interaction between these substances and nervous terminations at the skin level. The extreme variability of the degree of pruritus between patients, or even in the same patient, may have two explanations: (1) inter-individual or time variability of the systemic concentrations of the “pruritogenic” compounds, which are generally confined within the enterohepatic circulation; and (2) subjective variability of the perception of pruritus, mainly due to psycho-emotional factors. Increased serum concentrations of bile acids are associated with cholestasis by definition, and a direct causative relationship between increased bile acid concentrations and the occurrence of pruritus has been suggested<sup>[69]</sup>. Several observations support this hypothesis, including: (1) the presence of bile acids in the skin in cholestatic patients<sup>[70]</sup>; (2) the capability of bile acids to produce pruritus when injected subcutaneously<sup>[71,72]</sup>; (3) the relief of pruritus by external biliary drainage, and by cholestyramine which can bind bile acids and thus favours their fecal elimination<sup>[73-75]</sup>. However, this hypothesis has never been proven since no relationship was found between degree of pruritus and bile acid levels measured in cutaneous interstitial fluid<sup>[76-78]</sup>. In addition, it is possible that many other substances are eliminated during both biliary drainage and cholestyramine administration.

The hypothesis that pruritus in cholestatic liver disease may have a central origin has been suggested by the observation of an increased opioidergic activity in both experimental models of cholestasis<sup>[79-82]</sup> and in cholestatic patients<sup>[79-81,83]</sup>, and by the observation that opioid receptor ligands with agonist properties (morphine for example) mediate pruritus<sup>[84-86]</sup>. Therefore, there have been studies using opioid antagonists for the treatment of pruritus in cholestatic conditions with positive results<sup>[87-89]</sup>, thus confirming the hypothesis that an increased opioidergic activity plays a role in the occurrence of pruritus associated with cholestasis. In cholestatic conditions high concentrations of bile acids in the systemic circulation may alter several central regulatory systems such as the opioid-mediated system.

**Portal hypertension:** This may occur even before cirrhosis develops. However, usually, ascites, variceal bleeding, and hepatic encephalopathy complicate the course of PBC only in advanced stages. Similarly, the incidence of hepatocellular carcinoma is elevated among patients with long-standing histologically advanced PBC<sup>[90]</sup>.

### **Consequences of long-standing cholestasis**

Other common findings in advanced PBC include the consequences of long-standing cholestasis that can lead to hyperlipidemia, fat malabsorption, renal tubular acidosis, and osteopenia. However, the clinical relevance of hyperlipidemia in patients with PBC remains questionable since neither cardiovascular risk<sup>[91]</sup> nor more precocious signs of atherosclerosis<sup>[92]</sup> are associated with alterations of lipid metabolism in PBC. In addition, the wide use of therapeutic bile acids in the last decade may have modified the metabolic pattern of plasma lipids in PBC<sup>[93,94]</sup>.

Metabolic bone disease described in patients with PBC is the result of two different pathological processes: osteomalacia and osteoporosis. Osteomalacia which is a consequence of lipid malabsorption may be easily corrected by supplementation with calcium and vitamin D<sup>[95-97]</sup>. The changing spectrum of bone disease associated with cholestasis with a progressive disappearance of osteomalacic features over time may be due to the increasingly wide use of vitamin D and calcium supplementation in clinical practice<sup>[97,98]</sup>. Therefore, at present, osteoporosis is the predominant component of metabolic bone disease<sup>[98]</sup>. During end-stage liver disease, which is characterized by reduced physical activity, malnutrition, and, possibly, infectious complications, bone loss is a major clinical issue<sup>[99]</sup>. On the other hand there is no consensus on the clinical relevance of cholestasis in inducing bone loss at less advanced stages of liver disease<sup>[100]</sup>. In a recent longitudinal controlled study, we demonstrated that cholestasis was not an additional risk factor for bone demineralization in women with well-compensated PBC if adequate calcium and vitamin D supplementation had been provided<sup>[101]</sup>. These data are in accordance with several studies<sup>[102-104]</sup> but in contrast with others<sup>[105-107]</sup>. Different results may be due to: (1) the cross-sectional nature of many studies; (2) the lack of an adequate control group in the majority of the published studies so precluding the protection against confounding factors such as menopausal status, which is important in a population wherein perimenopausal women are largely represented; (3) the lack of adequate vitamin D and calcium supplementation in most of the published studies; and (4) the confounding effects of other concomitant medications.

Malabsorption, deficiencies of fat-soluble vitamins, and steatorrhea are uncommon except in the late stages of the disease<sup>[108]</sup>. Finally, the occurrence of renal tubular acidosis which was once thought to be quite frequent<sup>[109]</sup> was not found in a large population of PBC in the absence of complication of liver cirrhosis<sup>[110]</sup>, thus indicating that such a complication, if present at all, may be restricted to very late stages of the disease in association with multiorgan dysfunction.

### **Associated diseases of autoimmune type**

Symptoms of coexisting autoimmune diseases including Sjogren syndrome, scleroderma, rheumatoid arthritis autoimmune thrombocytopenia, and haemolytic anaemia may be present. Interestingly, liver disease was recently shown to have a slower progression when systemic

sclerosis is associated with PBC compared with matched patients with PBC alone<sup>[111]</sup>. Overlap syndromes with autoimmune hepatitis are described in another article in this issue.

## NATURAL HISTORY AND PROGNOSTIC MODELS

The natural history and prognosis of PBC have become more difficult to characterize given the rising number of asymptomatic cases which require long-term follow-up<sup>[1,43,109,112]</sup>. Furthermore, patients are more likely than in the past to be asymptomatic at diagnosis<sup>[1,43,112]</sup> and to receive medical treatment as soon as diagnosis is made. Hence, estimated survival has significantly improved compared to the past. Earlier data on survival suggesting a poor outcome were obtained from patients in whom the disease had been diagnosed many years ago when no effective treatment existed<sup>[1,43,112]</sup>. In addition, most of these patients were symptomatic<sup>[1,43,112]</sup>.

A different outcome of the disease has been reported for symptomatic *versus* asymptomatic patients. In 1983, the reported survival of asymptomatic PBC patients was similar to that of a normal U.S. population matched for age and sex<sup>[113]</sup>, but, when their survival data were extended for a longer duration, the asymptomatic patients had a shortened survival compared with controls<sup>[114]</sup>. In this latter study, 279 patients from the USA were observed for up to 24 years, and the median survival of asymptomatic PBC patients was significantly longer than symptomatic patients at presentation<sup>[114]</sup>. Additional studies confirmed that initially asymptomatic patients had a longer survival than symptomatic ones<sup>[109,115]</sup>. In one of these studies from Canada, asymptomatic PBC patients had a shortened survival compared with a healthy population<sup>[115]</sup>. The results described in a community-based study from the UK are at variance with all of the other reports<sup>[54]</sup>. Here 770 patients (61% asymptomatic) living in England were diagnosed between 1987 and 1994 and observed until death, transplantation, or until data were censored in January 2000. The median survival was similar in asymptomatic and symptomatic patients, and symptom development was not associated with shorter survival. However, the design of this study, in which patients were followed by regular interview and by examination of their medical records may be not as informative as a single centre cohort study to assess the natural history of PBC, even though it is sufficient for epidemiological purposes. In fact, these UK results are confounded by the fact that 45% of the deaths in asymptomatic patients occurred while these patients were remained asymptomatic, suggesting that many of these patients would have been died of non-hepatic causes and that age at diagnosis was a major determinant of survival. Since the prognostic relevance of the presence of symptoms is well documented, the higher proportions of asymptomatic patients enrolled in the more recent cohort studies explain, partly at least, the observed improvement in the natural history of PBC since 1980s.

**Table 3** Parameters independently associated with bad prognosis in different prognostic models based on a single point observation<sup>[16,113,119,121-123]</sup>

Parameters	Yale	European	Mayo	Glasgow	Oslo	London
Increase in serum bilirubin	+	+	+	+	+	+
Decrease in serum albumin		+	+			+
Increase in PT (INR)			+			
Advanced age	+	+	+	+		+
Hepatomegaly	+					+
Ascites, fluid retention			+	+		+
Esophageal varices						+
Gastrointestinal bleeding				+	+	
Cirrhosis	+	+		+		+
Cholestatic picture at histology		+		+		
Mallory bodies				+		

Most patients with PBC are now treated with UDCA<sup>[43]</sup> and the widely used administration of this drug has greatly changed the natural history of the disease<sup>[43,112]</sup>. At least 20% of patients treated with UDCA will have no histologic progression over four years, and some will have no progression over a decade or longer<sup>[116]</sup>. In a recent study, the survival rate of patients with stage 1 or 2 disease given UDCA long-term was similar to that of a healthy control population<sup>[117]</sup>. In the above-mentioned community-based study from the UK no improvement in survival was found in UDCA-treated patients<sup>[54]</sup>. We reiterate that such a study design albeit excellent for epidemiological purposes, is not adequate for the evaluation of the effects of medical treatment. In addition, there is sufficient evidence that UDCA treatment does prevent the development of esophageal varices<sup>[118]</sup>. Therefore, sufficient information is now available to indicate that, among the reasons for the improving prognosis of PBC, is the wide use of bile acid therapy. Detailed information on the effects of UDCA therapy on survival is described below.

Cox proportional hazards regression analysis has been used to develop prognostic models. There are different prognostic models for predicting survival for PBC patients. Of these models, the Mayo survival model is the most popular. The Mayo model was based on combined data from more than 400 patients who were observed at the Mayo Clinic and was then externally cross-validated using PBC patients from other medical centers<sup>[119,120]</sup>. The Mayo model uses five independent prognostic variables: age, total serum bilirubin, serum albumin, prothrombin time, and the severity of fluid retention. Serum bilirubin is the most heavily weighted among these variables, consistent with the presence of this index in all the proposed prognostic models<sup>[16,113,119,121-123]</sup> (Table 3). All these models are based on a single assessment but several have been modified to include repeated measures of prognostic indices<sup>[121,124,125]</sup>. The Mayo model has been widely used to assess the efficacy of medical treatment in clinical trials, but also serum bilirubin concentrations



have been similarly used as surrogate markers of disease improvement, due to the prognostic value of this index in PBC patients with more advanced disease<sup>[126]</sup>.

Recently, also an immune marker was shown to be of prognostic value since a particular specificity of antinuclear antibodies that directed against nuclear pore complex, identified patients destined to experience more rapid disease progression<sup>[127]</sup>.

## TREATMENT OF SYMPTOMS AND COMPLICATIONS

### Fatigue

No therapy that has been evaluated for the treatment of PBC has proven able to ameliorate fatigue<sup>[128-130]</sup>. However, this symptom is not specific, only indirect quantitative measurement is available, and there are no convincing data to support any organic pathophysiological mechanism with even a psychological basis possible in some cases<sup>[65-67]</sup>.

### Pruritus

Pruritus in several, albeit very rare, cases may severely affect the quality of life, leading to sleep disturbance and major depression. This is the reason why intractable pruritus has been considered an indication for orthotopic liver transplantation (OLT). A large number of pharmacological approaches have been tested on the basis of both pathophysiologic considerations and serendipitous observations. The heterogeneity of the treatments suggested reflects the difficulties in treating this symptom which is extremely variable in severity and type, influenced by subjective factors and not easily quantifiable. The administration of UDCA, the only approved treatment for PBC, was not associated with a consistent improvement of pruritus in most controlled clinical trials; however, since the majority of them were not designed specifically to test the effects of this drug on pruritus, no definite conclusion can be drawn. In addition, as reported above, epidemiological data indicate that the disease expression has changed during the last two decades towards less symptomatic disease<sup>[1,43,112]</sup>, and a possible effect of the widely administered UDCA in decreasing pruritus certainly cannot be ruled out.

The oral anion exchange resin cholestyramine has been the mainstay of therapy for pruritus associated with cholestasis<sup>[73-75]</sup>. The mechanism of action is related to binding of bile acids and other biliary molecules, with their subsequent fecal excretion. Dose of cholestyramine should start from 4 g daily and should be increased, in case of therapeutic failure, until a maximum of 16 g. The timing of administration is before meals. The drug is more effective in those patients with an intact gallbladder when taken before and after breakfast, because the greatest amount of bile is likely to be available for binding at this time. Since cholestyramine binds also other medications, notably UDCA, oral contraceptive hormones, digoxin and thyroxine, it is advisable that at least 4 h should elapse between the administration of cholestyramine and other medications. In the majority of cases this drug is effective within a few days from starting treatment, but in about

**Table 4 Pharmacological characteristics of the opiate antagonists investigated in clinical studies**

Pharmacological characteristics	
Naloxone	Very short half life Intravenous continuous infusion Dose: 0.2-0.4 µg/kg per minute
Nalmefene	Longer half life Oral administration 2 mg twice/d with a gradual increase until 20 mg twice/d
Naltrexone	Longer half life Oral administration 50 mg/d (in two divided doses the first day and subsequently in a unique dose)

10% to 20% of the patients it is ineffective. In addition, many patients find cholestyramine unpleasant to take and complain of dyspeptic symptoms or diarrhea or, alternatively, constipation so leading to poor compliance with treatment.

Rifampicin is an enzyme-inducing antibiotic which was serendipitously identified as an agent that improves pruritus in cholestasis<sup>[131]</sup>. A subsequent crossover trial indicated that the drug provided good control of pruritus in PBC at doses of 150 mg twice per day or three times per day<sup>[132]</sup>. In subsequent studies higher doses were used up to 600 mg/d<sup>[133]</sup> and 10 mg/kg per day<sup>[134]</sup>. Its mechanism of action remains unknown but it may alter bile acid composition<sup>[135,136]</sup> and stimulate the hepatobiliary transport systems<sup>[137,138]</sup>. When given long-term, rifampicin was shown to improve also the biochemical expression of PBC<sup>[139]</sup>. However, it is not effective in all patients and may cause side effects<sup>[140]</sup>. Two cases of acute hepatitis were reported (12.5% of treated patients) during long-term administration<sup>[139]</sup>, but this spontaneously resolved after discontinuation of treatment. In any case, the potential hepatotoxicity of rifampicin precludes long-term administration of this drug to patients with PBC.

Many studies endorse the use of opioid antagonists, given intravenously or orally, for the treatment of cholestasis-related pruritus<sup>[87-89]</sup>. The main pharmacological characteristics of the three compounds investigated clinically are reported in Table 4. Each compound was shown to be highly effective in improving pruritus, but the main limit on their use was the occurrence of withdrawal-like symptoms in several patients. In addition, after initial enthusiasm following elegant studies supporting the intriguing hypothesis of an increased opioidergic activity in cholestatic patients<sup>[79-82]</sup>, opioid antagonists have lapsed for the treatment of pruritus. Larger and longer studies are needed to fully assess the actual clinical value of opioid antagonists in controlling pruritus in PBC.

Since the serotonergic system participates in the mediation of nociception, it appears rational to use drugs acting on this system. Several studies suggested that a possible beneficial effect may be exerted by ondansetron a type III serotonin antagonist<sup>[141-143]</sup>, but subsequent studies showed only limited or no effects on pruritus<sup>[144-146]</sup>. Surprisingly, the results of a recently published small randomized, double-blind, placebo-controlled trial based

on a heterogeneous group of patients with pruritus and liver disease suggested a beneficial effect of sertraline, a serotonin reuptake inhibitor<sup>[147]</sup>. Finally, since the cannaboidergic system plays a role in the mediation of nociception, uncontrolled observations on the effects of dronabinol, a cannabinoid B1 receptor, suggested relief of pruritus in course of cholestasis<sup>[148]</sup>.

In conclusion, since UDCA is the only accepted therapy for PBC, this bile acid represents the treatment of choice for pruritus. If the symptom persists, cholestyramine be initiated. Only in the case of a lack of response to maximal doses of cholestyramine a therapeutic approach with rifampicin or opioid antagonists should be considered.

### Metabolic bone disease

Osteomalacia may be easily corrected by parenteral supplementation of vitamin D (vitamin D<sub>3</sub> 100 000 UI intramuscular monthly). Supplementation with calcium carbonate (1 g/d) has been largely recommended based on pathophysiological considerations and on data coming from experience in postmenopausal osteoporosis whereas only indirect evidence is available in PBC patients<sup>[97,149]</sup>.

As reported above, it is highly questionable whether osteoporosis during cholestatic conditions represents a separate clinical entity<sup>[100,101]</sup>. Therefore the available data on treatment of metabolic bone disease in PBC are similar to those reported for postmenopausal osteoporosis noting that most patients with PBC are females at a menopausal age. Various data indicate that hormone replacement therapy is effective and safe, contrary to previous beliefs<sup>[150-154]</sup>. Etidronate was suggested to be effective<sup>[155,156]</sup>, but not all studies reported positive results<sup>[157]</sup>, while alendronate was shown to be superior<sup>[158,159]</sup>. Calcitonin failed to improve bone mineral density in female patients with PBC<sup>[149]</sup>. The negligible improvement observed in one study<sup>[160]</sup>, is perhaps attributable to concomitant vitamin D and calcium supplementation. Several indications for the clinical management of metabolic bone disease associated with PBC are reported in Table 5. Finally it should be highlighted that UDCA, the specific treatment for PBC was shown to have no effects on the occurrence of bone loss<sup>[161]</sup>.

### Hyperlipidemia

It is still questionable if hypercholesterolaemia associated with PBC should be treated, and which patients need pharmacological treatment. Since increased cholesterol concentrations associated with cholestasis do not increase the atherosclerotic risk, it seems reasonable to treat hypercholesterolaemia only when hyperlipidemia of familial and nutritional origin probably coexists<sup>[162]</sup>. The extent of cholesterol reduction by UDCA administration<sup>[93]</sup> may be insufficient to protect this group of patients from cardiovascular risk. These patients probably would benefit from dietary modifications, weight loss, and the administration of specific lipid-lowering drugs. Cholestyramine may be indicated for its cholesterol lowering capacity in hypercholesterolaemic patients, especially if there is associated pruritus, while

**Table 5** Clinical management of metabolic bone disease associated with primary biliary cirrhosis

Clinical management	Efficacy	
	Moderate efficacy	Mild efficacy, insufficient data
Prevention		
1 Parenteral vitamin D3 supplementation	Indicated for all patients to prevent osteomalacic lesions	
2 Calcium carbonate supplementation		
Treatment		
1 Estrogen		Few data but effective and safe
2 Etidronate		Conflicting data Indicated in case of concomitant corticosteroid administration
3 Alendronate		Few data but effective and safe
4 Calcitonin		Probably ineffective

HMGC<sub>o</sub>A-reductase inhibitors should be limited to hypercholesterolaemic patients in whom serum levels of HDL are below the protective range, or if additional risk factors for cardiovascular disease are present<sup>[162]</sup>. In pilot studies, both simvastatin and atorvastatin proved to be safe and effective in reducing serum cholesterol levels in patients with PBC<sup>[163-165]</sup>.

### Malnutrition

During severe cholestasis, which occurs only at very advanced stages of PBC when liver transplantation is precluded, lipid malabsorption occurs with steatorrhea and weight loss. In such cases a reduction to 40 mg of the daily dietary fat intake is indicated and the same amount should be administered as medium chain triglycerides, which are digested and absorbed in the intestine even in the presence of low bile acid concentrations. In several cases administration of cholestyramine has to be discontinued.

Since malabsorption of lipophilic vitamins occurs even in the absence of clinically evident steatorrhea, preventive supplementation with vitamin D may be advisable in case of significant alterations of biochemical markers of cholestasis. Parenteral vitamin K supplementation should be given if prothrombin time is increased.

## SPECIFIC TREATMENT FOR PBC

Many therapeutic agents have been tested for PBC but difficulties have been encountered in establishing statistically significant long-term benefits for a disease with such a variable natural history. In addition, PBC surrogate markers of prognosis have several limitations: impairment of indices of liver synthetic function occurs only at very advanced phases of the disease, and the likelihood of sampling errors limits the value of liver histology. The only index which may be useful to assess prognosis is serum bilirubin, and this only in late phases of the disease. Randomized, controlled trials, recently

**Table 6** Efficacy and toxicity of the principal drugs investigated for the medical treatment of primary biliary cirrhosis

	Efficacy	Toxicity
D-penicillamine	-	+
Chlorambucil	+/-	+
Cyclosporine	+/-	+
Azathioprine	+/-	+
Methotrexate	+/-	+
Colchicine	+/-	-
Glucocorticoids	+/-	+/-
UDCA	+	-

re-evaluated by a meta-analysis<sup>[166]</sup>, have endorsed the failure of penicillamine. The only accepted treatment for PBC is UDCA that may delay but not halt the progression of the disease<sup>[167]</sup>. For several other agents, mainly immunosuppressive components, some interesting possibilities have been revealed but mainly in terms of combination treatment with UDCA. Data are summarised in Table 6. Regarding corticosteroid drugs, data are scanty mainly because bone demineralization represents a big concern in a population of female patients at postmenopausal age<sup>[168,169]</sup>. Corticosteroid monotherapy does not seem to offer a sufficient benefit *versus* side effects ratio for most PBC patients and its use should be limited to patients with other concomitant autoimmune diseases or with a PBC-autoimmune hepatitis overlap syndrome<sup>[170]</sup>. In such cases, co-administration of etidronate may prevent bone loss<sup>[156]</sup>.

Azathioprine administration should not be recommended on the ground of a limited efficacy and the substantial risk of side effects<sup>[121,171,172]</sup>. For chlorambucil, the frequency and potential severity of side effects outweighs potential benefits of this immunosuppressive drug, thus contraindicating its use in PBC<sup>[173]</sup>. After preliminary encouraging data coming from a pilot study<sup>[174]</sup>, Kaplan and colleagues have repeatedly reported biochemical and histological improvement after the administration of low dose of methotrexate (15 mg/wk), but no data on survival have been presented<sup>[175,176]</sup>. Aside from potentially serious complications<sup>[177]</sup>, the beneficial effects of methotrexate in the treatment of PBC, alone or in combination with UDCA, could not be confirmed by randomized, controlled trials performed by other groups<sup>[178-180]</sup>. There is no indication for the clinical use of cyclosporine in PBC, given the limited efficacy and known side effects<sup>[181]</sup>.

Available information indicates that colchicine with its anti-inflammatory and antifibrotic properties may exert limited beneficial effects on the natural history of PBC but without relevant side effects<sup>[182-185]</sup>. This is the reason why it has been largely tested in association with UDCA but showing no additional benefit in terms of clinically relevant end-points in comparison with UDCA monotherapy<sup>[186,187]</sup>.

### UDCA for the therapy of PBC

The rationale for the use of UDCA in the treatment of PBC depends on its ability in displacing and/or

diluting detergent and hepatotoxic bile acids from the bile acid pool. It is well known that in cholestatic conditions, endogenous bile acids are retained within hepatocytes, thus leading to the progressive deterioration of liver function. The beneficial effects of UDCA on indices of liver dysfunction have been attributed to its physicochemical properties, since UDCA is very hydrophilic and therefore non-toxic to biological membranes<sup>[188,189]</sup>. However, experimental data failed to support this hypothesis since a substantial shift towards hydrophilicity of the bile acid pool was not observed during UDCA administration<sup>[47]</sup>. It has been suggested that UDCA has a direct cytoprotective effect, and different molecular mechanisms may be responsible, such as regulation of cellular signalling systems and protection against apoptosis<sup>[190]</sup>. Immunomodulatory effects of UDCA have been also described<sup>[190]</sup>, although it is not conventionally used as an immunosuppressive drug in non-hepatic diseases.

A number of randomized controlled studies have been conducted to evaluate UDCA efficacy<sup>[43]</sup>. In all studies UDCA was well tolerated since no relevant side effects were reported. In all studies a significant improvement of serum liver enzymes markers of cholestasis and cytolysis occurred. Serum concentrations of bilirubin, the most important prognostic marker of the disease, were reduced by UDCA administration. A consistent reduction of IgM, which is an immunological marker of PBC was also reported.

Results of randomized placebo-controlled trials with a duration long enough to evaluate the effects on histology and on survival are summarized in Table 7<sup>[191-197]</sup>. Among the six studies that evaluated the effects of UDCA on pruritus<sup>[191-196]</sup>, an improvement was described in only three<sup>[191,194,196]</sup>, but these studies were not specifically designed to assess pruritus. In four studies a significant improvement of several histological indices was reported<sup>[191,192,194,196]</sup>. The Mayo Clinic group did not report any improvement of liver histology, but have suggested in a separate paper that UDCA delays the occurrence of esophageal varices<sup>[118]</sup>, thus indicating a positive effect on the progression of the disease.

To evaluate the effectiveness of a specific therapy for a severe life-threatening disease, the effects on survival should be explored. However, since PBC is a relatively uncommon disease with a long and variable natural history, a very large sample size and a very long follow-up are needed to obtain reliable data. No effect on survival was observed in any of the single studies reported in Table 7, and only after an extension of follow up was a positive effect on survival without OLT reported by the French and the Mayo Clinic studies<sup>[198,199]</sup>. During the 2-year extension of the French study all patients administered placebo were switched to UDCA, while in the Mayo Clinic study, UDCA was offered to all patients but, for the analysis, follow-up was censored at the end of the randomized phase for patients initially assigned to the placebo group, thus avoiding the limits of a switch-over design.

A combined analysis of three studies<sup>[167]</sup> and two meta-

**Table 7** Randomized, double-blind, placebo-controlled trials on ursodeoxycholic acid administration to patients with primary biliary cirrhosis

First author	No. of patients	Study design and duration of follow up	UDCA effects on		
			Pruritus	Histology	Survival
Poupon <sup>[191]</sup>	146	2 yr	Improved	Improved	No effect
Heathcote <sup>[192]</sup>	222	2 yr	No effect	Improved	No effect
Lindor <sup>[193]</sup>	180	Mean follow up: 2 yr	No effect	No effect	No effect
Combes <sup>[194]</sup>	151	2 yr	Improved	Improved (early stages)	No effect
Eriksson <sup>[195]</sup>	116	2 yr + 2 yr as open trial (UDCA)	No effect	No effect	No effect
Pares <sup>[196]</sup>	192	Mean follow up: 3.4 yr	Improved	Improved	No effect
Papatheodoritis <sup>[197]</sup>	86	Mean follow up 7.3 yr for UDCA 8.1 yr for controls	Not evaluated	No effect	No effect

analyses<sup>[200,201]</sup> have been performed, since the majority of the published studies had insufficient statistical power to explore the effects of UDCA on survival. The combined analysis was obtained by pooling of results from three trials with similar designs but dissimilar results. The analysis included 548 patients and a significant improvement of survival free from OLT was reported with the relative risk of death being 0.53 (0.36-0.77; 95% CI). A significant improvement of survival could be recorded only in patients with serum bilirubin higher than 1.4 mg/dL at baseline. The lack of an effect on survival in patients with less severe disease may well indicate that the time of observation was not sufficient to detect effects of UDCA in a population with a low probability of developing clinically relevant events. On the other hand, results of the two meta-analyses indicate no effects of UDCA on the natural history of the disease. Formal meta-analysis includes consideration of all relevant trials, justifies eventual exclusion of trials from the analysis, and explores heterogeneity between trials and the reason for variation in results. The main limit of a meta-analysis is that trials evaluated may be too different in their designs to be truly comparable. The reason for the opposite results reported by the combined analysis<sup>[167]</sup> and by the two meta-analyses<sup>[200,201]</sup> remains unclear. The main criticisms directed against the combined analysis were the limits of the switching over design, but the "intention to treat" basis of the analysis is protective against type I error, thus reducing the probability of demonstrating benefits of UDCA in the absence of a true beneficial effect. Conversely, the inclusion in the meta-analyses of studies using low doses of UDCA, and with a follow-up too short for assessment of effects on clinically relevant end-points, has been strongly criticized. The effects on surrogate markers of clinical outcome, such as serum bilirubin concentration, do indicate that UDCA may positively affect survival in PBC. In addition, the UDCA safety and its relatively low cost permit a wide scale use of this therapeutic bile acid.

So, in conclusion, our opinion is that UDCA does exert a favourable effect on the natural history of PBC, but since many studies had been characterized by an insufficient number of patients, insufficiently long follow-up periods, heterogeneity of evaluated indices, and inadequate study designs, an absolutely clear-cut demonstration of benefit was precluded. Indirect data on the beneficial effects of UDCA also in patients at the initial

stages of the disease are now available<sup>[117,202]</sup>. An excellent long-term survival, comparable to that observed in a control population, has been recently reported in patients with PBC showing biochemical response to UDCA<sup>[202]</sup>. These data were obtained by studying a cohort of 192 patients, mainly with stage 1 and 2 of the disease, who had been treated for a mean period of more than 6 years. In addition, in a recent study of 262 patients with PBC who received UDCA for a mean of 8 years, the survival rate of patients with stage 1 or 2 disease was similar to that of a healthy control population<sup>[117]</sup>. However, not all patients have a response to treatment, since in the same study, the probability of death or undergoing OLT in patients with stage 3 or 4 of PBC was significantly increased compared with a healthy population, despite UDCA treatment. Therefore strategies aimed at improving therapeutic agents for PBC are still needed, mainly by the use of associated treatments.

Several drugs have been tested in association with UDCA. The results obtained with colchicine, and budesonide are the more promising but none of the drugs studied was shown to provide any additional benefit, in terms of clinically relevant events, compared to UDCA monotherapy<sup>[186,187,203-205]</sup>.

### OLT for the therapy of PBC

Finally, OLT has greatly improved survival in patients with PBC since this is the only effective treatment in patients with very advanced disease. "The survival rates are 92% and 85% at 1 year and 5 years, respectively<sup>[206]</sup>. While the recurrence rate is 30% at 10 years<sup>[207]</sup>. Note that OLT is considered in detail in another article in this series.

## CONCLUSION

Data coming from the more recent epidemiological studies indicate that PBC is not a rare disease and its prevalence and incidence are apparently increasing. In addition, the clinical presentation of PBC has progressively changed from a highly symptomatic disorder with a bad prognosis to a slowly evolving disease. The changing methods used for the diagnosis, with an increasingly wide assessment of laboratory indices related to both cholestasis and immunology, together with improved case finding strategies, may explain these observations.

As a result, the recognition of earlier more indolent



cases led to the presence of a substantial proportion of asymptomatic patients within PBC cohorts. Therefore development of early prognostic indices may be useful to predict which patients are destined to develop a progressive disease thus requiring a more intensive follow-up.

UDCA does not act on the aetiology of the disease but reverses the detrimental effects of the retention of endogenous bile acids within the liver. Although several flaws of the available studies prevented a clear-cut demonstration of its efficacy, many indirect observations suggest that a beneficial effect occurs and we cannot exclude that the wide use of UDCA may have significantly changed the clinical course of the disease. However, UDCA is able to slow but not to halt the progression of the disease and, in advanced stages, when the large majority of bile ducts have been destroyed, OLT remains the only therapeutic option.

In the future, reliable epidemiological data to be obtained by screening the entire population at risk, will provide both a correct measurement of the real prevalence and incidence of PBC and a greater insight into aetiology and pathogenesis, thus leading to the possibility of a specifically targeted therapy.

## REFERENCES

- Lazaridis KN, Talwalkar JA. Clinical epidemiology of primary biliary cirrhosis: incidence, prevalence, and impact of therapy. *J Clin Gastroenterol* 2007; **41**: 494-500
- Triger DR, Berg PA, Rodes J. Epidemiology of primary biliary cirrhosis. *Liver* 1984; **4**: 195-200
- Danielsson A, Boqvist L, Uddenfeldt P. Epidemiology of primary biliary cirrhosis in a defined rural population in the northern part of Sweden. *Hepatology* 1990; **11**: 458-464
- Mysor M, James OF. The epidemiology of primary biliary cirrhosis in north-east England: an increasingly common disease? *Q J Med* 1990; **75**: 377-385
- Witt-Sullivan H, Heathcote J, Cauch K, Blendis L, Ghent C, Katz A, Milner R, Pappas SC, Rankin J, Wanless IR. The demography of primary biliary cirrhosis in Ontario, Canada. *Hepatology* 1990; **12**: 98-105
- Watson RG, Angus PW, Dewar M, Goss B, Sewell RB, Smallwood RA. Low prevalence of primary biliary cirrhosis in Victoria, Australia. Melbourne Liver Group. *Gut* 1995; **36**: 927-930
- Metcalfe JV, Bhopal RS, Gray J, Howel D, James OF. Incidence and prevalence of primary biliary cirrhosis in the city of Newcastle upon Tyne, England. *Int J Epidemiol* 1997; **26**: 830-836
- Kim WR, Lindor KD, Locke GR 3rd, Therneau TM, Homburger HA, Batts KP, Yawn BP, Petz JL, Melton LJ 3rd, Dickson ER. Epidemiology and natural history of primary biliary cirrhosis in a US community. *Gastroenterology* 2000; **119**: 1631-1636
- Sood S, Gow PJ, Christie JM, Angus PW. Epidemiology of primary biliary cirrhosis in Victoria, Australia: high prevalence in migrant populations. *Gastroenterology* 2004; **127**: 470-475
- Prince MI, James OF. The epidemiology of primary biliary cirrhosis. *Clin Liver Dis* 2003; **7**: 795-819
- Hamlyn AN, Sherlock S. The epidemiology of primary biliary cirrhosis: a survey of mortality in England and Wales. *Gut* 1974; **15**: 473-479
- Triger DR. Primary biliary cirrhosis: an epidemiological study. *Br Med J* 1980; **281**: 772-775
- Hamlyn AN, Macklon AF, James O. Primary biliary cirrhosis: geographical clustering and symptomatic onset seasonality. *Gut* 1983; **24**: 940-945
- Eriksson S, Lindgren S. The prevalence and clinical spectrum of primary biliary cirrhosis in a defined population. *Scand J Gastroenterol* 1984; **19**: 971-976
- Lofgren J, Jarnerot G, Danielsson D, Hemdal I. Incidence and prevalence of primary biliary cirrhosis in a defined population in Sweden. *Scand J Gastroenterol* 1985; **20**: 647-650
- Goudie BM, Burt AD, Macfarlane GJ, Boyle P, Gillis CR, MacSween RN, Watkinson G. Risk factors and prognosis in primary biliary cirrhosis. *Am J Gastroenterol* 1989; **84**: 713-716
- Almdal TP, Sorensen TI. Incidence of parenchymal liver diseases in Denmark, 1981 to 1985: analysis of hospitalization registry data. The Danish Association for the Study of the Liver. *Hepatology* 1991; **13**: 650-655
- Villeneuve JP, Fenyves D, Infante-Rivard C. Descriptive epidemiology of primary biliary cirrhosis in the province of Quebec. *Can J Gastroenterol* 1991; **5**: 174-178
- Ilan Y, Shouval D. Primary biliary cirrhosis in Israel. *Isr J Med Sci* 1992; **28**: 683-687
- Rommel T, Rommel H, Uibo R, Salupere V. Primary biliary cirrhosis in Estonia. With special reference to incidence, prevalence, clinical features, and outcome. *Scand J Gastroenterol* 1995; **30**: 367-371
- Anand AC, Elias E, Neuberger JM. End-stage primary biliary cirrhosis in a first generation migrant south Asian population. *Eur J Gastroenterol Hepatol* 1996; **8**: 663-666
- Byron D, Minuk GY. Clinical hepatology: profile of an urban, hospital-based practice. *Hepatology* 1996; **24**: 813-815
- van Dam GM, Gips CH. Primary biliary cirrhosis (PBC) in an European country--a description of death rates in The Netherlands (1979-1992). *Hepatogastroenterology* 1996; **43**: 906-913
- Balakrishnan V, Bhaskaran AS. Primary biliary cirrhosis with pruritus in India. *Indian J Gastroenterol* 1997; **16**: 121-122
- Boberg KM, Aadland E, Jahnsen J, Raknerud N, Stiris M, Bell H. Incidence and prevalence of primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune hepatitis in a Norwegian population. *Scand J Gastroenterol* 1998; **33**: 99-103
- Kingham JG, Parker DR. The association between primary biliary cirrhosis and coeliac disease: a study of relative prevalences. *Gut* 1998; **42**: 120-122
- James OF, Bhopal R, Howel D, Gray J, Burt AD, Metcalf JV. Primary biliary cirrhosis once rare, now common in the United Kingdom? *Hepatology* 1999; **30**: 390-394
- Ray-Chadhuri D, Rigney E, MacComack K. Epidemiology of PBC in Sheffield updated: demographics and relation to water supply. *Gut* 2001; **48**: 42
- Hurlburt KJ, McMahon BJ, Deubner H, Hsu-Trawinski B, Williams JL, Kowdley KV. Prevalence of autoimmune liver disease in Alaska Natives. *Am J Gastroenterol* 2002; **97**: 2402-2407
- Rautiainen H, Salomaa V, Niemela S, Karvonen AL, Nurmi H, Isoniemi H, Farkkila M. Prevalence and incidence of primary biliary cirrhosis are increasing in Finland. *Scand J Gastroenterol* 2007; **42**: 1347-1353
- Pla X, Vergara M, Gil M, Dalmau B, Cistero B, Bella RM, Real J. Incidence, prevalence and clinical course of primary biliary cirrhosis in a Spanish community. *Eur J Gastroenterol Hepatol* 2007; **19**: 859-864
- Invernizzi P, Miozzo M, Battezzati PM, Bianchi I, Grati FR, Simoni G, Selmi C, Watnik M, Gershwin ME, Podda M. Frequency of monosomy X in women with primary biliary cirrhosis. *Lancet* 2004; **363**: 533-535
- Bach N, Schaffner F. Familial primary biliary cirrhosis. *J Hepatol* 1994; **20**: 698-701
- Jones DE, Watt FE, Metcalf JV, Bassendine MF, James OF. Familial primary biliary cirrhosis reassessed: a geographically-based population study. *J Hepatol* 1999; **30**: 402-407

- 35 **Tsuji K**, Watanabe Y, Van De Water J, Nakanishi T, Kajiyama G, Parikh-Patel A, Coppel R, Gershwin ME. Familial primary biliary cirrhosis in Hiroshima. *J Autoimmun* 1999; **13**: 171-178
- 36 **Selmi C**, Mayo MJ, Bach N, Ishibashi H, Invernizzi P, Gish RG, Gordon SC, Wright HI, Zweiban B, Podda M, Gershwin ME. Primary biliary cirrhosis in monozygotic and dizygotic twins: genetics, epigenetics, and environment. *Gastroenterology* 2004; **127**: 485-492
- 37 **Ala A**, Stanca CM, Bu-Ghanim M, Ahmado I, Branch AD, Schiano TD, Odin JA, Bach N. Increased prevalence of primary biliary cirrhosis near Superfund toxic waste sites. *Hepatology* 2006; **43**: 525-531
- 38 **Prince MI**, Chetwynd A, Diggle P, Jarner M, Metcalf JV, James OF. The geographical distribution of primary biliary cirrhosis in a well-defined cohort. *Hepatology* 2001; **34**: 1083-1088
- 39 **Talwalkar JA**, Lazaridis KN. Polluting the pathogenesis of primary biliary cirrhosis. *Hepatology* 2006; **43**: 398-400
- 40 **Gershwin ME**, Selmi C, Worman HJ, Gold EB, Watnik M, Utts J, Lindor KD, Kaplan MM, Vierling JM. Risk factors and comorbidities in primary biliary cirrhosis: a controlled interview-based study of 1032 patients. *Hepatology* 2005; **42**: 1194-1202
- 41 **Howel D**, Fischbacher CM, Bhopal RS, Gray J, Metcalf JV, James OF. An exploratory population-based case-control study of primary biliary cirrhosis. *Hepatology* 2000; **31**: 1055-1060
- 42 **Parikh-Patel A**, Gold EB, Worman H, Krivy KE, Gershwin ME. Risk factors for primary biliary cirrhosis in a cohort of patients from the united states. *Hepatology* 2001; **33**: 16-21
- 43 **Kaplan MM**, Gershwin ME. Primary biliary cirrhosis. *N Engl J Med* 2005; **353**: 1261-1273
- 44 **Gershwin ME**, Mackay IR. Primary biliary cirrhosis: paradigm or paradox for autoimmunity. *Gastroenterology* 1991; **100**: 822-833
- 45 **Invernizzi P**, Crosignani A, Battezzati PM, Covini G, De Valle G, Larghi A, Zuin M, Podda M. Comparison of the clinical features and clinical course of antimitochondrial antibody-positive and -negative primary biliary cirrhosis. *Hepatology* 1997; **25**: 1090-1095
- 46 **Lawson AM**, Setchell KDR. Mass spectrometry of bile acids. In: Setchell KDR, Kritchinsky D, Nair P, eds. The bile acids: Methods and applications. New York: Plenum Press, 1988: 167-267
- 47 **Crosignani A**, Podda M, Battezzati PM, Bertolini E, Zuin M, Watson D, Setchell KD. Changes in bile acid composition in patients with primary biliary cirrhosis induced by ursodeoxycholic acid administration. *Hepatology* 1991; **14**: 1000-1007
- 48 **Desmet V**. Pathology of small duct cholangiopathies. AASLD 1996. Postgraduate Course, 1996: 91-97
- 49 **Scheuer PJ**. Primary biliary cirrhosis: diagnosis, pathology and pathogenesis. *Postgrad Med J* 1983; **59** Suppl 4: 106-115
- 50 **Sherlock S**, Scheuer PJ. The presentation and diagnosis of 100 patients with primary biliary cirrhosis. *N Engl J Med* 1973; **289**: 674-678
- 51 **James O**, Macklon AF, Watson AJ. Primary biliary cirrhosis--a revised clinical spectrum. *Lancet* 1981; **1**: 1278-1281
- 52 **Nyberg A**, Loof L. Primary biliary cirrhosis: clinical features and outcome, with special reference to asymptomatic disease. *Scand J Gastroenterol* 1989; **24**: 57-64
- 53 **Prince M**, Chetwynd A, Newman W, Metcalf JV, James OF. Survival and symptom progression in a geographically based cohort of patients with primary biliary cirrhosis: follow-up for up to 28 years. *Gastroenterology* 2002; **123**: 1044-1051
- 54 **Prince MI**, Chetwynd A, Craig WL, Metcalf JV, James OF. Asymptomatic primary biliary cirrhosis: clinical features, prognosis, and symptom progression in a large population based cohort. *Gut* 2004; **53**: 865-870
- 55 **Milkiewicz P**, Heathcote EJ. Fatigue in chronic cholestasis. *Gut* 2004; **53**: 475-477
- 56 **Goldblatt J**, Taylor PJ, Lipman T, Prince MI, Baragiotta A, Bassendine MF, James OF, Jones DE. The true impact of fatigue in primary biliary cirrhosis: a population study. *Gastroenterology* 2002; **122**: 1235-1241
- 57 **Poupon RE**, Chretien Y, Chazouilleres O, Poupon R, Chwalow J. Quality of life in patients with primary biliary cirrhosis. *Hepatology* 2004; **40**: 489-494
- 58 **Jones DE**, Bhala N, Burt J, Goldblatt J, Prince M, Newton JL. Four year follow up of fatigue in a geographically defined primary biliary cirrhosis patient cohort. *Gut* 2006; **55**: 536-541
- 59 **Newton JL**, Hudson M, Tachtatzis P, Sutcliffe K, Pairman J, Burt JA, Jones DE. Population prevalence and symptom associations of autonomic dysfunction in primary biliary cirrhosis. *Hepatology* 2007; **45**: 1496-1505
- 60 **Selmi C**, Gershwin ME, Lindor KD, Worman HJ, Gold EB, Watnik M, Utts J, Invernizzi P, Kaplan MM, Vierling JM, Bowlus CL, Silveira MG, Bossi I. Quality of life and everyday activities in patients with primary biliary cirrhosis. *Hepatology* 2007; **45**: 1836-1841
- 61 **Newton JL**, Allen J, Kerr S, Jones DE. Reduced heart rate variability and baroreflex sensitivity in primary biliary cirrhosis. *Liver Int* 2006; **26**: 197-202
- 62 **Newton JL**, Davidson A, Kerr S, Bhala N, Pairman J, Burt J, Jones DE. Autonomic dysfunction in primary biliary cirrhosis correlates with fatigue severity. *Eur J Gastroenterol Hepatol* 2007; **19**: 125-132
- 63 **Newton JL**, Gibson GJ, Tomlinson M, Wilton K, Jones D. Fatigue in primary biliary cirrhosis is associated with excessive daytime somnolence. *Hepatology* 2006; **44**: 91-98
- 64 **Forton DM**, Patel N, Prince M, Oatridge A, Hamilton G, Goldblatt J, Allsop JM, Hajnal JV, Thomas HC, Bassendine M, Jones DE, Taylor-Robinson SD. Fatigue and primary biliary cirrhosis: association of globus pallidus magnetisation transfer ratio measurements with fatigue severity and blood manganese levels. *Gut* 2004; **53**: 587-592
- 65 **Bjornsson E**, Simren M, Olsson R, Chapman RW. Fatigue is not a specific symptom in patients with primary biliary cirrhosis. *Eur J Gastroenterol Hepatol* 2005; **17**: 351-357
- 66 **Blackburn P**, Freeston M, Baker CR, Jones DE, Newton JL. The role of psychological factors in the fatigue of primary biliary cirrhosis. *Liver Int* 2007; **27**: 654-661
- 67 **Huet PM**, Deslauriers J, Tran A, Faucher C, Charbonneau J. Impact of fatigue on the quality of life of patients with primary biliary cirrhosis. *Am J Gastroenterol* 2000; **95**: 760-767
- 68 **Talwalkar JA**, Souto E, Jorgensen RA, Lindor KD. Natural history of pruritus in primary biliary cirrhosis. *Clin Gastroenterol Hepatol* 2003; **1**: 297-302
- 69 **Herndon JH Jr**. Pathophysiology of pruritus associated with elevated bile acid levels in serum. *Arch Intern Med* 1972; **130**: 632-637
- 70 **Schoenfield LF**, Sjoval J, Perman E. Bile acids on the skin of patients with pruritic hepatobiliary disease. *Nature* 1967; **213**: 93-94
- 71 **Kirby J**, Heaton KW, Burton JL. Pruritic effect of bile salts. *Br Med J* 1974; **4**: 693-695
- 72 **Varadi DP**. Pruritus induced by crude bile and purified bile acids. Experimental production of pruritus in human skin. *Arch Dermatol* 1974; **109**: 678-681
- 73 **Carey JB**. Lowering of serum bile acid concentrations and relief of pruritus in jaundiced patients fed a bile acid sequestering resin. *J Lab Clin Med* 1960; **56**: 797-798
- 74 **Carey JB**. Relief of pruritus of jaundiced patients with a bile sequestering resin. *JAMA* 1962; **176**: 432-435
- 75 **Datta DV**, Sherlock S. Cholestyramine for long term relief of the pruritus complicating intrahepatic cholestasis. *Gastroenterology* 1966; **50**: 323-332
- 76 **Bartholomew TC**, Summerfield JA, Billing BH, Lawson AM, Setchell KD. Bile acid profiles of human serum and skin interstitial fluid and their relationship to pruritus studied by gas chromatography-mass spectrometry. *Clin Sci (Lond)* 1982; **63**: 65-73

- 77 **Freedman MR**, Holzbach RT, Ferguson DR. Pruritus in cholestasis: no direct causative role for bile acid retention. *Am J Med* 1981; **70**: 1011-1016
- 78 **Ghent CN**, Bloomer JR, Klatskin G. Elevations in skin tissue levels of bile acids in human cholestasis: relation to serum levels and to pruritus. *Gastroenterology* 1977; **73**: 1125-1130
- 79 **Bergasa NV**, Jones EA. The pruritus of cholestasis. *Semin Liver Dis* 1993; **13**: 319-327
- 80 **Bergasa NV**, Jones EA. The pruritus of cholestasis: potential pathogenic and therapeutic implications of opioids. *Gastroenterology* 1995; **108**: 1582-1588
- 81 **Jones EA**, Bergasa NV. The pruritus of cholestasis: from bile acids to opiate agonists. *Hepatology* 1990; **11**: 884-887
- 82 **Swain MG**, Rothman RB, Xu H, Vergalla J, Bergasa NV, Jones EA. Endogenous opioids accumulate in plasma in a rat model of acute cholestasis. *Gastroenterology* 1992; **103**: 630-635
- 83 **Thornton JR**, Losowsky MS. Opioid peptides and primary biliary cirrhosis. *BMJ* 1988; **297**: 1501-1504
- 84 **Justins DM**, Reynolds F. Intraspinal opiates and itching: a new reflex? *Br Med J (Clin Res Ed)* 1982; **284**: 1401
- 85 **Scott PV**, Fischer HB. Spinal opiate analgesia and facial pruritus: a neural theory. *Postgrad Med J* 1982; **58**: 531-535
- 86 **Thomas DA**, Williams GM, Iwata K, Kenshalo DR Jr, Dubner R. Effects of central administration of opioids on facial scratching in monkeys. *Brain Res* 1992; **585**: 315-317
- 87 **Bergasa NV**, Alling DW, Talbot TL, Swain MG, Yurdaydin C, Turner ML, Schmitt JM, Walker EC, Jones EA. Effects of naloxone infusions in patients with the pruritus of cholestasis. A double-blind, randomized, controlled trial. *Ann Intern Med* 1995; **123**: 161-167
- 88 **Bergasa NV**, Schmitt JM, Talbot TL, Alling DW, Swain MG, Turner ML, Jenkins JB, Jones EA. Open-label trial of oral nalmefene therapy for the pruritus of cholestasis. *Hepatology* 1998; **27**: 679-684
- 89 **Wolfhagen FH**, Sternieri E, Hop WC, Vitale G, Bertolotti M, Van Buuren HR. Oral naltrexone treatment for cholestatic pruritus: a double-blind, placebo-controlled study. *Gastroenterology* 1997; **113**: 1264-1269
- 90 **Nijhawan PK**, Therneau TM, Dickson ER, Boynton J, Lindor KD. Incidence of cancer in primary biliary cirrhosis: the Mayo experience. *Hepatology* 1999; **29**: 1396-1398
- 91 **Longo M**, Crosignani A, Battezzati PM, Squarcia Giussani C, Invernizzi P, Zuin M, Podda M. Hyperlipidaemic state and cardiovascular risk in primary biliary cirrhosis. *Gut* 2002; **51**: 265-269
- 92 **Allocca M**, Crosignani A, Gritti A, Ghilardi G, Gobatti D, Caruso D, Zuin M, Podda M, Battezzati PM. Hypercholesterolaemia is not associated with early atherosclerotic lesions in primary biliary cirrhosis. *Gut* 2006; **55**: 1795-1800
- 93 **Poupon RE**, Ouguerram K, Chretien Y, Verneau C, Eschwege E, Magot T, Poupon R. Cholesterol-lowering effect of ursodeoxycholic acid in patients with primary biliary cirrhosis. *Hepatology* 1993; **17**: 577-582
- 94 **Miettinen TA**, Farkkila M, Vuoristo M, Karvonen AL, Leino R, Lehtola J, Friman C, Seppala K, Tuominen J. Serum cholestanol, cholesterol precursors, and plant sterols during placebo-controlled treatment of primary biliary cirrhosis with ursodeoxycholic acid or colchicine. *Hepatology* 1995; **21**: 1261-1268
- 95 **Compston JE**, Thompson RP. Intestinal absorption of 25-hydroxyvitamin D and osteomalacia in primary biliary cirrhosis. *Lancet* 1977; **1**: 721-724
- 96 **Bengoa JM**, Sitrin MD, Meredith S, Kelly SE, Shah N, Baker AL, Rosenberg IH. Intestinal calcium absorption and vitamin D status in chronic cholestatic liver disease. *Hepatology* 1984; **4**: 261-265
- 97 **Heathcote EJ**. Management of primary biliary cirrhosis. The American Association for the Study of Liver Diseases practice guidelines. *Hepatology* 2000; **31**: 1005-1013
- 98 **Talwalkar JA**, Lindor KD. Primary biliary cirrhosis. *Lancet* 2003; **362**: 53-61
- 99 **Eastell R**, Dickson ER, Hodgson SF, Wiesner RH, Porayko MK, Wahner HW, Cedel SL, Riggs BL, Krom RA. Rates of vertebral bone loss before and after liver transplantation in women with primary biliary cirrhosis. *Hepatology* 1991; **14**: 296-300
- 100 **Leslie WD**, Bernstein CN, Leboff MS. AGA technical review on osteoporosis in hepatic disorders. *Gastroenterology* 2003; **125**: 941-966
- 101 **Benetti A**, Crosignani A, Varenna M, Giussani CS, Allocca M, Zuin M, Podda M, Battezzati PM. Primary biliary cirrhosis is not an additional risk factor for bone loss in women receiving regular calcium and vitamin D supplementation: a controlled longitudinal study. *J Clin Gastroenterol* 2008; **42**: 306-311
- 102 **Hodgson SF**, Dickson ER, Wahner HW, Johnson KA, Mann KG, Riggs BL. Bone loss and reduced osteoblast function in primary biliary cirrhosis. *Ann Intern Med* 1985; **103**: 855-860
- 103 **Newton J**, Francis R, Prince M, James O, Bassendine M, Rawlings D, Jones D. Osteoporosis in primary biliary cirrhosis revisited. *Gut* 2001; **49**: 282-287
- 104 **Ormarsdottir S**, Ljunggren O, Mallmin H, Brahm H, Loof L. Low body mass index and use of corticosteroids, but not cholestasis, are risk factors for osteoporosis in patients with chronic liver disease. *J Hepatol* 1999; **31**: 84-90
- 105 **Menon KV**, Angulo P, Weston S, Dickson ER, Lindor KD. Bone disease in primary biliary cirrhosis: independent indicators and rate of progression. *J Hepatol* 2001; **35**: 316-323
- 106 **Van Berkum FN**, Beukers R, Birkenhager JC, Kooij PP, Schalm SW, Pols HA. Bone mass in women with primary biliary cirrhosis: the relation with histological stage and use of glucocorticoids. *Gastroenterology* 1990; **99**: 1134-1139
- 107 **Guanabens N**, Pares A, Ros I, Caballeria L, Pons F, Vidal S, Monegal A, Peris P, Rodes J. Severity of cholestasis and advanced histological stage but not menopausal status are the major risk factors for osteoporosis in primary biliary cirrhosis. *J Hepatol* 2005; **42**: 573-577
- 108 **Lanspa SJ**, Chan AT, Bell JS 3rd, Go VL, Dickson ER, DiMagno EP. Pathogenesis of steatorrhea in primary biliary cirrhosis. *Hepatology* 1985; **5**: 837-842
- 109 **Pares A**, Rodes J. Natural history of primary biliary cirrhosis. *Clin Liver Dis* 2003; **7**: 779-794
- 110 **Allocca M**, Crosignani A, Gritti A, Benetti A, Zuin M, Podda M, Battezzati PM. Inadequate dietary intake but not renal tubular acidosis is associated with bone demineralization in primary biliary cirrhosis. *Aliment Pharmacol Ther* 2007; **25**: 219-227
- 111 **Rigamonti C**, Shand LM, Feudjo M, Bunn CC, Black CM, Denton CP, Burroughs AK. Clinical features and prognosis of primary biliary cirrhosis associated with systemic sclerosis. *Gut* 2006; **55**: 388-394
- 112 **Lee YM**, Kaplan MM. The natural history of PBC: has it changed? *Semin Liver Dis* 2005; **25**: 321-326
- 113 **Roll J**, Boyer JL, Barry D, Klatskin G. The prognostic importance of clinical and histologic features in asymptomatic and symptomatic primary biliary cirrhosis. *N Engl J Med* 1983; **308**: 1-7
- 114 **Mahl TC**, Shockcor W, Boyer JL. Primary biliary cirrhosis: survival of a large cohort of symptomatic and asymptomatic patients followed for 24 years. *J Hepatol* 1994; **20**: 707-713
- 115 **Springer J**, Cauch-Dudek K, O'Rourke K, Wanless IR, Heathcote EJ. Asymptomatic primary biliary cirrhosis: a study of its natural history and prognosis. *Am J Gastroenterol* 1999; **94**: 47-53
- 116 **Locke GR 3rd**, Therneau TM, Ludwig J, Dickson ER, Lindor KD. Time course of histological progression in primary biliary cirrhosis. *Hepatology* 1996; **23**: 52-56
- 117 **Corpechot C**, Carrat F, Bahr A, Chretien Y, Poupon RE, Poupon R. The effect of ursodeoxycholic acid therapy on the natural course of primary biliary cirrhosis. *Gastroenterology* 2005; **128**: 297-303
- 118 **Lindor KD**, Jorgensen RA, Therneau TM, Malinchoc M, Dickson ER. Ursodeoxycholic acid delays the onset of esophageal varices in primary biliary cirrhosis. *Mayo Clin*

- Proc* 1997; **72**: 1137-1140
- 119 **Dickson ER**, Grambsch PM, Fleming TR, Fisher LD, Langworthy A. Prognosis in primary biliary cirrhosis: model for decision making. *Hepatology* 1989; **10**: 1-7
  - 120 **Grambsch PM**, Dickson ER, Kaplan M, LeSage G, Fleming TR, Langworthy AL. Extramural cross-validation of the Mayo primary biliary cirrhosis survival model establishes its generalizability. *Hepatology* 1989; **10**: 846-850
  - 121 **Christensen E**, Neuberger J, Crowe J, Altman DG, Popper H, Portmann B, Doniach D, Ranek L, Tygstrup N, Williams R. Beneficial effect of azathioprine and prediction of prognosis in primary biliary cirrhosis. Final results of an international trial. *Gastroenterology* 1985; **89**: 1084-1091
  - 122 **Rydning A**, Schrupf E, Abdelnoor M, Elgjo K, Jenssen E. Factors of prognostic importance in primary biliary cirrhosis. *Scand J Gastroenterol* 1990; **25**: 119-126
  - 123 **Biagini MR**, Guardascione M, Raskino C, McIntyre N, Surrenti C, Burroughs AK. Poor prognostication for survival in individual PBC patients with Cox models. *J Hepatol* 1990; **11**: S7
  - 124 **Hughes MD**, Raskino CL, Pocock SJ, Biagini MR, Burroughs AK. Prediction of short-term survival with an application in primary biliary cirrhosis. *Stat Med* 1992; **11**: 1731-1745
  - 125 **Murtaugh PA**, Dickson ER, Van Dam GM, Malinchoc M, Grambsch PM, Langworthy AL, Gips CH. Primary biliary cirrhosis: prediction of short-term survival based on repeated patient visits. *Hepatology* 1994; **20**: 126-134
  - 126 **Shapiro JM**, Smith H, Schaffner F. Serum bilirubin: a prognostic factor in primary biliary cirrhosis. *Gut* 1979; **20**: 137-140
  - 127 **Wesierska-Gadek J**, Penner E, Battezzati PM, Selmi C, Zuin M, Hitchman E, Worman HJ, Gershwin ME, Podda M, Invernizzi P. Correlation of initial autoantibody profile and clinical outcome in primary biliary cirrhosis. *Hepatology* 2006; **43**: 1135-1144
  - 128 **Prince MI**, Mitchison HC, Ashley D, Burke DA, Edwards N, Bramble MG, James OF, Jones DE. Oral antioxidant supplementation for fatigue associated with primary biliary cirrhosis: results of a multicentre, randomized, placebo-controlled, cross-over trial. *Aliment Pharmacol Ther* 2003; **17**: 137-143
  - 129 **Talwalkar JA**, Donlinger JJ, Gossard AA, Keach JC, Jorgensen RA, Petz JC, Lindor KD. Fluoxetine for the treatment of fatigue in primary biliary cirrhosis: a randomized, double-blind controlled trial. *Dig Dis Sci* 2006; **51**: 1985-1991
  - 130 **Theal JJ**, Toosi MN, Giralan L, Heslegrave RJ, Huet PM, Burak KW, Swain M, Tomlinson GA, Heathcote EJ. A randomized, controlled crossover trial of ondansetron in patients with primary biliary cirrhosis and fatigue. *Hepatology* 2005; **41**: 1305-1312
  - 131 **Hoensch HP**, Balzer K, Dylewicz P, Kirch W, Goebell H, Ohnhaus EE. Effect of rifampicin treatment on hepatic drug metabolism and serum bile acids in patients with primary biliary cirrhosis. *Eur J Clin Pharmacol* 1985; **28**: 475-477
  - 132 **Ghent CN**, Carruthers SG. Treatment of pruritus in primary biliary cirrhosis with rifampin. Results of a double-blind, crossover, randomized trial. *Gastroenterology* 1988; **94**: 488-493
  - 133 **Podesta A**, Lopez P, Terg R, Villamil F, Flores D, Mastai R, Udaondo CB, Compagn JP. Treatment of pruritus of primary biliary cirrhosis with rifampin. *Dig Dis Sci* 1991; **36**: 216-220
  - 134 **Bachs L**, Pares A, Elena M, Piers C, Rodes J. Comparison of rifampicin with phenobarbitone for treatment of pruritus in biliary cirrhosis. *Lancet* 1989; **1**: 574-576
  - 135 **Ghent CN**. Pruritus of cholestasis is related to effects of bile salts on the liver, not the skin. *Am J Gastroenterol* 1987; **82**: 117-118
  - 136 **Nakashima T**, Sano A, Seto Y, Nakajima T, Shima T, Sakamoto Y, Okuno T, Kashima K, Hasegawa T. Unusual trihydroxy bile acids in the urine of patients treated with chenodeoxycholate, ursodeoxycholate or rifampicin and those with cirrhosis. *Hepatology* 1990; **11**: 255-260
  - 137 **Wietholtz H**, Marshall HU, Sjoval J, Matern S. Stimulation of bile acid 6 alpha-hydroxylation by rifampin. *J Hepatol* 1996; **24**: 713-718
  - 138 **Marshall HU**, Wagner M, Zollner G, Fickert P, Diczfalussy U, Gumhold J, Silbert D, Fuchsichler A, Benthin L, Grundstrom R, Gustafsson U, Sahlin S, Einarsson C, Trauner M. Complementary stimulation of hepatobiliary transport and detoxification systems by rifampicin and ursodeoxycholic acid in humans. *Gastroenterology* 2005; **129**: 476-485
  - 139 **Bachs L**, Pares A, Elena M, Piers C, Rodes J. Effects of long-term rifampicin administration in primary biliary cirrhosis. *Gastroenterology* 1992; **102**: 2077-2080
  - 140 **Prince MI**, Burt AD, Jones DE. Hepatitis and liver dysfunction with rifampicin therapy for pruritus in primary biliary cirrhosis. *Gut* 2002; **50**: 436-439
  - 141 **Raderer M**, Muller C, Scheithauer W. Ondansetron for pruritus due to cholestasis. *N Engl J Med* 1994; **330**: 1540
  - 142 **Schworer H**, Hartmann H, Ramadori G. Relief of cholestatic pruritus by a novel class of drugs: 5-hydroxytryptamine type 3 (5-HT<sub>3</sub>) receptor antagonists: effectiveness of ondansetron. *Pain* 1995; **61**: 33-37
  - 143 **Schworer H**, Ramadori G. Improvement of cholestatic pruritus by ondansetron. *Lancet* 1993; **341**: 1277
  - 144 **Jones EA**, Molenaar HA, Oosting J. Ondansetron and pruritus in chronic liver disease: a controlled study. *Hepato-gastroenterology* 2007; **54**: 1196-1199
  - 145 **Muller C**, Pongratz S, Pidlich J, Penner E, Kaider A, Schemper M, Raderer M, Scheithauer W, Ferenci P. Treatment of pruritus in chronic liver disease with the 5-hydroxytryptamine receptor type 3 antagonist ondansetron: a randomized, placebo-controlled, double-blind cross-over trial. *Eur J Gastroenterol Hepatol* 1998; **10**: 865-870
  - 146 **O'Donohue JW**, Pereira SP, Ashdown AC, Haigh CG, Wilkinson JR, Williams R. A controlled trial of ondansetron in the pruritus of cholestasis. *Aliment Pharmacol Ther* 2005; **21**: 1041-1045
  - 147 **Mayo MJ**, Handem I, Saldana S, Jacobe H, Getachew Y, Rush AJ. Sertraline as a first-line treatment for cholestatic pruritus. *Hepatology* 2007; **45**: 666-674
  - 148 **Neff GW**, O'Brien CB, Reddy KR, Bergasa NV, Regev A, Molina E, Amaro R, Rodriguez MJ, Chase V, Jeffers L, Schiff E. Preliminary observation with dronabinol in patients with intractable pruritus secondary to cholestatic liver disease. *Am J Gastroenterol* 2002; **97**: 2117-2119
  - 149 **Camisasca M**, Crosignani A, Battezzati PM, Albisetti W, Grandinetti G, Pietrogrande L, Biffi A, Zuin M, Podda M. Parenteral calcitonin for metabolic bone disease associated with primary biliary cirrhosis. *Hepatology* 1994; **20**: 633-637
  - 150 **Boone RH**, Cheung AM, Giralan LM, Heathcote EJ. Osteoporosis in primary biliary cirrhosis: a randomized trial of the efficacy and feasibility of estrogen/progestin. *Dig Dis Sci* 2006; **51**: 1103-1112
  - 151 **Crippin JS**, Jorgensen RA, Dickson ER, Lindor KD. Hepatic osteodystrophy in primary biliary cirrhosis: effects of medical treatment. *Am J Gastroenterol* 1994; **89**: 47-50
  - 152 **Ormarsdottir S**, Ljunggren O, Mallmin H, Olsson R, Prytz H, Loof L. Longitudinal bone loss in postmenopausal women with primary biliary cirrhosis and well-preserved liver function. *J Intern Med* 2002; **252**: 537-541
  - 153 **Ormarsdottir S**, Mallmin H, Naessen T, Petren-Mallmin M, Broome U, Hultcrantz R, Loof L. An open, randomized, controlled study of transdermal hormone replacement therapy on the rate of bone loss in primary biliary cirrhosis. *J Intern Med* 2004; **256**: 63-69
  - 154 **Pereira SP**, O'Donohue J, Moniz C, Phillips MG, Abrahama H, Buxton-Thomas M, Williams R. Transdermal hormone replacement therapy improves vertebral bone density in primary biliary cirrhosis: results of a 1-year controlled trial. *Aliment Pharmacol Ther* 2004; **19**: 563-570
  - 155 **Guanabens N**, Pares A, Monegal A, Peris P, Pons F, Alvarez



- L, de Osaba MJ, Roca M, Torra M, Rodes J. Etidronate versus fluoride for treatment of osteopenia in primary biliary cirrhosis: preliminary results after 2 years. *Gastroenterology* 1997; **113**: 219-224
- 156 **Wolfhagen FH**, van Buuren HR, den Ouden JW, Hop WC, van Leeuwen JP, Schalm SW, Pols HA. Cyclical etidronate in the prevention of bone loss in corticosteroid-treated primary biliary cirrhosis. A prospective, controlled pilot study. *J Hepatol* 1997; **26**: 325-330
  - 157 **Lindor KD**, Jorgensen RA, Tiegs RD, Khosla S, Dickson ER. Etidronate for osteoporosis in primary biliary cirrhosis: a randomized trial. *J Hepatol* 2000; **33**: 878-882
  - 158 **Guanabens N**, Pares A, Ros I, Alvarez L, Pons F, Caballeria L, Monegal A, Martinez de Osaba MJ, Roca M, Peris P, Rodes J. Alendronate is more effective than etidronate for increasing bone mass in osteopenic patients with primary biliary cirrhosis. *Am J Gastroenterol* 2003; **98**: 2268-2274
  - 159 **Zein CO**, Jorgensen RA, Clarke B, Wenger DE, Keach JC, Angulo P, Lindor KD. Alendronate improves bone mineral density in primary biliary cirrhosis: a randomized placebo-controlled trial. *Hepatology* 2005; **42**: 762-771
  - 160 **Floreani A**, Chiamonte M, Giannini S, Malvasi L, Lodetti MG, Castrignano R, Giacomini A, D'Angelo A, Naccarato R. Longitudinal study on osteodystrophy in primary biliary cirrhosis (PBC) and a pilot study on calcitonin treatment. *J Hepatol* 1991; **12**: 217-223
  - 161 **Lindor KD**, Janes CH, Crippin JS, Jorgensen RA, Dickson ER. Bone disease in primary biliary cirrhosis: does ursodeoxycholic acid make a difference? *Hepatology* 1995; **21**: 389-392
  - 162 **Longo M**, Crosignani A, Podda M. Hyperlipidemia in Chronic Cholestatic Liver Disease. *Curr Treat Options Gastroenterol* 2001; **4**: 111-114
  - 163 **Del Puppo M**, Galli Kienle M, Crosignani A, Petroni ML, Amati B, Zuin M, Podda M. Cholesterol metabolism in primary biliary cirrhosis during simvastatin and UDCA administration. *J Lipid Res* 2001; **42**: 437-441
  - 164 **Ritzel U**, Leonhardt U, Nuther M, Schufer G, Armstrong VW, Ramadori G. Simvastatin in primary biliary cirrhosis: effects on serum lipids and distinct disease markers. *J Hepatol* 2002; **36**: 454-458
  - 165 **Stojakovic T**, Putz-Bankuti C, Fauler G, Scharnagl H, Wagner M, Stadlbauer V, Gurakuqi G, Stauber RE, Murz W, Trauner M. Atorvastatin in patients with primary biliary cirrhosis and incomplete biochemical response to ursodeoxycholic acid. *Hepatology* 2007; **46**: 776-784
  - 166 **Gong Y**, Klingenberg SL, Glud C. Systematic review and meta-analysis: D-Penicillamine vs. placebo/no intervention in patients with primary biliary cirrhosis--Cochrane Hepato-Biliary Group. *Aliment Pharmacol Ther* 2006; **24**: 1535-1544
  - 167 **Poupon RE**, Lindor KD, Cauch-Dudek K, Dickson ER, Poupon R, Heathcote EJ. Combined analysis of randomized controlled trials of ursodeoxycholic acid in primary biliary cirrhosis. *Gastroenterology* 1997; **113**: 884-890
  - 168 **Mitchison HC**, Bassendine MF, Malcolm AJ, Watson AJ, Record CO, James OF. A pilot, double-blind, controlled 1-year trial of prednisolone treatment in primary biliary cirrhosis: hepatic improvement but greater bone loss. *Hepatology* 1989; **10**: 420-429
  - 169 **Mitchison HC**, Palmer JM, Bassendine MF, Watson AJ, Record CO, James OF. A controlled trial of prednisolone treatment in primary biliary cirrhosis. Three-year results. *J Hepatol* 1992; **15**: 336-344
  - 170 **Chazouilleres O**, Wendum D, Serfaty L, Montembault S, Rosmorduc O, Poupon R. Primary biliary cirrhosis-autoimmune hepatitis overlap syndrome: clinical features and response to therapy. *Hepatology* 1998; **28**: 296-301
  - 171 **Heathcote J**, Ross A, Sherlock S. A prospective controlled trial of azathioprine in primary biliary cirrhosis. *Gastroenterology* 1976; **70**: 656-660
  - 172 **Roll J**. A new treatment for primary biliary cirrhosis? *Gastroenterology* 1985; **89**: 1195-1199
  - 173 **Hoofnagle JH**, Davis GL, Schafer DF, Peters M, Avigan MI, Pappas SC, Hanson RG, Minuk GY, Dusheiko GM, Campbell G. Randomized trial of chlorambucil for primary biliary cirrhosis. *Gastroenterology* 1986; **91**: 1327-1334
  - 174 **Kaplan MM**, Knox TA. Treatment of primary biliary cirrhosis with low-dose weekly methotrexate. *Gastroenterology* 1991; **101**: 1332-1338
  - 175 **Kaplan MM**, DeLellis RA, Wolfe HJ. Sustained biochemical and histologic remission of primary biliary cirrhosis in response to medical treatment. *Ann Intern Med* 1997; **126**: 682-688
  - 176 **Kaplan MM**, Schmid C, Provenzale D, Sharma A, Dickstein G, McKusick A. A prospective trial of colchicine and methotrexate in the treatment of primary biliary cirrhosis. *Gastroenterology* 1999; **117**: 1173-1180
  - 177 **Sharma A**, Provenzale D, McKusick A, Kaplan MM. Interstitial pneumonitis after low-dose methotrexate therapy in primary biliary cirrhosis. *Gastroenterology* 1994; **107**: 266-270
  - 178 **Combes B**, Emerson SS, Flye NL, Munoz SJ, Luketic VA, Mayo MJ, McCashland TM, Zetterman RK, Peters MG, Di Bisceglie AM, Benner KG, Kowdley KV, Carithers RL Jr, Rosoff L Jr, Garcia-Tsao G, Boyer JL, Boyer TD, Martinez EJ, Bass NM, Lake JR, Barnes DS, Bonacini M, Lindsay KL, Mills AS, Markin RS, Rubin R, West AB, Wheeler DE, Contos MJ, Hofmann AF. Methotrexate (MTX) plus ursodeoxycholic acid (UDCA) in the treatment of primary biliary cirrhosis. *Hepatology* 2005; **42**: 1184-1193
  - 179 **Gonzalez-Koch A**, Brahm J, Antezana C, Smok G, Cumsille MA. The combination of ursodeoxycholic acid and methotrexate for primary biliary cirrhosis is not better than ursodeoxycholic acid alone. *J Hepatol* 1997; **27**: 143-149
  - 180 **Hendrickse MT**, Rigney E, Giaffer MH, Soomro I, Triger DR, Underwood JC, Gleeson D. Low-dose methotrexate is ineffective in primary biliary cirrhosis: long-term results of a placebo-controlled trial. *Gastroenterology* 1999; **117**: 400-407
  - 181 **Lombard M**, Portmann B, Neuberger J, Williams R, Tygstrup N, Ranek L, Ring-Larsen H, Rodes J, Navasa M, Trepo C. Cyclosporin A treatment in primary biliary cirrhosis: results of a long-term placebo controlled trial. *Gastroenterology* 1993; **104**: 519-526
  - 182 **Bodenheimer H Jr**, Schaffner F, Pezzullo J. Evaluation of colchicine therapy in primary biliary cirrhosis. *Gastroenterology* 1988; **95**: 124-129
  - 183 **Kaplan MM**, Alling DW, Zimmerman HJ, Wolfe HJ, Sepersky RA, Hirsch GS, Elta GH, Glick KA, Eagen KA. A prospective trial of colchicine for primary biliary cirrhosis. *N Engl J Med* 1986; **315**: 1448-1454
  - 184 **Warnes TW**, Smith A, Lee FI, Haboubi NY, Johnson PJ, Hunt L. A controlled trial of colchicine in primary biliary cirrhosis. Trial design and preliminary report. *J Hepatol* 1987; **5**: 1-7
  - 185 **Zifroni A**, Schaffner F. Long-term follow-up of patients with primary biliary cirrhosis on colchicine therapy. *Hepatology* 1991; **14**: 990-993
  - 186 **Battezzati PM**, Zuin M, Crosignani A, Allocca M, Invernizzi P, Selmi C, Villa E, Podda M. Ten-year combination treatment with colchicine and ursodeoxycholic acid for primary biliary cirrhosis: a double-blind, placebo-controlled trial on symptomatic patients. *Aliment Pharmacol Ther* 2001; **15**: 1427-1434
  - 187 **Poupon RE**, Huet PM, Poupon R, Bonnand AM, Nhieu JT, Zafrani ES. A randomized trial comparing colchicine and ursodeoxycholic acid combination to ursodeoxycholic acid in primary biliary cirrhosis. UDCA-PBC Study Group. *Hepatology* 1996; **24**: 1098-1103
  - 188 **Attili AF**, Angelico M, Cantafora A, Alvaro D, Capocaccia L. Bile acid-induced liver toxicity: relation to the hydrophobic-hydrophilic balance of bile acids. *Med Hypotheses* 1986; **19**: 57-69
  - 189 **Hofmann AF**, Popper H. Ursodeoxycholic acid for primary biliary cirrhosis. *Lancet* 1987; **2**: 398-399
  - 190 **Paumgartner G**, Beuers U. Ursodeoxycholic acid in cholestatic

- liver disease: mechanisms of action and therapeutic use revisited. *Hepatology* 2002; **36**: 525-531
- 191 **Poupon RE**, Balkau B, Eschwege E, Poupon R. A multicenter, controlled trial of ursodiol for the treatment of primary biliary cirrhosis. UDCA-PBC Study Group. *N Engl J Med* 1991; **324**: 1548-1554
  - 192 **Heathcote EJ**, Cauch-Dudek K, Walker V, Bailey RJ, Blendis LM, Ghent CN, Michieletti P, Minuk GY, Pappas SC, Scully LJ. The Canadian Multicenter Double-blind Randomized Controlled Trial of ursodeoxycholic acid in primary biliary cirrhosis. *Hepatology* 1994; **19**: 1149-1156
  - 193 **Lindor KD**, Dickson ER, Baldus WP, Jorgensen RA, Ludwig J, Murtaugh PA, Harrison JM, Wiesner RH, Anderson ML, Lange SM. Ursodeoxycholic acid in the treatment of primary biliary cirrhosis. *Gastroenterology* 1994; **106**: 1284-1290
  - 194 **Combes B**, Carithers RL Jr, Maddrey WC, Lin D, McDonald MF, Wheeler DE, Eigenbrodt EH, Munoz SJ, Rubin R, Garcia-Tsao G. A randomized, double-blind, placebo-controlled trial of ursodeoxycholic acid in primary biliary cirrhosis. *Hepatology* 1995; **22**: 759-766
  - 195 **Eriksson LS**, Olsson R, Glauman H, Prytz H, Befrits R, Ryden BO, Einarsson K, Lindgren S, Wallerstedt S, Weden M. Ursodeoxycholic acid treatment in patients with primary biliary cirrhosis. A Swedish multicentre, double-blind, randomized controlled study. *Scand J Gastroenterol* 1997; **32**: 179-186
  - 196 **Pares A**, Caballeria L, Rodes J, Bruguera M, Rodrigo L, Garcia-Plaza A, Berenguer J, Rodriguez-Martinez D, Mercader J, Velicia R. Long-term effects of ursodeoxycholic acid in primary biliary cirrhosis: results of a double-blind controlled multicentric trial. UDCA-Cooperative Group from the Spanish Association for the Study of the Liver. *J Hepatol* 2000; **32**: 561-566
  - 197 **Papatheodoridis GV**, Hadziyannis ES, Deutsch M, Hadziyannis SJ. Ursodeoxycholic acid for primary biliary cirrhosis: final results of a 12-year, prospective, randomized, controlled trial. *Am J Gastroenterol* 2002; **97**: 2063-2070
  - 198 **Poupon RE**, Poupon R, Balkau B. Ursodiol for the long-term treatment of primary biliary cirrhosis. The UDCA-PBC Study Group. *N Engl J Med* 1994; **330**: 1342-1347
  - 199 **Lindor KD**, Therneau TM, Jorgensen RA, Malinchoc M, Dickson ER. Effects of ursodeoxycholic acid on survival in patients with primary biliary cirrhosis. *Gastroenterology* 1996; **110**: 1515-1518
  - 200 **Gluud C**, Christensen E. Ursodeoxycholic acid for primary biliary cirrhosis. *Cochrane Database Syst Rev* 2002; CD000551
  - 201 **Goulis J**, Leandro G, Burroughs AK. Randomised controlled trials of ursodeoxycholic-acid therapy for primary biliary cirrhosis: a meta-analysis. *Lancet* 1999; **354**: 1053-1060
  - 202 **Pares A**, Caballeria L, Rodes J. Excellent long-term survival in patients with primary biliary cirrhosis and biochemical response to ursodeoxycholic Acid. *Gastroenterology* 2006; **130**: 715-720
  - 203 **Leuschner M**, Maier KP, Schlichting J, Strahl S, Herrmann G, Dahm HH, Ackermann H, Happ J, Leuschner U. Oral budesonide and ursodeoxycholic acid for treatment of primary biliary cirrhosis: results of a prospective double-blind trial. *Gastroenterology* 1999; **117**: 918-925
  - 204 **Angulo P**, Jorgensen RA, Keach JC, Dickson ER, Smith C, Lindor KD. Oral budesonide in the treatment of patients with primary biliary cirrhosis with a suboptimal response to ursodeoxycholic acid. *Hepatology* 2000; **31**: 318-323
  - 205 **Rautiainen H**, Karkkainen P, Karvonen AL, Nurmi H, Pikkarainen P, Nuutinen H, Farkkila M. Budesonide combined with UDCA to improve liver histology in primary biliary cirrhosis: a three-year randomized trial. *Hepatology* 2005; **41**: 747-752
  - 206 **MacQuillan GC**, Neuberger J. Liver transplantation for primary biliary cirrhosis. *Clin Liver Dis* 2003; **7**: 941-956, ix
  - 207 **Neuberger J**. Liver transplantation for primary biliary cirrhosis: indications and risk of recurrence. *J Hepatol* 2003; **39**: 142-148

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## TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, Series Editors

# Etiopathogenesis of primary biliary cirrhosis

Ana Lleo, Pietro Invernizzi, Ian R Mackay, Harry Prince, Ren-Qian Zhong, M Eric Gershwin

Ana Lleo, Pietro Invernizzi, Division of Internal Medicine and Liver Unit, San Paolo Hospital School of Medicine, University of Milan, Milan 20142, Italy

Ana Lleo, Pietro Invernizzi, M Eric Gershwin, Division of Rheumatology, Allergy, and Clinical Immunology, University of California at Davis, Davis, CA 95616, United States

Ian R Mackay, Department of Biochemistry & Molecular Biology, Monash University, Clayton Victoria 3800, Australia

Harry Prince, Focus Diagnostics, 5785 Corporate Avenue, Cypress, CA 90630, United States

Ren-Qian Zhong, Laboratory Diagnostics of Changzheng Hospital, Second Military Medical University and Clinical Immunology Center of PLA, Shanghai 200003, China

Correspondence to: M Eric Gershwin, MD, Division of Rheumatology, Allergy and Clinical Immunology, University of California at Davis School of Medicine, Genome and Biomedical Sciences Facility, 451 E Health Sciences Drive, suite 6510, Davis, CA 95616, United States. [megershwin@ucdavis.edu](mailto:megershwin@ucdavis.edu)  
Telephone: +1-530-7522884 Fax: +1-530-7524669

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proclivity to express the antigen PDC-E2 in the course of apoptosis, undergoes a multilineage immune attack comprised of CD4<sup>+</sup> and CD8<sup>+</sup> T cells and antibody. In this article, we critically review the available evidence on etiopathogenesis of PBC and present interpretations of complex data, new developments and theories, and nominate directions for future research.

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**Key words:** Autoantibodies; Autoreactive T cells; 2-oxoacid dehydrogenase; Biliary epithelial cells; Primary biliary cirrhosis

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## Abstract

Primary biliary cirrhosis (PBC) is an autoimmune disease of the liver characterized by progressive bile duct destruction eventually leading to cirrhosis and liver failure. The serological hallmark of the disease is the presence of circulating antimitochondrial antibodies (AMA). These reflect the presence of autoreactive T and B cells to the culprit antigens, the E2 subunits of mitochondrial 2-oxo-acid dehydrogenase enzymes, chiefly pyruvate dehydrogenase (PDC-E2). The disease results from a combination of genetic and environmental risk factors. Genetic predisposition is indicated by the higher familial incidence of the disease particularly among siblings and the high concordance rate among monozygotic twins. Environmental triggering events appear crucial to disrupt a pre-existing unstable immune tolerance of genetic origin allowing, after a long latency, the emergence of clinical disease. Initiating mimotopes of the vulnerable epitope of the PDC-E2 autoantigen can be derived from microbes that utilize the PDC enzyme or, alternatively, environmental xenobiotics/chemical compounds that modify the structure of native proteins to make them immunogenic. A further alternative as a source of antigen is PDC-E2 derived from apoptotic cells. In the effector phase the biliary ductular cell, by reason of its

## INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease of autoimmune origin characterized by a striking female predominance, high titer serum anti-mitochondrial autoantibodies (AMA), disease-specific antinuclear autoantibodies (ANAs), and an autoimmune-mediated destruction of the small and medium size intrahepatic bile ducts<sup>[1]</sup>. PBC is a peculiar, yet representative, organ-specific autoimmune disease. The presence of serum AMA and autoreactive T and B cells, in conjunction with the co-occurrence of other autoimmune diseases, all point to an autoimmune pathogenesis for PBC. Although most patients with PBC have AMA against the E2 subunit of the mitochondrial pyruvate dehydrogenase complex (PDC), there is no direct correlation between the titer of AMAs and disease severity. However, certain disease-specific antinuclear antibodies (ANAs) are present in about one third of patients and these carry a risk for more severe and progressive disease<sup>[2]</sup>.

A multifactorial genetic background is suggested by a higher incidence of the disease among first-degree relatives<sup>[3]</sup>, by the high concordance rate among monozygotic twins<sup>[4]</sup>, and by an apparent role for X chromosome defects in PBC, based on the observation that women with PBC have preferential loss of one X chromosome

in peripheral white blood cells<sup>[5,6]</sup>. A vital question in the pathogenesis of PBC is why biliary epithelial cells (BEC) in particular are the primary target of pathology despite the ubiquitous presence of the PDC autoantigen in all tissue cells. Recent studies suggest that enhanced apoptosis in BEC is a critical step in ductular destruction in PBC<sup>[7,8]</sup>, and some clues exist on mechanisms by which apoptosis in BECs cause the tissue-specific autoimmune reactivity characteristic of PBC.

## GENETICS IN PBC

It is currently accepted that PBC pathogenesis is multifactorial, with genetic and environmental factors interplaying to determine disease onset and progression. Although the etiology of PBC remains enigmatic, there are several items of data indicating that genetic predisposition contributes strongly to the overall pathogenesis of PBC. The lines of evidence are these: (1) Data from monozygotic twins indicate that the concordance rate of PBC in monozygotic twins is 63%<sup>[4]</sup>, among the highest reported for autoimmunity; (2) Approximately 6% of patients with PBC have a first-degree relative that also suffers from PBC<sup>[3]</sup>; (3) There is a high female:male disease incidence ratio (8:1), with suggestions of a significant role for X chromosome defects in PBC, based on the observation that women with PBC have a significantly enhanced monosomy X frequency in peripheral white blood cells compared to age-matched healthy women<sup>[5]</sup> and that the X chromosome loss is preferential<sup>[6]</sup>. Interestingly, similar genetic defects were also found in women with systemic sclerosis and autoimmune thyroid disease<sup>[9]</sup>, but not with systemic lupus erythematosus<sup>[10]</sup>. Future studies should assess whether haploinsufficiency for specific X-linked genes may lead to loss of tolerance; (4) PBC is exceptional among autoimmune diseases in having controversially variable associations with alleles of the major histocompatibility complex (MHC, HLA); only a weak and regional association with HLA *DRB1\*08* has been widely confirmed<sup>[11]</sup>, although there is growing evidence on a protective association with HLA *DRB1\*11* and *\*13*<sup>[12,13]</sup>.

Several association studies have attempted to identify gene loci associated with PBC but no family study of genetic linkage has been performed. Associations are often not applicable to all populations but available evidence suggests that a “multi-hit” genetic model might apply to PBC, with different genetic variants conferring initial susceptibility, and others influencing subsequent disease progression. Genetic influences operative in PBC may reflect mutations transmitted through germline genes, or conceivably, somatic mutations in hemopoietic precursor cells<sup>[14]</sup>.

In summary, a susceptible genetic background is considered to be necessary, but is not sufficient to explain either PBC onset or the strong female predominance<sup>[15]</sup>. Thus several environmental factors have been invoked as additional elements in tolerance breakdown.

## ENVIRONMENTAL FACTORS

Bacterial infection in various settings has been repeatedly invoked in the etiopathogenesis of PBC. This etiology is usually linked to the concept of molecular (epitope) mimicry. The cross-reactivity of AMA with prokaryotic antigens has been reported for a number of microbes. This cross-reactivity is not particularly surprising given the conserved sequence of PDC-E2 across all species, from eubacteria to mammals.

We provided experimental evidence suggesting that *Novosphingobium aromaticivorans*, a ubiquitous xenobiotic-metabolizing Gram-negative bacterium, is the best microbial candidate yet for the induction of PBC<sup>[16,17]</sup>. Briefly, we can extrapolate theories on microbial molecular mimicry in PBC as follows. The microbial motif CpG enhances IgM production in peripheral blood mononuclear cell cultures, with CD27+ memory B cells in PBC patients being responsible for this IgM production through Toll-like receptor (TLR) 9 signaling. Also, CpG can stimulate AMA production and expression of TLR9, CD86, and one of the potassium channels, KCa3.1, in B cells of PBC patients. Moreover upregulated expression of TLR9 and CD86, and AMA secretion induced by CpG, can be suppressed by a specific blocker of the KCa3.1 channel, namely TRAM. These data indicate that B-cell immunity of PBC patients depends on an enhanced innate immune response and imply that TRAM-34 can influence B-cell autoimmunity in PBC<sup>[18]</sup>.

Another source of antigenic mimicry is xenobiotics. These are foreign compounds that may either alter or complex to defined self or non-self proteins, inducing a change in the molecular structure of the native protein sufficient to induce an immune response<sup>[19]</sup>. Such immune responses may then result in the cross-recognition of the self molecule, which could in turn perpetuate the immune response, thus leading to chronic autoimmunity. Interestingly, most xenobiotics are metabolized in the liver, thereby increasing the potential for liver-specific alteration of proteins. Recent data demonstrate that certain chemical/xenobiotic compounds can induce AMA and are in fact recognized by PBC sera with higher affinity compared to the analogous self protein and that such compounds are found in products in common use as food flavorings and cosmetics<sup>[20-23]</sup>. This implicit involvement of cosmetics could contribute to the female predisposition to PBC.

## ROLE OF BEC

PBC is characterized by destruction of the small and medium size intrahepatic bile ducts, lined by BECs (cholangiocytes). BECs express cell surface adhesion molecules which permit adhesion and recognition of lymphocytes. Moreover, several studies have demonstrated that BECs of both healthy and diseased liver have the capacity to increase the expression of adhesion molecules, ICAM-1 and others, MHC class I and II, TNF-alpha, interferon (IFN) -gamma



**Table 1 Immunopathological characteristics of biliary epithelium in PBC<sup>[90]</sup>**

	Normal	PBC
Expression level of PDC-E2	+	+++
Adhesion molecules		
- ICAM-1	+	++
- VCAM-1	-/+	+
- LFA-1	-/+	+
- E-selectin		++
Biliary intra-epithelial lymphocytes	Large bile ducts, few CD4 <sup>+</sup>	Small bile ducts, increased CD4 <sup>+</sup> CD28 <sup>+</sup>
Apoptosis-related molecules		
- Fas (CD95)	-	+
- granzyme B	-	-/+
- perforin	-	-/+
- bcl-2	++	-
BEC phagocytosis of apoptotic BECs	-	++
Cytokines		
- INF- $\gamma$	-	++
- IL-2	-	++
- IL-6	-	++
- IL-6 receptor	-	-/+
- TNF- $\alpha$	-/+	++
- TNF receptor	-/+	++

and IL-1<sup>[24-26]</sup> upon stimulation with proinflammatory cytokines<sup>[27]</sup>. Up-regulation of VCAM-1 and LFA-1 can also be identified<sup>[28]</sup>. Adhesion molecules expressed on the BEC surface, and the up-regulation by proinflammatory cytokines, which are abundant in the course of inflammatory reactions, allow BECs to modulate the intensity and localization of inflammatory reactions. The other immune feature attributed to the BECs is a capacity to act as APCs. Several studies demonstrate that BECs express HLA class II<sup>[27,29]</sup>, and such expression is increased after injury and after stimulation with IFN- $\gamma$  and IL-1. BECs also express accessory molecules responsible for the second (co-stimulatory) signal to T cells, CD80, 86 (B7-1, B7-2)<sup>[30]</sup>. These interactions with T cells might also be responsible for bile duct loss, one of the fundamental characteristics of progression of disease.

Data obtained in recent years point towards apoptosis as a leading mechanism for ductopenia. Years ago, Harada and colleagues demonstrated susceptibility to apoptosis via the perforin/granzyme B pathway, and this was enhanced by interaction of CD95 (Fas) with CD178 (FasL) in BECs of patients with PBC<sup>[31]</sup>. The hypothesis was further confirmed when apoptotic BECs were shown to express CD40, and Fas and FasL, with transcriptional up regulation of the latter molecules after stimulation with CD154 (CD40L), culminating in apoptosis<sup>[32]</sup>. Odin and colleagues discovered that glutathiolation of the lysine-lipoic acid moiety of PDC-E2 was dramatically reduced by serum AMA<sup>[33]</sup>. Recently it has been demonstrated that apoptotic cells are phagocytosed by BECs and consequently could be an endogenous source of autoantigens from BECs<sup>[34-36]</sup>. Importantly, these findings support the concept that

tissue specific damage in PBC is due to cell type-specific differences in apoptosis, and phagocytosis of apoptotic cells.

Antigenicity of BEC self-molecules, or highly homologous epitopes, could also be related to their role in mucosal immunity. Like other epithelial cells, BECs actively transfer IgAs, and in PBC these IgAs have specificity for PDC-E2. These specific IgA-type AMA can be detected in almost all body fluids of patients with PBC, including saliva, urine and bile<sup>[37,38]</sup>. Further, Fukushima and colleagues<sup>[39]</sup> detected deposits representing co-localization of such antibodies with PDC-E2 (or a highly homologous molecule) at the apical surface and in the cytoplasm of BECs, and also detected their presence in liver allografts in patients with recurrent PBC after receiving a liver transplant. To assess the direct pathogenicity of the IgA antibody class, Matsumura and colleagues exposed canine kidney cells transfected with the human polymeric Ig receptor to highly purified AMA-IgAs, thereby inducing caspase up-regulation, and thus providing evidence for direct toxic effects<sup>[40]</sup>. The immunogenic characteristics of BECs in PBC are summarized in Table 1. Finally, the still unknown role of autophagy in autoimmunity could in the future provide interesting data for the pathogenesis of PBC<sup>[41]</sup>.

## B CELLS AND AUTOANTIBODIES

As mentioned, the presence of serum AMA and autoreactive B cells strongly endorses the concept of an autoimmune pathogenesis of PBC<sup>[42-44]</sup>.

AMA is highly specific for PBC and can be detected in nearly 100% of patients, when sensitive diagnostic methodologies based on recombinant antigens are used<sup>[45]</sup>. They are directed against members of the 2-oxoacid dehydrogenase complexes (2-OADC) existing in the inner membrane of mitochondria. Among them, the major autoantigen is the E2 subunit of the pyruvate dehydrogenase complex (PDC-E2). The epitopes for this antibody to the E-2 subunit localize to three domains of PDC-E2 component: (1) the inner (and outer) lipoic acid (lipoyl) domains; (2) the E3 binding domain; and (3) the catalytic and E2-binding domain<sup>[46]</sup>. Reactivity at lower frequency is also found against other 2-oxoacid dehydrogenase complexes (2-OADC), the 2-oxo glutarate dehydrogenase (OGDC-E2) and the branched-chain 2-oxo acid dehydrogenase (BCOADC-E2), involved respectively in the citric acid cycle and in amino acid catabolism. The 2-OADC autoantigens in PBC are summarized in Table 2.

Although the E-2 subunits of the three E-2 subunits are structurally similar, immunochemical studies have shown that the reactivities are independent, and do not depend on cross-reactivities, at least at the antibody level<sup>[47]</sup>. Antibodies to PDC-E2 and to the other 2-OADC enzymes are capable of inhibiting PDC-E2 enzyme activity *in vitro*, but this has not been shown to occur *in vivo*. Targeting of enzymes is a common feature of autoantibodies detected in patients with autoimmune

**Table 2 Mitochondrial and nuclear autoantigens in PBC**

Autoantigens		
Mitochondrial antigens	E2 subunits of 2-OADC	PDC-E2
		OGDC-E2
		BCOADC-E2
		E3BP
Nuclear antigens	Pyruvate dehydrogenase complex	PDC E1 $\alpha$
	Nuclear pore complex	gp210
		nucleoporin 62
	Multiple nuclear dots	Sp100
	Anticentromere	PML

2-OADC: 2-oxo-acid dehydrogenase complex; PDC: Pyruvate dehydrogenase complex; OGDC: Oxoglutarate dehydrogenase complex; BCOADC: Branched chain 2-oxo-acid dehydrogenase complex; E3BP: Dihyrolipoamide dehydrogenase (E3)-binding protein.

diseases, as is the inhibition of their activity by these autoantibodies. A pathogenic role for AMA is uncertain, since no clinical correlations with levels of AMA can be found, and in certain experimental animal models there is occurrence of serum AMA but no overt PBC-like liver lesions<sup>[14]</sup>. The role of the lipoic acid co-factor attached to lysine<sub>173</sub> (K<sub>173</sub>) in the composition of the epitope recognized by AMA is unclear. Both lipoylated and non-lipoylated PBC-E2 react with AMA and the question is to what degree does lipoic acid serve to enhance antigenicity.

In addition to AMA, PBC sera can present other disease-specific autoantibodies, particularly anti-nuclear (ANA) specificities<sup>[2]</sup>. PBC-specific ANA reactants include nuclear pore glycoproteins of the inner nuclear membrane, gp210<sup>[48]</sup> and p62<sup>[49]</sup>, with a detection rate up to about 30% and with an apparently higher prevalence among AMA-negative PBC. This subtype of PBC-specific ANA has been shown to correlate with disease severity and progression<sup>[50,51]</sup>. Other PBC-specific nucleoprotein reactants include the Sp100-promyelocytic leukemia (PML) autoantigen antigen that gives the characteristic fine nuclear dot pattern by immunofluorescence<sup>[52]</sup>; both appear specific for PBC, but the prevalence differs, being from about 20% to 30%. Finally anti-centromere antibodies occur in PBC (~10% of cases) often in association with a limited scleroderma syndrome. Recently, it has been demonstrated that anti-centromere antibodies were a significant predictive factor in PBC for the development of portal hypertension<sup>[51,53]</sup>. Table 2 specifies the nuclear autoantigens in PBC.

There seems no way of incorporating the co-occurrence of AMA and ANA into a unifying theory of pathogenesis of PBC, other than specifying both reactivities as reflecting a systemic failure of maintenance of immune tolerance.

## T CELLS

Autoreactive CD4<sup>+</sup> and CD8<sup>+</sup> T cells are demonstrably involved in the pathogenesis of PBC and, histologically, infiltration of presumably autoreactive T cells in the liver

and periductular spaces is one of the major features of the disease<sup>[54,55]</sup>. Both CD4<sup>+</sup> and CD8<sup>+</sup> T lymphocytes can be purified from biopsy samples of PBC patients and both subsets recognize epitopes of PDC-E2<sup>[56]</sup>; moreover, using recombinant fragments of PDC-E2 it has been demonstrated that there is a sequence overlap in the PDC-E2 specific T and B cell epitopes<sup>[57]</sup>. The minimal T-cell epitope for CD4<sup>+</sup> T cells was identified as amino acid residues 163 to 176 (GDLLAEIETDKATI), within the inner lipoyl domain of PDC-E2<sup>[57]</sup>. Phenotypically, the PDC-E2<sub>163-176</sub> T-cell clones were positive for CD4, CD45RO, and T cell receptor (TCR)  $\alpha\beta$ . The MHC Class II human leukocyte antigen (HLA) -restriction molecules for this epitope have been identified as HLA-DR53 (B4\*0101)<sup>[58]</sup>. In addition, PDC-E2<sub>163-176</sub>-specific CD4<sup>+</sup> T-cell clones recognize other functionally related mitochondrial autoantigens, including OGDC-E2 and BCOADC-E2, and also E3BP<sup>[59]</sup>. More specifically, these T-cell clones were cross-reactive with the amino acid residues 100 to 113 of OGDC-E2, residues 90 to 103 of BCOADC-E2, and residues 34 to 47 of E3BP, all located in the respective E2-lipoyl domain of these enzymes; thus the suggestion is that OGDC-E2<sub>100-113</sub>, BCOADC-E2<sub>90-103</sub>, and E3BP<sub>34-47</sub> all represent CD4<sup>+</sup> T-cell epitopes.

CD8<sup>+</sup> T cells (CTLs) from peripheral blood of patients with PBC have been studied in the context of MHC Class I HLA-A2.1 restriction, and have been found to identify amino-acid residues 159-167 and 165-174 of PDC-E2<sup>[57]</sup>. Specific MHC class I restricted CTLs can also be generated by *in vitro* stimulation with antigen pulsed dendritic cells<sup>[60]</sup> from blood of patients with PBC, but not from healthy controls, indicative of the presence in PBC of specific precursors of PDC-E2 -reactive T cell clones in peripheral blood. Interestingly, there was a greater increase in numbers of CTL precursors in blood in early *versus* advanced stages of PBC, and in the same study there was a 10 -fold increase in specific CTLs in the liver compared to the peripheral blood, supporting the role of these cells and their specific recruitment in the evolution of bile duct injury in PBC. Thus the two major subsets of T cells recognize the same or very close amino acid sequences within the same epitope regions in the lipoyl domain, thus supporting the hypothesis of a common etiological trigger mechanism, potentially molecular mimicry, associated with other particular immune modifications.

Coming now to CD4<sup>+</sup>CD25<sup>high</sup> natural regulatory T cells (Tregs), a decreased reactivity appears to contribute to a number of human autoimmune diseases<sup>[61-65]</sup> including PBC. A relative reduction of Tregs compared with healthy controls was detected and, as well, the ratio of hepatic Tregs over hepatic CD8<sup>+</sup> cells in PBC patients was lower than that in patients with chronic hepatitis C or autoimmune hepatitis<sup>[66,67]</sup>.

## INNATE IMMUNITY IN PBC

Innate immunity is a first line of defense against

infections and neoplasms, but its importance for adaptive immunity has been appreciated only recently, and its role in the induction of autoimmunity is only partially known<sup>[68]</sup>. The cellular components of innate immunity, including dendritic cells (DC) and other professional APCs<sup>[69]</sup>, and natural killer T cells (NKT), are known to have a regulatory function by modulating the quality and quantity of subsequent adaptive immune responses, including antigen-specific antibody and T cell responses. Innate immunity in PBC patients is characterized by an increased response to pathogen-associated stimuli, as indicated by higher levels of pro-inflammatory cytokines secreted *in vitro* by monocytes after exposure to micro-organisms<sup>[70]</sup>.

NK/NKT cells have been linked to autoimmune diseases in murine models, including autoimmune diabetes in NOD mice and experimental autoimmune encephalomyelitis, a model of multiple sclerosis<sup>[71]</sup>, and the role of such cells in autoimmunity in general is attracting increasing attention. In PBC, Chuang and colleagues recently demonstrated a marked increase in the frequency and absolute number in blood and liver of NK cells. Moreover, in the same study, the cytotoxic activity and perforin expression by isolated NK cells were significantly increased, associated with increased levels of plasma IL-8 and the expression of CD128a (IL-8 receptor) on such cells. In contrast, the levels of IFN- $\gamma$ , IL-6 and IL-8 synthesized by NK cells were significantly decreased in PBC compared to controls<sup>[72]</sup>.

Hyper-responsiveness of the innate immune system of itself would be insufficient to account for the breakdown of natural immune tolerance, but these alterations might come to influence the initiation and perpetuation of the subsequent adaptive autoimmune response.

## CYTOKINES

In PBC, a Th1 cytokine predominance has been reported in serum and liver<sup>[73]</sup>, and a high prevalence of INF- $\gamma$ , a Th1 cytokine, has been detected as a transcriptional up-regulation<sup>[74]</sup>. Moreover, BECs of patients with PBC overexpress TNF- $\alpha$  and the corresponding receptor, thus favoring the idea of a paracrine activity of, and effect on these cells, leading to their proliferation and, potentially, to apoptosis<sup>[75]</sup>. Recent findings further suggest the involvement of cytokine-cytokine receptor interactions in the effector stages of the pathogenesis of PBC<sup>[72]</sup>. Whilst T cells and NKT cells are major sources of cytokines, B cells, endothelial cells, macrophages and other cell types also contribute to cytokine production. Furthermore, different types of APC, genetic background, availability of costimulator molecules, and types and amounts of antigenic stimuli may also influence the differentiation of Th0 cells into either the Th1 or Th2 cell pathways, each with their particular cytokine profiles. Of course, cytokines also come into play in the earlier inductive stages of PBC, in particular transforming growth factor-beta (TGF- $\beta$ ). Deficiency of TGF- $\beta$  is

prejudicial to immunoregulatory functions, as illustrated by recent mouse models of PBC (see below).

## ANIMAL MODELS

The occurrence of a spontaneous animal model would be extremely helpful in elucidating causation and progression of PBC, but none has been identified, and there is some element of "artificiality" with induced models. However, recently, there have been developed three informative genetically manipulated mouse strains that simulate features of human PBC<sup>[76]</sup>.

The first of these mouse models is a congenic variant of the non-obese diabetic (NOD) mouse designated NOD.c3c4 that presents as an autoimmune larger bile duct cholangiopathy and PBC-like serology, with AMA positivity of 50%-60% and ANA positivity of 80%-90%<sup>[77]</sup>. Histologically, there is lymphocytic infiltration within portal tracts with appearances of chronic nonsuppurative destructive cholangitis and epithelioid granuloma formation, although certain features of the bile duct lesions differ from those in human PBC, particularly the occurrence of cystic changes<sup>[77]</sup>. Detailed analysis of the introgressed genetic intervals that determine the autoimmune switch from pancreatic insulinitis to cholangitis is awaited.

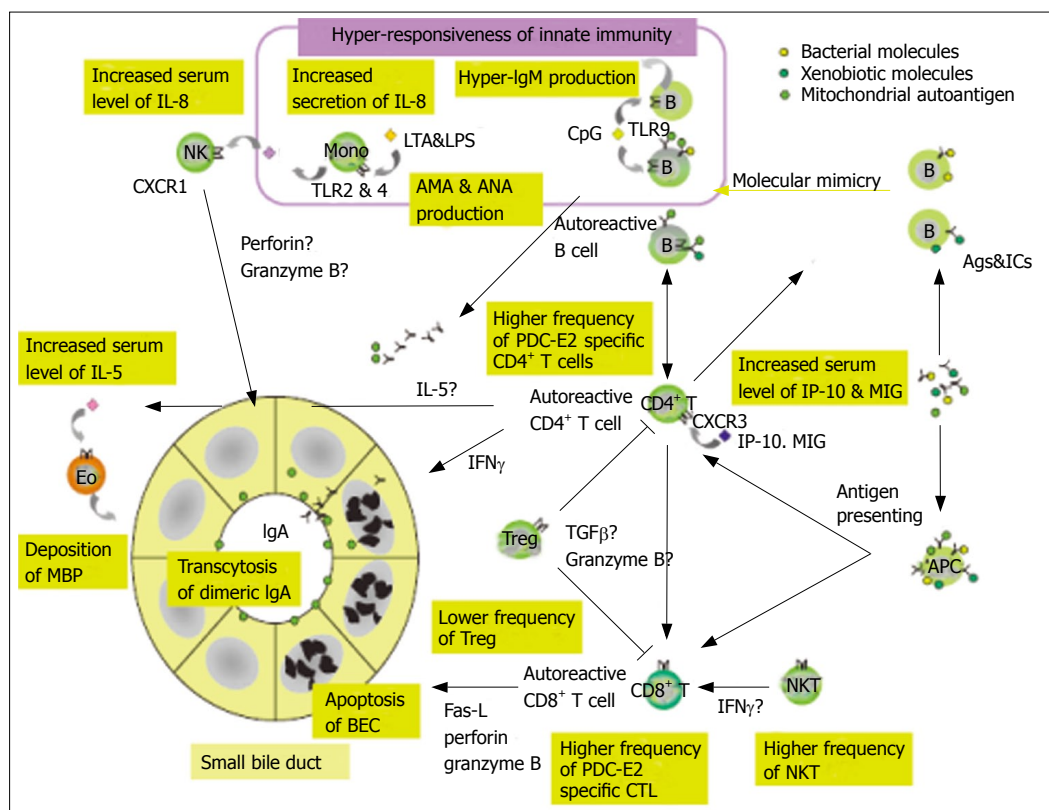
The second of the mouse models was derived by transgenic introduction of a dominant negative form of TGF- $\beta$  receptor II (dnTGF- $\beta$ R II)<sup>[78]</sup>. These mice have inflammatory cholangitis and show 100% AMA positivity against PDC-E2. TGF- $\beta$  receptor II is essential for signal transduction of TGF- $\beta$ , which regulates activation of lymphocytes. This model suggests a specific dysfunction of T cells with impaired TGF- $\beta$  signaling which, in the presence or absence of B cells, is implicated in the pathogenesis of a PBC-like disease, at least in mice<sup>[78]</sup>. Interestingly, it has been demonstrated that CD1d-restricted NKT cells in these mice are a critical factor in liver injury<sup>[79]</sup>.

The third of the mouse models depends on knockout of the gene for the IL-2 receptor (IL-2R $\alpha$  knockout mouse)<sup>[54]</sup>. These mice have inflammatory cholangitis with lymphocyte infiltration around the portal tracts accompanied by cholangiocyte injury, and show 100% AMA positivity against PDC-E2 and 80% ANA positivity. The IL-2R $\alpha$  is the CD25 molecule which, when highly expressed on CD4<sup>+</sup> T cells, is a marker for cells with immunoregulatory activity. This model further implicates deficiency of TGF- $\beta$ -dependent regulatory pathways in the pathogenesis of PBC.

Another useful model has been developed by experimental immunization with xenobiotically modified molecular variants of the PDC-E2 epitope region. Such immunization appears promising in that AMA have thus been elicited in different animal species, rabbits, guinea pigs and mice, as recently reviewed<sup>[14]</sup>.

## PATHOGENIC MECHANISMS

Several theories have been proposed for the



**Figure 1** Model of pathogenic mechanisms in primary biliary cirrhosis (PBC). PBC is initiated by an autoantigenic stimulus (upper, right) provided either by a bacterial mimic of the autoepitope of PDC-E2, a xenobiotically modified PDC-E2, or “spillage” of native mitochondrial autoantigens derived perhaps from apoptotic cells. Hyper-responsiveness of innate immunity (top, centre) can facilitate autoantigenicity; bacterial CpG enhances IgM production and cellular expression of TLR9. Genetic susceptibility is critical overall, and depends particularly on multiple inherited deficits in immune tolerance, mostly as yet undefined. APCs that become activated (lower, right) by stimulation through TLRs present immunogenic self peptides (or mimics) via MHC Class II molecules to autoreactive CD4<sup>+</sup> T lymphocytes (centre) which in turn activate CD8<sup>+</sup> cytotoxic T lymphocytes and B lymphocytes that produce AMA. Treg lymphocytes (lower, centre) that normally restrain activated autoreactive T cells are deficient in PBC, thus further impeding T cell tolerance. Effector mechanisms converge on the target cell in PBC, the BEC (lower left), which can be damaged by injurious cytokines (IFN- $\gamma$ ) from CD4<sup>+</sup> T cells, direct cytotoxicity (Fas-L, perforin, granzyme B) from CD8<sup>+</sup> T cells, or transcytosis of IgA-AMA. A toxic effect might even be supplied by activated eosinophils (centre, left) by release of eosinophil MBP. BECs thus undergo apoptosis and in doing so contribute immunogenic mitochondrial PDC-E2 autoantigen to sustain a self-perpetuating autoimmunization process and, by reason of a BEC-specific anomaly of apoptosis retain PBC-E2 intact in apoptotic blebs (see text), so conferring particular vulnerability on these cells. Ags: Antigens; AMA: Antimitochondrial antibodies; ANA: Antinuclear antibodies; APC: Antigen-presenting cell; BEC: Biliary epithelial cells; CTL: Cytotoxic T lymphocytes; ICs: Immune complexes; IL: Interleukin; IFN: Interferon; IP-10: Interferon- $\gamma$ -inducible protein 10; LTA: Lipoteichoic acid; LPS: Lipopolysaccharide; MIG: Monokine induced by  $\gamma$ -interferon; MBP: Major basic protein (primary cytotoxic granule protein); NKT: Natural killer T cells; PDC-E2: Pyruvate dehydrogenase complex E2; TGF: Transforming growth factor; TLR: Toll-like receptor; Treg: Regulatory T cells.

etiopathogenesis of the immune-mediated tissue injury observed in PBC (Figure 1). Such theories are not necessarily independent, but rather each may be directed to different phases of etiopathogenesis. In other words, we need to consider processes particular to initiation; processes particular to perpetuation, notably deficiencies in immune tolerance; and processes particular to the selective destruction of BECs, with the assumption that these express the target of the disease in an accessible form, namely the AMA autoantigen PDC-E2.

### Initiation

Initiation has been considered already and possibilities include microbial infection or chemical-xenobiotic modification of the PDC-E2 epitope sequence with tolerance-breaking effects due to molecular mimicry. Alternatively mere spillage of autoantigen after cellular injury and apoptosis could suffice, as shown recently in our laboratory<sup>[80]</sup>.

### Perpetuation

Perpetuation involves particular consideration of genetically-based tolerance deficits in PBC, and here more data are sorely needed. Consideration was given to this aspect in a recent review from this laboratory<sup>[14]</sup>. We can refer here to studies on the critical role of CD4<sup>+</sup> CD25<sup>high</sup> regulatory T cells (Tregs) in the prevention of autoimmune disease in murine models. It is postulated that Tregs are important for the prevention of autoimmunity and maintenance of self-tolerance, and studies have demonstrated that the transfer of T cells lacking the Treg subset into athymic nude mice results in the development of various T cell-mediated autoimmune diseases<sup>[61,81]</sup>. PBC patients display significantly lower frequencies of Tregs as percentages of total TCR- $\alpha\beta^+$ /CD4<sup>+</sup> T cells, which may contribute to the failure in tolerance in PBC<sup>[66,82,83]</sup>.

### Destruction

Destruction involves a multilineage attack by CD4<sup>+</sup> and CD8<sup>+</sup> T cells, and B cells, on the vulnerable biliary



ductile, and here our hypothesis would state that the immunodominant AMA autoantigen PDC-E2, which is normally located in the mitochondrial inner membrane, is aberrantly expressed on the cell surface of the BEC and thus is immunologically recognized. Several possibilities have been visualized. First, although *in situ* hybridization studies of *PDC-E2* mRNA showed no significant difference in the amount of PDC-E2 transcript present in PBC liver compared with other liver diseases, PDC-E2 may be selectively overexpressed in small bile duct BECs perhaps as a result of aberrations of apoptosis<sup>[14]</sup>. Second, variants of PDC-E2 may cause an abnormal turnover of the molecule, leading to the accumulation of PDC-E2 in these subpopulations of cells. It is possible that toxic substances disposed of by the liver may accumulate in the biliary epithelium and potentially modify the PDC-E2 molecule locally, leading to the production of such variants. Third, altered *PDC-E2* mRNA could be produced by the abnormal transcription of PDC-E2. For example, it is possible that abnormal splicing during synthesis of *PDC-E2* mRNA would substitute an endoplasmic reticulum targeting signal instead of a mitochondrial targeting signal, thereby enabling PDC-E2 to be delivered into the endoplasmic reticulum and Golgi apparatus via a secretory route to be expressed on the cell surface of biliary ducts, instead of into mitochondria. Although direct evidence supporting these mechanisms is currently lacking, it remains possible that the molecules that are expressed and identified on the ductular surface of BECs, and recognized by anti-PDC-E2 antibodies, may not be PDC-E2 itself, but are PDC-E2 mimics that cross-react with human PDC-E2. Some experimental data seem to support this hypothesis.

Another hypothesis that might explain the selective targeting of bile ducts in PBC is that the autoantigen-specific immunoglobulin A (IgA) antibody plays a role. IgA is the principal isotype of immunoglobulin in epithelial surfaces, including biliary epithelium. If AMA-IgA autoantibodies are responsible for the specific destruction of BECs in PBC, it is possible that this occurs by disrupting cell metabolism of the cells i.e. the AMA-IgA bound to the mitochondrial antigen induces cellular dysfunction and so accounts for the tissue specificity. Interestingly, IgA from PBC patients colocalized with PDC-E2 inside the cells and on the apical membrane of BECs<sup>[84]</sup>. These data support the idea that both the aberrant polar expression of PDC-E2 and the trafficking of IgA in BEC are possible mechanisms for selective damage of BECs. Thus, the apical staining of BECs revealed by anti-PDC-E2 monoclonal antibodies could also be accounted for by the presence of an immune complex formed from secreted IgA and mitochondrial enzyme autoantigens.

## CONCLUSIONS AND FUTURE PERSPECTIVES

There have been many substantial advances in the understanding of PBC since the molecular identification

in 1988 of PDC-E2 as the major reactant for characteristic AMA response. Possible initiators of PBC have emerged as environmental chemical xenobiotics, or microorganisms that utilize the shared culprit autoantigen PDC-E2. Strong genetic predisposition is certain from case study data, noting here the female predisposition and family clustering, but formal genome wide studies are not yet available. It is highly likely that multiple deficits in immune tolerance will prove important, and that these will be genetically based; recent mouse models are certainly pointing in this direction. Other unexplained features of PBC include susceptibility to infections, likely associated with the aberrant humoral and cellular reactivities<sup>[85,86]</sup>. It is crucial to ascertain whether there is a pathogenic role of AMA in the bile duct damage of PBC and, if so, how this is mediated. Further investigation of innate immune mechanisms in PBC is called for, as is the role of the BEC itself in stimulation and perpetuation of the peribiliary inflammatory process.

It is almost a truism that only knowledge of the etiopathogenetic mechanisms will open the door to effective therapies for diseases such as PBC<sup>[87]</sup>, yet the major advance in the therapy of PBC with UDCA<sup>[88]</sup> has come without insight into primary causes of the disease, just as successful biotherapies of rheumatoid arthritis have emerged without knowledge of the primary cause of that disease. Currently, we should press on with development and study of animal models, encourage the application of predictably informative genomic studies, and seize on the clues we have to environmental provocations.

At the practical level, clinicians should be alert to the need for early diagnosis, during the long latent period of the disease, in the susceptible middle-aged female population to ensure that such subjects do gain benefit from disease-retarding therapy, at whatever stage their disease may be<sup>[88]</sup>. To a degree, this is already happening if we compare what PBC was like 20 years ago<sup>[89]</sup> to what it is today<sup>[90]</sup>.

## REFERENCES

- 1 **Kaplan MM**, Gershwin ME. Primary biliary cirrhosis. *N Engl J Med* 2005; **353**: 1261-1273
- 2 **Invernizzi P**, Selmi C, Ranftler C, Podda M, Wiesierska-Gadek J. Antinuclear antibodies in primary biliary cirrhosis. *Semin Liver Dis* 2005; **25**: 298-310
- 3 **Gershwin ME**, Selmi C, Worman HJ, Gold EB, Watnik M, Utts J, Lindor KD, Kaplan MM, Vierling JM. Risk factors and comorbidities in primary biliary cirrhosis: a controlled interview-based study of 1032 patients. *Hepatology* 2005; **42**: 1194-1202
- 4 **Selmi C**, Mayo MJ, Bach N, Ishibashi H, Invernizzi P, Gish RG, Gordon SC, Wright HL, Zweiban B, Podda M, Gershwin ME. Primary biliary cirrhosis in monozygotic and dizygotic twins: genetics, epigenetics, and environment. *Gastroenterology* 2004; **127**: 485-492
- 5 **Invernizzi P**, Miozzo M, Battezzati PM, Bianchi I, Grati FR, Simoni G, Selmi C, Watnik M, Gershwin ME, Podda M. Frequency of monosomy X in women with primary biliary cirrhosis. *Lancet* 2004; **363**: 533-535

- 6 **Miozzo M**, Selmi C, Gentilin B, Grati FR, Sirchia S, Oertelt S, Zuin M, Gershwin ME, Podda M, Invernizzi P. Preferential X chromosome loss but random inactivation characterize primary biliary cirrhosis. *Hepatology* 2007; **46**: 456-462
- 7 **Peng Y**, Martin DA, Kenkel J, Zhang K, Ogden CA, Elkon KB. Innate and adaptive immune response to apoptotic cells. *J Autoimmun* 2007; **29**: 303-309
- 8 **Salunga TL**, Cui ZG, Shimoda S, Zheng HC, Nomoto K, Kondo T, Takano Y, Selmi C, Alpini G, Gershwin ME, Tsuneyama K. Oxidative stress-induced apoptosis of bile duct cells in primary biliary cirrhosis. *J Autoimmun* 2007; **29**: 78-86
- 9 **Invernizzi P**, Miozzo M, Selmi C, Persani L, Battezzati PM, Zuin M, Lucchi S, Meroni PL, Marasini B, Zeni S, Watnik M, Grati FR, Simoni G, Gershwin ME, Podda M. X chromosome monosomy: a common mechanism for autoimmune diseases. *J Immunol* 2005; **175**: 575-578
- 10 **Invernizzi P**, Miozzo M, Oertelt-Prigione S, Meroni PL, Persani L, Selmi C, Battezzati PM, Zuin M, Lucchi S, Marasini B, Zeni S, Watnik M, Tabano S, Maitz S, Pasini S, Gershwin ME, Podda M. X monosomy in female systemic lupus erythematosus. *Ann N Y Acad Sci* 2007; **1110**: 84-91
- 11 **Invernizzi P**, Selmi C, Mackay IR, Podda M, Gershwin ME. From bases to basis: linking genetics to causation in primary biliary cirrhosis. *Clin Gastroenterol Hepatol* 2005; **3**: 401-410
- 12 **Invernizzi P**, Battezzati PM, Crosignani A, Perego F, Poli F, Morabito A, De Arias AE, Scalapogno M, Zuin M, Podda M. Peculiar HLA polymorphisms in Italian patients with primary biliary cirrhosis. *J Hepatol* 2003; **38**: 401-406
- 13 **Donaldson PT**, Baragiotta A, Heneghan MA, Floreani A, Venturi C, Underhill JA, Jones DE, James OF, Bassendine MF. HLA class II alleles, genotypes, haplotypes, and amino acids in primary biliary cirrhosis: a large-scale study. *Hepatology* 2006; **44**: 667-674
- 14 **Gershwin ME**, Mackay IR. The causes of primary biliary cirrhosis: Convenient and inconvenient truths. *Hepatology* 2008; **47**: 737-745
- 15 **Gleicher N**, Barad DH. Gender as risk factor for autoimmune diseases. *J Autoimmun* 2007; **28**: 1-6
- 16 **Selmi C**, Balkwill DL, Invernizzi P, Ansari AA, Coppel RL, Podda M, Leung PS, Kenny TP, Van De Water J, Nantz MH, Kurth MJ, Gershwin ME. Patients with primary biliary cirrhosis react against a ubiquitous xenobiotic-metabolizing bacterium. *Hepatology* 2003; **38**: 1250-1257
- 17 **Padgett KA**, Selmi C, Kenny TP, Leung PS, Balkwill DL, Ansari AA, Coppel RL, Gershwin ME. Phylogenetic and immunological definition of four lipoylated proteins from *Novosphingobium aromaticivorans*, implications for primary biliary cirrhosis. *J Autoimmun* 2005; **24**: 209-219
- 18 **Moritoki Y**, Lian ZX, Wulff H, Yang GX, Chuang YH, Lan RY, Ueno Y, Ansari AA, Coppel RL, Mackay IR, Gershwin ME. AMA production in primary biliary cirrhosis is promoted by the TLR9 ligand CpG and suppressed by potassium channel blockers. *Hepatology* 2007; **45**: 314-322
- 19 **Rieger R**, Gershwin ME. The X and why of xenobiotics in primary biliary cirrhosis. *J Autoimmun* 2007; **28**: 76-84
- 20 **Amano K**, Leung PS, Rieger R, Quan C, Wang X, Marik J, Suen YF, Kurth MJ, Nantz MH, Ansari AA, Lam KS, Zeniya M, Matsuura E, Coppel RL, Gershwin ME. Chemical xenobiotics and mitochondrial autoantigens in primary biliary cirrhosis: identification of antibodies against a common environmental, cosmetic, and food additive, 2-octynoic acid. *J Immunol* 2005; **174**: 5874-5883
- 21 **Leung PS**, Quan C, Park O, Van de Water J, Kurth MJ, Nantz MH, Ansari AA, Coppel RL, Lam KS, Gershwin ME. Immunization with a xenobiotic 6-bromohexanoate bovine serum albumin conjugate induces antimitochondrial antibodies. *J Immunol* 2003; **170**: 5326-5332
- 22 **Long SA**, Quan C, Van de Water J, Nantz MH, Kurth MJ, Barsky D, Colvin ME, Lam KS, Coppel RL, Ansari A, Gershwin ME. Immunoreactivity of organic mimeotopes of the E2 component of pyruvate dehydrogenase: connecting xenobiotics with primary biliary cirrhosis. *J Immunol* 2001; **167**: 2956-2963
- 23 **Rieger R**, Leung PS, Jeddeloh MR, Kurth MJ, Nantz MH, Lam KS, Barsky D, Ansari AA, Coppel RL, Mackay IR, Gershwin ME. Identification of 2-nonynoic acid, a cosmetic component, as a potential trigger of primary biliary cirrhosis. *J Autoimmun* 2006; **27**: 7-16
- 24 **Leon MP**, Bassendine MF, Wilson JL, Ali S, Thick M, Kirby JA. Immunogenicity of biliary epithelium: investigation of antigen presentation to CD4+ T cells. *Hepatology* 1996; **24**: 561-567
- 25 **Wu CT**, Davis PA, Luketic VA, Gershwin ME. A review of the physiological and immunological functions of biliary epithelial cells: targets for primary biliary cirrhosis, primary sclerosing cholangitis and drug-induced ductopenias. *Clin Dev Immunol* 2004; **11**: 205-213
- 26 **Scholz M**, Cinatl J, Blaheta RA, Kornhuber B, Markus BH, Doerr HW. Expression of human leukocyte antigens class I and class II on cultured biliary epithelial cells after cytomegalovirus infection. *Tissue Antigens* 1997; **49**: 640-643
- 27 **Ayres RC**, Neuberger JM, Shaw J, Joplin R, Adams DH. Intercellular adhesion molecule-1 and MHC antigens on human intrahepatic bile duct cells: effect of pro-inflammatory cytokines. *Gut* 1993; **34**: 1245-1249
- 28 **Yasoshima M**, Nakanuma Y, Tsuneyama K, Van de Water J, Gershwin ME. Immunohistochemical analysis of adhesion molecules in the micro-environment of portal tracts in relation to aberrant expression of PDC-E2 and HLA-DR on the bile ducts in primary biliary cirrhosis. *J Pathol* 1995; **175**: 319-325
- 29 **Saidman SL**, Duquesnoy RJ, Zeevi A, Fung JJ, Starzl TE, Demetris AJ. Recognition of major histocompatibility complex antigens on cultured human biliary epithelial cells by alloreactive lymphocytes. *Hepatology* 1991; **13**: 239-246
- 30 **Tsuneyama K**, Harada K, Yasoshima M, Kaji K, Gershwin ME, Nakanuma Y. Expression of co-stimulatory factor B7-2 on the intrahepatic bile ducts in primary biliary cirrhosis and primary sclerosing cholangitis: an immunohistochemical study. *J Pathol* 1998; **186**: 126-130
- 31 **Harada K**, Ozaki S, Gershwin ME, Nakanuma Y. Enhanced apoptosis relates to bile duct loss in primary biliary cirrhosis. *Hepatology* 1997; **26**: 1399-1405
- 32 **Afford SC**, Ahmed-Choudhury J, Randhawa S, Russell C, Youster J, Crosby HA, Eliopoulos A, Hubscher SG, Young LS, Adams DH. CD40 activation-induced, Fas-dependent apoptosis and NF-kappaB/AP-1 signaling in human intrahepatic biliary epithelial cells. *FASEB J* 2001; **15**: 2345-2354
- 33 **Odin JA**, Huebert RC, Casciola-Rosen L, LaRusso NF, Rosen A. Bcl-2-dependent oxidation of pyruvate dehydrogenase-E2, a primary biliary cirrhosis autoantigen, during apoptosis. *J Clin Invest* 2001; **108**: 223-232
- 34 **Allina J**, Hu B, Sullivan DM, Fiel MI, Thung SN, Bronk SF, Huebert RC, van de Water J, LaRusso NF, Gershwin ME, Gores GJ, Odin JA. T cell targeting and phagocytosis of apoptotic biliary epithelial cells in primary biliary cirrhosis. *J Autoimmun* 2006; **27**: 232-241
- 35 **Allina J**, Stanca CM, Garber J, Hu B, Sautes-Fridman C, Bach N, Odin JA. Anti-CD16 autoantibodies and delayed phagocytosis of apoptotic cells in primary biliary cirrhosis. *J Autoimmun* 2008; **30**: 238-245
- 36 **O'Brien BA**, Geng X, Orteu CH, Huang Y, Ghoreishi M, Zhang Y, Bush JA, Li G, Finegood DT, Dutz JP. A deficiency in the in vivo clearance of apoptotic cells is a feature of the NOD mouse. *J Autoimmun* 2006; **26**: 104-115
- 37 **Tanaka A**, Nalbandian G, Leung PS, Benson GD, Munoz S, Findor JA, Branch AD, Coppel RL, Ansari AA, Gershwin ME. Mucosal immunity and primary biliary cirrhosis: presence of antimitochondrial antibodies in urine. *Hepatology* 2000; **32**: 910-915
- 38 **Reynoso-Paz S**, Leung PS, Van De Water J, Tanaka A, Munoz S, Bass N, Lindor K, Donald PJ, Coppel RL, Ansari

- AA, Gershwin ME. Evidence for a locally driven mucosal response and the presence of mitochondrial antigens in saliva in primary biliary cirrhosis. *Hepatology* 2000; **31**: 24-29
- 39 **Fukushima N**, Nalbandian G, Van De Water J, White K, Ansari AA, Leung P, Kenny T, Kamita SG, Hammock BD, Coppel RL, Stevenson F, Ishibashi H, Gershwin ME. Characterization of recombinant monoclonal IgA anti-PDC-E2 autoantibodies derived from patients with PBC. *Hepatology* 2002; **36**: 1383-1392
  - 40 **Matsumura S**, Van De Water J, Leung P, Odin JA, Yamamoto K, Gores GJ, Mostov K, Ansari AA, Coppel RL, Shiratori Y, Gershwin ME. Caspase induction by IgA antimitochondrial antibody: IgA-mediated biliary injury in primary biliary cirrhosis. *Hepatology* 2004; **39**: 1415-1422
  - 41 **Lleo A**, Invernizzi P, Selmi C, Coppel RL, Alpini G, Podda M, Mackay IR, Gershwin ME. Autophagy: highlighting a novel player in the autoimmunity scenario. *J Autoimmun* 2007; **29**: 61-68
  - 42 **Pasquali JL**, Soulas-Sprauel P, Korganow AS, Martin T. Auto-reactive B cells in transgenic mice. *J Autoimmun* 2007; **29**: 250-256
  - 43 **Rowley B**, Tang L, Shinton S, Hayakawa K, Hardy RR. Autoreactive B-1 B cells: constraints on natural autoantibody B cell antigen receptors. *J Autoimmun* 2007; **29**: 236-245
  - 44 **Invernizzi P**, Lleo A, Podda M. Interpreting serological tests in diagnosing autoimmune liver diseases. *Semin Liver Dis* 2007; **27**: 161-172
  - 45 **Oertelt S**, Rieger R, Selmi C, Invernizzi P, Ansari AA, Coppel RL, Podda M, Leung PS, Gershwin ME. A sensitive bead assay for antimitochondrial antibodies: Chipping away at AMA-negative primary biliary cirrhosis. *Hepatology* 2007; **45**: 659-665
  - 46 **Van de Water J**, Gershwin ME, Leung P, Ansari A, Coppel RL. The autoepitope of the 74-kD mitochondrial autoantigen of primary biliary cirrhosis corresponds to the functional site of dihydrolipoamide acetyltransferase. *J Exp Med* 1988; **167**: 1791-1799
  - 47 **Mackay IR**, Whittingham S, Fida S, Myers M, Ikuno N, Gershwin ME, Rowley MJ. The peculiar autoimmunity of primary biliary cirrhosis. *Immunol Rev* 2000; **174**: 226-237
  - 48 **Courvalin JC**, Lassoued K, Bartnik E, Blobel G, Wozniak RW. The 210-kD nuclear envelope polypeptide recognized by human autoantibodies in primary biliary cirrhosis is the major glycoprotein of the nuclear pore. *J Clin Invest* 1990; **86**: 279-285
  - 49 **Wesierska-Gadek J**, Hohenauer H, Hitchman E, Penner E. Autoantibodies against nucleoporin p62 constitute a novel marker of primary biliary cirrhosis. *Gastroenterology* 1996; **110**: 840-847
  - 50 **Wesierska-Gadek J**, Penner E, Battezzati PM, Selmi C, Zuin M, Hitchman E, Worman HJ, Gershwin ME, Podda M, Invernizzi P. Correlation of initial autoantibody profile and clinical outcome in primary biliary cirrhosis. *Hepatology* 2006; **43**: 1135-1144
  - 51 **Nakamura M**, Kondo H, Mori T, Komori A, Matsuyama M, Ito M, Takii Y, Koyabu M, Yokoyama T, Migita K, Daikoku M, Abiru S, Yatsushashi H, Takezaki E, Masaki N, Sugi K, Honda K, Adachi H, Nishi H, Watanabe Y, Nakamura Y, Shimada M, Komatsu T, Saito A, Saoshiro T, Harada H, Sodeyama T, Hayashi S, Masumoto A, Sando T, Yamamoto T, Sakai H, Kobayashi M, Muro T, Koga M, Shums Z, Norman GL, Ishibashi H. Anti-gp210 and anti-centromere antibodies are different risk factors for the progression of primary biliary cirrhosis. *Hepatology* 2007; **45**: 118-127
  - 52 **Szosteki C**, Guldner HH, Will H. Autoantibodies against "nuclear dots" in primary biliary cirrhosis. *Semin Liver Dis* 1997; **17**: 71-78
  - 53 **Yang WH**, Yu JH, Nakajima A, Neuberger D, Lindor K, Bloch DB. Do antinuclear antibodies in primary biliary cirrhosis patients identify increased risk for liver failure? *Clin Gastroenterol Hepatol* 2004; **2**: 1116-1122
  - 54 **Wakabayashi K**, Lian ZX, Moritoki Y, Lan RY, Tsuneyama K, Chuang YH, Yang GX, Ridgway W, Ueno Y, Ansari AA, Coppel RL, Mackay IR, Gershwin ME. IL-2 receptor alpha(-/-) mice and the development of primary biliary cirrhosis. *Hepatology* 2006; **44**: 1240-1249
  - 55 **Aoki CA**, Roifman CM, Lian ZX, Bowlus CL, Norman GL, Shoenfeld Y, Mackay IR, Gershwin ME. IL-2 receptor alpha deficiency and features of primary biliary cirrhosis. *J Autoimmun* 2006; **27**: 50-53
  - 56 **Van de Water J**, Ansari A, Prindiville T, Coppel RL, Ricalton N, Kotzin BL, Liu S, Roche TE, Krams SM, Munoz S, Gershwin ME. Heterogeneity of autoreactive T cell clones specific for the E2 component of the pyruvate dehydrogenase complex in primary biliary cirrhosis. *J Exp Med* 1995; **181**: 723-733
  - 57 **Shimoda S**, Nakamura M, Ishibashi H, Hayashida K, Niho Y. HLA DRB4 0101-restricted immunodominant T cell autoepitope of pyruvate dehydrogenase complex in primary biliary cirrhosis: evidence of molecular mimicry in human autoimmune diseases. *J Exp Med* 1995; **181**: 1835-1845
  - 58 **Shimoda S**, Van de Water J, Ansari A, Nakamura M, Ishibashi H, Coppel RL, Lake J, Keeffe EB, Roche TE, Gershwin ME. Identification and precursor frequency analysis of a common T cell epitope motif in mitochondrial autoantigens in primary biliary cirrhosis. *J Clin Invest* 1998; **102**: 1831-1840
  - 59 **Shimoda S**, Nakamura M, Ishibashi H, Kawano A, Kamihira T, Sakamoto N, Matsushita S, Tanaka A, Worman HJ, Gershwin ME, Harada M. Molecular mimicry of mitochondrial and nuclear autoantigens in primary biliary cirrhosis. *Gastroenterology* 2003; **124**: 1915-1925
  - 60 **Kita H**, Lian ZX, Van de Water J, He XS, Matsumura S, Kaplan M, Luketic V, Coppel RL, Ansari AA, Gershwin ME. Identification of HLA-A2-restricted CD8(+) cytotoxic T cell responses in primary biliary cirrhosis: T cell activation is augmented by immune complexes cross-presented by dendritic cells. *J Exp Med* 2002; **195**: 113-123
  - 61 **Alvarado-Sanchez B**, Hernandez-Castro B, Portales-Perez D, Baranda L, Layseca-Espinosa E, Abud-Mendoza C, Cubillas-Tejeda AC, Gonzalez-Amaro R. Regulatory T cells in patients with systemic lupus erythematosus. *J Autoimmun* 2006; **27**: 110-118
  - 62 **Ban Y**, Tozaki T, Tobe T, Ban Y, Jacobson EM, Concepcion ES, Tomer Y. The regulatory T cell gene FOXP3 and genetic susceptibility to thyroid autoimmunity: an association analysis in Caucasian and Japanese cohorts. *J Autoimmun* 2007; **28**: 201-207
  - 63 **Lan RY**, Mackay IR, Gershwin ME. Regulatory T cells in the prevention of mucosal inflammatory diseases: patrolling the border. *J Autoimmun* 2007; **29**: 272-280
  - 64 **Sharma R**, Jarjour WN, Zheng L, Gaskin F, Fu SM, Ju ST. Large functional repertoire of regulatory T-cell suppressible autoimmune T cells in scurfy mice. *J Autoimmun* 2007; **29**: 10-19
  - 65 **Sharma R**, Zheng L, Guo X, Fu SM, Ju ST, Jarjour WN. Novel animal models for Sjogren's syndrome: expression and transfer of salivary gland dysfunction from regulatory T cell-deficient mice. *J Autoimmun* 2006; **27**: 289-296
  - 66 **Lan RY**, Cheng C, Lian ZX, Tsuneyama K, Yang GX, Moritoki Y, Chuang YH, Nakamura T, Saito S, Shimoda S, Tanaka A, Bowlus CL, Takano Y, Ansari AA, Coppel RL, Gershwin ME. Liver-targeted and peripheral blood alterations of regulatory T cells in primary biliary cirrhosis. *Hepatology* 2006; **43**: 729-737
  - 67 **Abbas AK**, Lohr J, Knoechel B. Balancing autoaggressive and protective T cell responses. *J Autoimmun* 2007; **28**: 59-61
  - 68 **Lang KS**, Burow A, Kurrer M, Lang PA, Recher M. The role of the innate immune response in autoimmune disease. *J Autoimmun* 2007; **29**: 206-212
  - 69 **Papadimitraki ED**, Bertsias GK, Boumpas DT. Toll like receptors and autoimmunity: a critical appraisal. *J Autoimmun* 2007; **29**: 310-318
  - 70 **Mao TK**, Lian ZX, Selmi C, Ichiki Y, Ashwood P, Ansari AA,

- Coppel RL, Shimoda S, Ishibashi H, Gershwin ME. Altered monocyte responses to defined TLR ligands in patients with primary biliary cirrhosis. *Hepatology* 2005; **42**: 802-808
- 71 **Hammond KJ**, Kronenberg M. Natural killer T cells: natural or unnatural regulators of autoimmunity? *Curr Opin Immunol* 2003; **15**: 683-689
- 72 **Chuang YH**, Lian ZX, Tsuneyama K, Chiang BL, Ansari AA, Coppel RL, Eric Gershwin M. Increased killing activity and decreased cytokine production in NK cells in patients with primary biliary cirrhosis. *J Autoimmun* 2006; **26**: 232-240
- 73 **Nagano T**, Yamamoto K, Matsumoto S, Okamoto R, Tagashira M, Ibuki N, Matsumura S, Yabushita K, Okano N, Tsuji T. Cytokine profile in the liver of primary biliary cirrhosis. *J Clin Immunol* 1999; **19**: 422-427
- 74 **Martinez OM**, Villanueva JC, Gershwin ME, Krams SM. Cytokine patterns and cytotoxic mediators in primary biliary cirrhosis. *Hepatology* 1995; **21**: 113-119
- 75 **Yasoshima M**, Kono N, Sugawara H, Katayanagi K, Harada K, Nakanuma Y. Increased expression of interleukin-6 and tumor necrosis factor-alpha in pathologic biliary epithelial cells: in situ and culture study. *Lab Invest* 1998; **78**: 89-100
- 76 **Oertelt S**, Ridgway WM, Ansari AA, Coppel RL, Gershwin ME. Murine models of primary biliary cirrhosis: Comparisons and contrasts. *Hepatol Res* 2007; **37** Suppl 3: S365-S369
- 77 **Irie J**, Wu Y, Wicker LS, Rainbow D, Nalesnik MA, Hirsch R, Peterson LB, Leung PS, Cheng C, Mackay IR, Gershwin ME, Ridgway WM. NOD.c3c4 congenic mice develop autoimmune biliary disease that serologically and pathogenetically models human primary biliary cirrhosis. *J Exp Med* 2006; **203**: 1209-1219
- 78 **Oertelt S**, Lian ZX, Cheng CM, Chuang YH, Padgett KA, He XS, Ridgway WM, Ansari AA, Coppel RL, Li MO, Flavell RA, Kronenberg M, Mackay IR, Gershwin ME. Anti-mitochondrial antibodies and primary biliary cirrhosis in TGF-beta receptor II dominant-negative mice. *J Immunol* 2006; **177**: 1655-1660
- 79 **Chuang YH**, Lian ZX, Yang GX, Shu SA, Moritoki Y, Ridgway WM, Ansari AA, Kronenberg M, Flavell RA, Gao B, Gershwin ME. Natural killer T cells exacerbate liver injury in a transforming growth factor beta receptor II dominant-negative mouse model of primary biliary cirrhosis. *Hepatology* 2008; **47**: 571-580
- 80 **Leung PS**, Rossaro L, Davis PA, Park O, Tanaka A, Kikuchi K, Miyakawa H, Norman GL, Lee W, Gershwin ME. Antimitochondrial antibodies in acute liver failure: implications for primary biliary cirrhosis. *Hepatology* 2007; **46**: 1436-1442
- 81 **Lan RY**, Ansari AA, Lian ZX, Gershwin ME. Regulatory T cells: development, function and role in autoimmunity. *Autoimmun Rev* 2005; **4**: 351-363
- 82 **Wrenshall LE**, Smith DR, Stevens ET, Miller JD. Influence of interleukin-2 deficiency on the generation of autoimmune B cells. *J Autoimmun* 2007; **29**: 125-133
- 83 **Nagayama Y**, Horie I, Saitoh O, Nakahara M, Abiru N. CD4+CD25+ naturally occurring regulatory T cells and not lymphopenia play a role in the pathogenesis of iodide-induced autoimmune thyroiditis in NOD-H2h4 mice. *J Autoimmun* 2007; **29**: 195-202
- 84 **Malmberg AC**, Shultz DB, Luton F, Mostov KE, Richly E, Leung PS, Benson GD, Ansari AA, Coppel RL, Gershwin ME, Van de Water J. Penetration and co-localization in MDCK cell mitochondria of IgA derived from patients with primary biliary cirrhosis. *J Autoimmun* 1998; **11**: 573-580
- 85 **Youinou P**. B cell conducts the lymphocyte orchestra. *J Autoimmun* 2007; **28**: 143-151
- 86 **Blank M**, Shoenfeld Y. B cell targeted therapy in autoimmunity. *J Autoimmun* 2007; **28**: 62-68
- 87 **Ross CJ**, Katzov H, Carleton B, Hayden MR. Pharmacogenomics and its implications for autoimmune disease. *J Autoimmun* 2007; **28**: 122-128
- 88 **Corpechot C**, Poupon R. Geotherapeutics of primary biliary cirrhosis: bright and sunny around the Mediterranean but still cloudy and foggy in the United Kingdom. *Hepatology* 2007; **46**: 963-965
- 89 **Christensen E**, Crowe J, Doniach D, Popper H, Ranek L, Rodes J, Tygstrup N, Williams R. Clinical pattern and course of disease in primary biliary cirrhosis based on an analysis of 236 patients. *Gastroenterology* 1980; **78**: 236-246
- 90 **Gershwin ME**, Nishio A, Ishibashi H, Lindor K. Primary biliary cirrhosis. In: Gershwin ME, Vierling JM, Manns MP, editors. *Liver Immunology*. Philadelphia: Hanley & Belfus, Inc, 2003: 311-328

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## TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, Series Editors

# Clinical features and management of primary sclerosing cholangitis

Marina G Silveira, Keith D Lindor

Marina G Silveira, Keith D Lindor, Division of Gastroenterology and Hepatology, Miles and Shirley Fiterman Center for Digestive Diseases, Mayo Clinic and Foundation, Rochester, Minnesota, United States

Correspondence to: Keith D Lindor, MD, Division of Gastroenterology and Hepatology, Miles and Shirley Fiterman Center for Digestive Diseases, Mayo Clinic and Foundation, 200 First Street, SW, Rochester, MN 55905, United States. [lindor.keith@mayo.edu](mailto:lindor.keith@mayo.edu)

Telephone: +1-507-2842969 Fax: +1-507-2664531

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and Experimental Medicine, Videnska 1958/9, Praha 4, 14000, Czech; Richard M Green, MD, Associate Professor of Medicine, Chief, Division of Hepatology, Northwestern University, Searle 10-541, 303 E. Chicago Avenue, Chicago, IL 60611 United States

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## Abstract

Primary sclerosing cholangitis is a chronic cholestatic liver disease characterized by inflammation and fibrosis of the bile ducts, resulting in cirrhosis and need for liver transplantation and reduced life expectancy. The majority of cases occur in young and middle-aged men, often in association with inflammatory bowel disease. The etiology of primary sclerosing cholangitis includes immune-mediated components and elements of undefined nature. No effective medical therapy has been identified. The multiple complications of primary sclerosing cholangitis include metabolic bone disease, dominant strictures, bacterial cholangitis, and malignancy, particularly cholangiocarcinoma, which is the most lethal complication of primary sclerosing cholangitis. Liver transplantation is currently the only life-extending therapeutic alternative for patients with end-stage disease, although recurrence in the allografted liver has been described. A PSC-like variant attracting attention is cholangitis marked by raised levels of the immunoglobulin G4 subclass, prominence of plasma cells within the lesions, and steroid responsiveness.

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**Key words:** Sclerosing cholangitis; Diagnosis; Therapy; Cholestasis; Cholangiocarcinoma; Liver transplantation

**Peer reviewers:** Dr. Limas Kupcinskas, Professor, Gastroenterology of Kaunas University of Medicine, Mickeviciaus 9, Kaunas LT 44307, Lithuania; Dr. Milan Jirsa, Laboratory of Experimental Medicine-building Z1, Institute for Clinical

## INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease characterized by fibrosing inflammatory destruction of the intrahepatic and/or extrahepatic bile ducts<sup>[1]</sup>, leading to bile stasis, hepatic fibrosis, and ultimately to cirrhosis, end-stage liver disease, and need for liver transplantation. The majority of cases occur in association with inflammatory bowel disease (IBD), which often precedes the development of PSC<sup>[2]</sup>. The etiology of PSC is undefined, apart from an increasing body of evidence that points to an immunologic disturbance as a component of the disease. However, PSC lacks the features of a typical autoimmune disease and responds poorly, if at all, to typical immunosuppressive therapies<sup>[3]</sup>. No effective medical therapy for halting disease progression has been identified, but ursodeoxycholic acid is being assessed. A median duration of 12 to 18 years from the time of diagnosis before patients develop end-stage liver disease has been observed. Among eligible patients, liver transplantation (LT) is currently the only life-extending therapy for patients with end-stage PSC, although the disease can recur in the allografted liver and be a cause of morbidity post-transplant<sup>[4]</sup>.

## CLINICAL FEATURES

### Clinical manifestations

PSC affects primarily young and middle-aged men, especially patients with underlying inflammatory bowel disease. Approximately 70% to 80% of PSC patients in the United States have ulcerative colitis (UC)<sup>[5-10]</sup>.

Conversely, approximately 2% to 7.5% of patients with UC<sup>[11]</sup> and 1.4% to 3.4% of patients with Crohn's disease<sup>[12]</sup> develop PSC. IBD can be diagnosed at any time during the course of PSC, and PSC can occur at any time during the course of IBD<sup>[13]</sup>. In general, however, IBD is diagnosed several years earlier than PSC<sup>[13]</sup>. PSC may also develop many years after proctocolectomy for colitis and IBD can develop many years after liver transplantation due to advanced PSC<sup>[11]</sup>. Whether PSC is a distinct entity in patients with and without IBD might be a clinically significant issue<sup>[14,15]</sup>, but at present, there are not sufficient data to conclude that PSC occurring in patients without IBD is an entity separate from PSC found in association with IBD<sup>[11,16]</sup>.

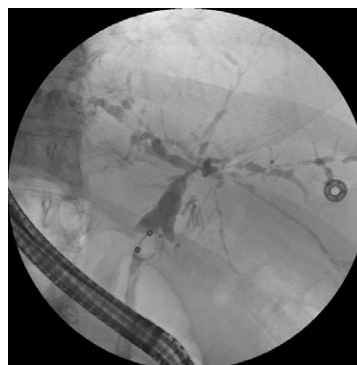
Asymptomatic patients represent about 15% to 40% of the patients at time of diagnosis in early studies<sup>[2]</sup>. More recently, more patients are identified at an earlier stage of the disease with fewer symptoms. One study showed that the majority of patients (greater than 55%) initially present with asymptotically elevated liver enzymes<sup>[17]</sup>. Due to its close association to IBD, many cases come to medical attention when patients with IBD are screened for liver disease.

The clinical course of PSC is typically one of insidious worsening of cholestasis and eventual development of jaundice and end-stage liver disease<sup>[3]</sup>. As such, asymptomatic patients with PSC are at increased risk for developing symptoms over time. Fatigue and pruritus are reported as the most common symptoms. Jaundice, pain, fever and weight loss, cholangiocarcinoma, or manifestations of portal hypertension in advanced stages of liver disease are uncommon initial manifestations. In one recent study, the most common presenting symptoms were described as abdominal pain (20%), pruritus (10%), diarrhea (8%), jaundice (6%), fatigue (6%) and fever (4%)<sup>[17]</sup>. Another recent study from Sweden suggested that more patients without IBD are identified and the patients are older at diagnosis<sup>[18]</sup>. Symptoms of bacterial cholangitis usually are not manifested until patients undergo endoscopic intervention or surgical exploration of the biliary tract<sup>[19]</sup>. Cholangiocarcinoma develops in up to 23% of patients<sup>[20]</sup> and can occur relatively early and before onset of cirrhosis<sup>[3]</sup>.

Impairments in health-related quality of life among individuals with PSC compared to the general population were confirmed in two independent populations<sup>[21,22]</sup>. Patients with cirrhosis form primary hepatocellular disease, however, reported lower health-related quality of life scores compared to patients with cholestatic liver disease<sup>[21]</sup>.

### Biochemical features

A cholestatic picture of liver function with elevations in serum alkaline phosphatase values are the biochemical hallmark of PSC. Increases between 3 and 10 times the upper limit of normal occur in 95% of cases. Serum alanine and aspartate aminotransferase levels are usually 2-3 fold higher than normal levels. The serum total bilirubin level is normal in 60% of individuals at diagnosis<sup>[2]</sup>. The liver function tests, however, may



**Figure 1** Cholangiographic finding in PSC. Cholangiogram demonstrating multifocal strictures with intervening saccular dilatation of both intrahepatic and extrahepatic bile duct characteristic of PSC. (Photograph courtesy of Dr. Rahul Pannala).

be normal and can fluctuate during the course of the disease<sup>[23]</sup>.

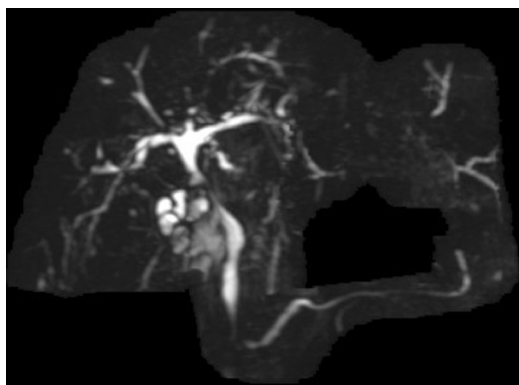
Several prognostic models for PSC have been developed, most of which include age, serum bilirubin and histologic staging<sup>[5,7,9,10,24]</sup>. Most recently, a Mayo model for predicting the survival has been refined<sup>[25]</sup>. This uses the age of the patient, total serum bilirubin, aspartate aminotransferase levels, presence or absence of variceal bleeding and serum albumin as independent variables, and can be used in early stages of PSC, before onset of cirrhosis. The limitations of prognostic models include the inability to account for the development of cholangiocarcinoma and health-related quality of life<sup>[19]</sup>. Once decompensated cirrhosis is present, the Model for End-Stage Liver Disease (MELD) score<sup>[26]</sup> more accurately predicts survival and is more appropriately used in prioritizing patients for liver transplantation<sup>[3]</sup>.

### Serologic features

Currently, testing for specific autoimmune antibodies does not contribute to the diagnosis of PSC. The prevalent autoantibody reactivity is a perinuclear antineutrophilic autoantibody (pANCA), present in approximately 80% of patients but lacking in diagnostic specificity<sup>[27-30]</sup>. The unidentified antigenic reactant is not the proteinase (myeloperoxidase) of conventional pANCA. Other autoantibodies such as antinuclear antibodies and smooth muscle antibodies occur in 20% to 60% of patients, usually in lower titers than those observed in autoimmune hepatitis<sup>[31]</sup>; their fine antigenic specificity has not been established. Antimitochondrial antibodies are rarely found in patients with PSC<sup>[6]</sup>, in keeping with the lack of overlap between PSC and primary biliary cirrhosis (PBC). The serological markers of autoimmune liver diseases are covered in more detail in other articles in this series.

### Radiographic features

Cholangiography is considered to be the gold standard for the diagnosis of PSC<sup>[6]</sup>. In experienced hands, endoscopic retrograde cholangiopancreatography (ERCP) is successful in demonstrating the intra- and extra-hepatic biliary tree in 95% of the cases<sup>[3]</sup>. Segmental fibrosis of intrahepatic and/or extrahepatic bile ducts with saccular dilatation of normal intervening areas results in the characteristic beads-on-a-string appearance (Figure 1). Intrahepatic duct involvement is nearly universal with most patients affected



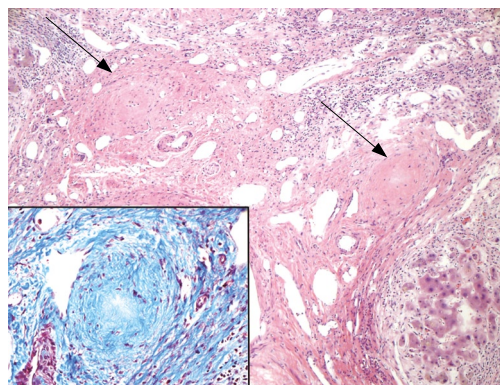
**Figure 2** Cholangiographic findings in PSC. Magnetic resonance cholangiography demonstrating findings of PSC.

by intrahepatic and extrahepatic disease<sup>[19]</sup>. Procedure-related complications from ERCP can occur in 3% to 8% of patients and include abdominal pain, pancreatitis, bleeding, common bile duct perforation, biliary sepsis and death<sup>[32-34]</sup>.

The use of magnetic resonance cholangiography (MRC) for detecting PSC has been evaluated as a rapid, noninvasive examination of the biliary tract (Figure 2). MRC has no significant morbidity when performed in appropriately selected individuals, and avoids the potential adverse effects of radiation exposure and contrast media associated with ERCP<sup>[35]</sup>. For the detection of PSC, MRC has been found to be accurate and comparable to ERCP<sup>[36-39]</sup>. Moreover, one study has suggested it also results in cost savings when used as the initial test strategy for diagnosing PSC<sup>[34]</sup>. Factors that lead to difficulties in interpreting the MRC compared to ERCP include the presence of cirrhosis and PSC limited to the peripheral intrahepatic bile ducts<sup>[36]</sup>. The major disadvantage of MRC is that it is a purely diagnostic examination, although it can be used to identify patients who would benefit from subsequent therapeutic ERCP<sup>[19]</sup>. Although biliary tree changes on MRC aid in the diagnosis of PSC, they do not correlate with survival, as predicted by the Mayo Risk Score<sup>[38]</sup>.

### Histologic features

PSC is histologically characterized by damage, atrophy, and, ultimately, loss of medium- and large-sized bile ducts, within or outside the liver<sup>[40,41]</sup>. These are not typically captured in a percutaneous liver biopsy. The histological picture is complicated by the fact that separation of the disease process itself from the effects of distal obstruction of bile ducts can be challenging<sup>[41]</sup>. The smaller ducts are affected by the resultant obstruction and gradually disappear (ductopenia). The characteristic pathologic features of PSC are concentric periductal fibrosis ("onion-skinning") that progresses to a narrowing and then obliteration of the small bile ducts leaving a bile duct scar (Figure 3), but this is found in less than 15% of the patients with PSC<sup>[42]</sup>. Many of the biopsy changes, such as bile stasis, pseudoxanthomatous changes, Mallory bodies and copper accumulation, lack specificity for diagnostic



**Figure 3** Fibro-obliterative lesions in PSC. Image shows an expanded portal area without two distinct fibro-obliterative lesions (arrows) in end-stage primary sclerosing cholangitis. There is no intact bile duct present in this portal area, only cross-sections of portal vein and hepatic artery branches (H&E, original magnification, 100 ×). Inset: Higher magnification of the fibro-obliterative lesion (Masson trichrome, × 400). (Photograph courtesy of Dr. Schuyler Sanderson).

purposes<sup>[41]</sup>, and can occur with chronic extra-hepatic bile duct obstruction from any cause<sup>[43]</sup>. Several stages can be recognized histologically, ranging from stages I to IV: cholangitis and portal hepatitis (stage I); periportal fibrosis or periportal hepatitis (stage II); septal fibrosis, bridging necrosis or both (stage III); and biliary cirrhosis (stage IV)<sup>[44]</sup>. Sampling error is a significant limitation of liver biopsy<sup>[41]</sup>.

Liver biopsy in patients with radiographic evidence of PSC is not needed for diagnosis, although it may help in excluding other diseases<sup>[45]</sup>. Histological staging may be complementary to ERCP evaluation, but most times is not necessary. Liver biopsy should probably be limited to patients with a challenging presentation or those being investigated for small duct PSC or possible overlap syndrome with autoimmune hepatitis<sup>[17]</sup>. The presence of biliary dysplasia on liver histology has been proposed as a marker for eventual cholangiocarcinoma in PSC<sup>[46,47]</sup>, but its use in clinical practice is limited by poor reproducibility of findings on pathologic interpretation<sup>[47]</sup>.

## VARIANT FORMS OF PSC

### Small duct PSC

Small duct PSC refers to disease that affects bile ducts that are too small to be identified by ERCP. This entity is characterized by a consistent liver histology and radiographically normal bile ducts<sup>[48,49]</sup>. The proportion of small duct PSC to "large duct PSC" has been described as approximately 5%-15%<sup>[50,51]</sup>. Small duct PSC is believed to be a distinct entity from the large duct form, with a less aggressive course and less likely to lead to cholangiocarcinoma<sup>[50,52]</sup>.

### Overlap with autoimmune hepatitis

PSC with overlap features of autoimmune hepatitis has been reported both in the pediatric and adult populations of patients with PSC. In adults, the therapeutic response to immunosuppressants, in particular the autoimmune

hepatitis- or hepatocellular component of the overlap syndrome, can be excellent, and can lead to complete remission of disease activity<sup>[53]</sup>. The response to therapy might be dependent on the predominance of AIH or PSC features. The overlap syndromes of autoimmune liver diseases, including overlap of AIH and PSC in childhood are covered in more detail in other articles in this series.

### ***IgG4-related sclerosing cholangitis***

During the past decade, patients with steroid responsive sclerosing cholangitis have been described, often but not always associated with autoimmune pancreatitis<sup>[54]</sup>. Histological findings of lymphoplasmacytic infiltration and infiltration of IgG4-bearing plasma cells, and high serum IgG4 levels have been consistently observed and frequently required for diagnosis of autoimmune pancreatitis<sup>[55,56]</sup>, and have also been observed in the hepatobiliary system. Described hepatobiliary changes have included stenosis of the bile ducts, biliary duct wall thickening, stenosis of the portal vein, portal fibrosis<sup>[57]</sup>, and hepatic inflammatory pseudotumor<sup>[58]</sup>. Hepatobiliary involvement without pancreatic involvement suggests that IgG4-related sclerosing cholangitis could be a distinct entity from autoimmune pancreatitis<sup>[58]</sup>. IgG4-related sclerosing cholangitis is similar to PSC with regard to cholangiographic features, but, in contrast to PSC, is susceptible to steroid therapy and is reversible<sup>[58]</sup>. Therefore, identifying patients with IgG4-related sclerosing cholangitis and distinguishing them from patients with PSC could have major therapeutic implications<sup>[59]</sup>. Although very limited data exists on the prognosis and natural history of IgG4-related sclerosing cholangitis, it seems that the prognosis of these patients is more favorable than that of patients with PSC<sup>[54]</sup>.

## **DISEASE-MODIFYING TREATMENTS**

Different forms of medical treatment have been tried, but until now, no treatment has been proven efficient in randomized controlled studies.

### ***Ineffective or unproven therapies***

Several drugs, such as penicillamine<sup>[60]</sup>, methotrexate<sup>[61]</sup>, budesonide<sup>[62]</sup>, colchicine<sup>[63]</sup>, cladribine<sup>[64]</sup>, cyclosporine<sup>[65]</sup>, mycophenolate mofetil<sup>[66,67]</sup>, etanercept<sup>[68]</sup>, oral and transdermal nicotine<sup>[69,70]</sup>, silymarin<sup>[71]</sup>, pirfenidone<sup>[72]</sup>, and pentoxifylline<sup>[73]</sup>, have been evaluated in the treatment of this condition, but none of them has demonstrated convincing evidence of benefit and some are associated with significant side effects<sup>[62,66]</sup>.

Tacrolimus was shown in a pilot study to cause significant improvement in serum liver biochemistries including alkaline phosphatase<sup>[74]</sup>. A subsequent study from Mayo Clinic supports previous observations that oral tacrolimus is associated with significant reductions in alkaline phosphatase levels in PSC<sup>[75]</sup>. However, in this study, the drug was not well tolerated, and the clinical benefit with oral tacrolimus with respect to disease activity in PSC appears to be limited.

Biologic therapy has been a major advancement in the current therapy of inflammatory bowel disease. There are no controlled trials evaluating the role of biologic therapy (e.g. infliximab and adalimumab) in the management of PSC.

### ***Ursodeoxycholic acid***

Multiple controlled studies have suggested that ursodeoxycholic acid (UDCA) has beneficial effects on liver biochemistries of patients with PSC<sup>[76-85]</sup>. A few studies have documented an improvement in liver histological appearance<sup>[76,79,82]</sup>. Other studies have not included liver histology as an outcome mainly because of sampling issues<sup>[85]</sup>. However, UDCA has not yet proven to prolong survival or improve outcome of PSC. All the trials performed to date have been limited by small number of patients and relatively short follow-up periods.

In an open label study performed at Mayo Clinic, 30 patients with PSC received high-dose (25-30 mg/kg per day) UDCA<sup>[83]</sup>, and substantial reduction not only in serum hepatic biochemistries but also Mayo risk score were observed after 12 mo of therapy. Differences in expected 4-year survival based on Mayo Risk Score were substantial between historical placebo and high-dose UDCA groups. A previous placebo-controlled study conducted at Mayo Clinic in which 51 patients received lower doses of UDCA (13-15 mg/kg per day)<sup>[77]</sup> had showed beneficial effects limited to serum hepatic biochemistries, but no difference in predicted survival. An independent, double-blind, placebo-controlled trial<sup>[82]</sup> of UDCA at 20 mg/kg per day involving 102 patients observed improvement in liver biochemistries, cholangiographic appearance and liver histology after 2 years of therapy, but failed to have any significant effect on survival. No significant UDCA related adverse events were reported from either study. On the other hand, a European study<sup>[85]</sup> with 219 patients who were randomized to receive either high-dose UDCA (17-23 mg/kg per day) or placebo for 5 years did not observe any significant decrease of serum alkaline phosphatase in the UDCA-treated patients. There was no significant benefit from UDCA on survival without liver transplantation or prevention of cholangiocarcinoma, but the study was too small to exclude a significant beneficial effect on survival. A large, multicenter National Institutes of Health sponsored randomized trial of high-dose UDCA is currently underway<sup>[86]</sup>.

### ***Combination therapy***

Combination therapy is a relatively new avenue of clinical research in the treatment of chronic cholestatic diseases. Drugs in monotherapy are often limited by efficacy and dose-related toxicity; combination therapy may hold the potential for improved efficacy through additive or synergistic effects, with the potential minimization of drug toxicities<sup>[87]</sup>. A controlled but nonrandomized study with 12 patients treated with a combination of low-dose prednisolone and colchicine failed to find any benefit in PSC<sup>[88]</sup>. The combination of UDCA and methotrexate was studied in 19 patients with



PSC and no changes in biochemistries from baseline values were seen compared to patients receiving UDCA alone<sup>[89]</sup>. An 8-wk pilot study evaluating the combination of prednisone or budesonide combined with UDCA failed to demonstrate significant beneficial effects to justify its use in patients with PSC<sup>[90]</sup>. In a small study with 15 patients, positive results were obtained with the combination of UDCA, prednisolone and azathioprine in decreasing liver biochemistry values<sup>[91]</sup>, but evidence supporting long-term use of this therapy is lacking. Most recently, a study with 80 PSC patients randomized to either UDCA alone or the combination of metronidazole and UDCA showed that combination therapy led to improved serum alkaline phosphatase and Mayo Risk Score, but no significant effect on disease progression compared to UDCA alone<sup>[92]</sup>.

### **Innovative approaches to medical therapy**

Trials of antibiotics such as metronidazole and minocycline have been promising but inconclusive. A small study of docosahexaenoic acid (DHA) which improves CFTR function<sup>[93]</sup> is currently underway. Most promising for the near future are inhibitors of TNF action, antifibrotic agents (such as angiotensin-converting enzyme [ACE] inhibitors, sirolimus/rapamycin), and inhibitors of formation of toxic bile (such as 24-norursodeoxycholic acid)<sup>[94]</sup>.

### **Endoscopic therapy**

Some patients present with clinical and biochemical deterioration and exhibit a dominant stricture that involves the larger extrahepatic biliary ducts. Such lesions may be amenable to endoscopic or radiologic dilatation with or without a biliary drainage procedure, such as sphincterotomy and stenting<sup>[45]</sup>. This leads to improvement of clinical symptoms, liver biochemistries and cholangiographic findings. However, the endoscopic treatment of PSC has generated controversy, not only with regard to optimal management, but also its overall influence on survival. The use of endobiliary stents has been compared to balloon dilatation alone in patients with PSC<sup>[95,96]</sup>, and a greater frequency of intervention-related complications including acute cholangitis was observed in patients with endobiliary stent placement. Repeated balloon dilatations of dominant biliary strictures resulted in improved actual survival rates compared to survival rates predicted by Mayo risk score<sup>[97,98]</sup>.

### **Biliary surgery**

Dominant strictures can also be managed surgically by dilatation or choledochojejunostomy, but this treatment has become uncommon with more recent advancement of endoscopic techniques and growing success of liver transplantation. At present, biliary surgery in patients with PSC, other than simple cholecystectomy, should be minimized and reserved for the selected rare noncirrhotic patients who have marked cholestasis or recurrent cholangitis caused by a dominant extrahepatic or hilar stricture not amenable to endoscopic or percutaneous dilatation<sup>[45]</sup>. In patients who may undergo

liver transplantation, prior biliary surgery has been associated with a significantly longer operation time, greater intraoperative blood loss, and a higher incidence of biliary complications post-liver transplantation compared with those patients with no history of biliary surgery<sup>[99-103]</sup>.

### **Liver transplantation**

Although PSC is an uncommon disease, advanced-stage PSC remains among the most common indications for LT in the United States and in Europe<sup>[20]</sup>. Unique circumstances that require evaluation for possible LT include recurrent bacterial cholangitis despite intensive medical and endoscopic therapy, severe extrahepatic biliary obstruction that precludes operative repair, and uncontrolled peristomal variceal bleeding. Intractable pruritus may also be an indication for liver transplantation. Liver transplantation should be considered before the disease is too advanced, in order to enhance the long-term survival rates post-liver transplantation<sup>[104]</sup>. Prognostic models can aid in the timing of liver transplantation. Reports from single centers performing LT in PSC patients have demonstrated excellent survival rates of 90%-97% at one year, and 83%-88% at 5 years<sup>[105,106]</sup>. However, retransplantation rates seem to be higher for patients with PSC than other diagnoses<sup>[3]</sup>.

Recurrence of PSC in the liver graft has been documented. Diagnosis of recurrence can be challenging, as non-specific bile duct injuries and strictures caused by allograft reperfusion injury, ischemia, rejection and recurrent biliary sepsis can mimic the findings of PSC post-transplantation and need to be carefully excluded before the diagnosis of recurrence can be established<sup>[107,108]</sup>. The frequency of recurrent PSC after liver transplantation remains controversial. The frequency of recurrent disease is estimated between 10% to 20% of patients<sup>[109]</sup>, but a recent systematic review has indicated that publication bias might be a concern regarding this topic<sup>[4]</sup>. PSC might recur earlier at a higher ratio after living donor liver transplantation, particularly when the liver graft is obtained from a biologically related living donor<sup>[110]</sup>. Proposed risk factors for recurrent PSC include inflammatory bowel disease, prolonged cold ischemia time, number of cellular rejection episodes, previous biliary surgery, cytomegalovirus infection, and lymphocytotoxic cross-match<sup>[4]</sup> but these require further investigation. As more liver transplant recipients survive longer, the recurrence of disease may become the primary cause of morbidity and mortality in PSC<sup>[4]</sup>.

Liver transplantation in autoimmune liver diseases is covered in more detail in another article in this series.

## **DISEASE-RELATED COMPLICATIONS**

### **Fatigue and pruritus**

Pruritus is a prevalent problem in patients with chronic cholestatic disease<sup>[111]</sup>. Cholestyramine is effective in 80% to 90% of the patients, and represents the first-line of treatment<sup>[112]</sup>. Other drugs that have been commonly used for the treatment of pruritus include rifampin<sup>[113]</sup>,

opioid antagonists<sup>[114,115]</sup> and ondansetron<sup>[116]</sup>. Sertraline has been demonstrated to have positive effects on pruritus in cholestatic liver disease in a small study<sup>[117]</sup>. Etanercept has also had positive effects on pruritus in patients with PSC in a small study not originally designed to primarily assess the effect of that drug on pruritus<sup>[68]</sup>. In controlled trials, UDCA has not been associated with improvement in pruritus, but those studies were not specifically designed for that purpose. Intractable pruritus may be an indication for LT<sup>[119]</sup>.

Fatigue is also noted to be a prevalent problem in patients with chronic cholestatic disease, and a major determinant of impaired health related quality of life<sup>[118]</sup>. However, no medical therapy is available for treatment.

### Metabolic bone disease

Severe bone disease in PSC patients is more common than expected, but less frequent than that reported in primary biliary cirrhosis<sup>[119]</sup>. Patients with longer duration of IBD, and more advanced liver disease were found to be at higher risk of severe osteoporosis<sup>[119]</sup>. In this same study, Angulo *et al.*<sup>[119]</sup> found that the severity of osteopenic disease in PSC seems to increase as liver disease advances, but this finding was not confirmed in a subsequent study by Campbell *et al.*<sup>[120]</sup>. Bone mineral densitometry measurements are the only test helpful in evaluating progression of osteopenia in patients with PSC, and the presence, severity and progression of the bone disease cannot be accurately evaluated by routine clinical, biochemical, or histological variables<sup>[119]</sup>. This is important since most patients with PSC and advanced liver disease undergo liver transplantation. Early post-transplant bone loss remains a clinically significant problem and frequently leads to fracturing in a third of patients with PSC when the pre-transplant bone mineral density is below the fracture threshold<sup>[119,121]</sup>.

For treatment of osteoporosis and osteopenia, calcium and vitamin D supplementation are recommended, and in selected cases, bisphosphonates may be indicated. Many new drugs have become available for the treatment of post-menopausal osteoporosis, and more studies are needed to determine the role of these treatments in primary sclerosing cholangitis.

### Gallbladder stones and polyps

Cholelithiasis has been noted in 26% of individuals with PSC, with the majority being asymptomatic<sup>[122]</sup>. Consideration should be given to performing a cholecystectomy if cross-sectional imaging results in the identification of gallbladder polyps given the potential for neoplastic transformation in PSC<sup>[123]</sup>.

### Peristomal varices

A special complication of portal hypertension in PSC patients with an ileal stoma is the development of peristomal varices<sup>[45]</sup>. Those develop within the adhesions between the ileal (portal) veins and the anterior abdominal wall (systemic) veins. Patients bleeding from peristomal varices often present with recurrent hemorrhagic

episodes. Bleeding from the peristomal varices is more difficult to treat than bleeding from esophageal varices. Local treatment to control and prevent bleeding is usually unsuccessful in the long term. Liver transplantation should be considered for treatment. If that is not possible, peristomal variceal bleeding can be controlled with a portosystemic shunt<sup>[2]</sup>.

### Dominant stricture

A dominant stricture, defined by Stiehl *et al.*<sup>[124]</sup> as a diameter in the common duct of less than 1.5 mm and in the hepatic duct of less than 1 mm, is a frequent finding and occurs in 45% to 58% of patients during follow-up. Stenotic lesions in PSC are thus far more often benign than malignant in nature<sup>[124]</sup>. Endoscopic treatment of dominant stenoses improves cholestasis and prolongs survival in comparison to predicted survival<sup>[97,125]</sup>. Prophylactic antibiotic administration prior to endoscopic manipulation of the biliary tree is recommended by the American Society for Gastrointestinal Endoscopy in the setting of bile duct obstruction to prevent contamination during the cannulation of the bile duct<sup>[126]</sup>.

### Bacterial cholangitis

Bacteriobilia is found in the majority of PSC patients<sup>[127]</sup>, but, as previously mentioned, bacterial cholangitis usually is not manifested until patients undergo endoscopic intervention or surgical exploration of the biliary tract. Bacterial cholangitis is common in patients with dominant stricture and requires antibiotic treatment<sup>[45]</sup>. It may also occur after endoscopic procedures or in patients with bile duct stones or tight strictures<sup>[128]</sup>, warranting prophylactic antibiotic administration prior to endoscopic manipulation of the biliary tree. Most biliary infections in patients with obstructive disease of the biliary tract are caused by aerobic enteric organisms such as *Escheria coli*, *Klebsiella* species, and *E. faecalis*<sup>[129]</sup>. Recurrent episodes of bacterial cholangitis can be an indication for liver transplantation in patients with otherwise preserved liver function. Prophylaxis with antibiotics has not been proven to be of benefit<sup>[128]</sup>, but patients with recurrent cholangitis should be advised to seek medical attention rapidly and start antibiotics at the first sign of biliary infection.

### Malignancy

**Cholangiocarcinoma:** Primary sclerosing cholangitis carries an increased risk of hepatobiliary malignancy, especially cholangiocarcinoma (CCA)<sup>[8,130]</sup>. The development of CCA is the most lethal complication of PSC. Cholangiocarcinoma can arise at any stage of PSC, although, in general, the incidence is higher in more advanced disease<sup>[5,131]</sup>. There are no clinical features that predict the diagnosis of CCA, and diagnosis can be challenging.

The cumulative life-time incidence of CCA is estimated as 6% to 23%<sup>[20]</sup>. The reported prevalence of CCA in explanted livers and autopsy is much higher, approximately 30% to 42%<sup>[9,132]</sup>. Overall, up to 50%

of CCA cases are detected synchronous with the PSC diagnosis or within one year of diagnosis of PSC<sup>[130,133-136]</sup>. Based upon the same reported series, the incidence of CCA during follow-up, starting at 1 year after the diagnosis of PSC, can be calculated as being between 0.5% and 1.5% per year. The malignancy usually develops in the fourth decade of life, whereas CCA in patients without PSC usually develops much later in life, in their seventh decade of life<sup>[20]</sup>.

Risk factors for the development of CCA in PSC have not been clearly identified, but older age, longer duration of IBD and smoking behavior have been associated with an increased risk for development of CCA in patients with PSC. Finding biliary dysplasia on liver histology has also been proposed as a precursor in the development of cholangiocarcinoma<sup>[46,47]</sup>.

The diagnosis of CCA can be challenging. The role of serum CA19-9 level in the diagnosis of CCA is controversial. There are no tumor markers which are specific for cholangiocarcinoma. In the context of PSC, a serum CA19-9 level greater than 100 U/mL has been reported to have a sensitivity of 75% and a specificity of 80% for presence of cholangiocarcinoma<sup>[137,138]</sup>. A recent study from the Mayo Clinic found that a serum level greater than 129 U/mL provided a sensitivity of 78.6% and specificity of 98.5% for CCA in PSC<sup>[139]</sup>. Even though these studies suggest CA 19-9 is an accurate test to diagnose cholangiocarcinoma, CA 19-9 was only found to identify patients with advanced, unresectable CCA, and thus its use is not appropriate as a screening test<sup>[139]</sup>. Ultrasonography, computed tomography, and magnetic resonance have inadequate sensitivity to distinguish CCA from PSC. Endoscopic biopsy and biliary brushing for cytology, digital image analysis, and fluorescent *in situ* hybridization are noted for good specificity but poor sensitivity in detecting CCA<sup>[19]</sup>.

Patients with PSC and CCA have a very poor outcome, with median survival of approximately 5 to 11 mo<sup>[20,135,140]</sup>. Even though survival of patients in whom CCA was found incidentally by histological examination of the explanted liver has been reported to be good<sup>[5]</sup>, in general, LT for patients with CCA results in a low success rate<sup>[141-144]</sup>. However, more recent data from investigational protocols have suggested better outcomes in highly selected individuals. The use of pretreatment radiotherapy and subsequent capecitabine for 2 to 3 wk prior to LT at Mayo Clinic has yielded a 3- and 5-year actuarial survival of 82%<sup>[145]</sup>. The use of brachytherapy and continuous 5-fluoracil infusion before liver transplantation in Nebraska resulted in 45% long-term cancer-free survival after follow-up for a median of 7.5 years<sup>[146]</sup>. Curative resection among individuals with early-stage cholangiocarcinoma may also be of benefit in PSC<sup>[133]</sup>, although recent data suggest that transplant with neoadjuvant chemoradiation with localized, node-negative hilar CCA may achieve better survival with less recurrence than conventional resection<sup>[145,147]</sup>.

Recent studies have suggested that the incidence of CCA in patients with PSC treated with UDCA is lower

than expected and decreases with time of therapy<sup>[141,148]</sup>. Further studies are needed to confirm this finding.

**Colonic dysplasia and carcinoma:** Whether the presence of PSC increases the risk of colonic dysplasia and carcinoma in ulcerative colitis is controversial<sup>[149,150]</sup>. Patients with PSC and UC have been found to have an increased incidence of colonic carcinomas compared to patients with ulcerative colitis alone in a few studies<sup>[136,151-153]</sup>, however, contradictory results have also been presented<sup>[154,155]</sup>. The size, design, end-points, and populations involved in these studies have varied, and critical review suggests that colorectal cancer is more common in the setting of PSC<sup>[150]</sup>. Furthermore, PSC patients with UC remain at an increased risk for developing colorectal dysplasia and carcinoma after they have undergone liver transplantation<sup>[11,156]</sup>. The immunosuppressive treatment after liver transplantation may have an impact on the development of cancer.

Two studies have indicated that UDCA reduced the incidence of colonic dysplasias and/or carcinomas<sup>[157,158]</sup>.

**Gallbladder neoplasia:** Dysplasia, adenomas and carcinoma of the gallbladder have been described in PSC but are less common than cholangiocarcinoma. PSC is recognized as one of the major risk factors for both gallbladder and bile duct carcinoma<sup>[159]</sup>. A recent study reported statistically significant association between hilar/intrahepatic biliary neoplasia and gallbladder neoplasia, suggesting a “field effect” in the intrahepatic and extrahepatic biliary tree in PSC<sup>[160]</sup>. Identification of gallbladder polyps on cross-sectional imaging should lead to consideration for cholecystectomy<sup>[123]</sup>. Large studies on this subject have not been performed.

**Hepatocellular carcinoma:** Although patients with cirrhotic stage PSC may also be at risk for developing hepatocellular carcinoma, this malignancy occurs infrequently<sup>[131,136,161]</sup>.

## PREGNANCY AND PSC

Little is known regarding the natural history and potential complications of pregnancy in patients with PSC. There are very few case reports<sup>[162,163]</sup> and one small series of thirteen pregnancies in 10 patients with PSC<sup>[164]</sup> that describe the fetal and maternal outcome of pregnancy in PSC. Although previously described, it appears that hepatic disease activity is not significantly worsened during the gestational period<sup>[2]</sup>. Nonetheless, patients with PSC who become pregnant require close monitoring<sup>[162]</sup>. Regular blood tests, including serum bilirubin and aminotransferase levels, are essential. In the event that the patient develops symptoms worrisome for obstruction, an ultrasound is a safe diagnostic test and may detect the presence of dominant strictures or stones; it lacks sensitivity however. MRC might have an emerging role in pregnancy, and more invasive tests such as ERCP might be required.

## CONCLUSION

Primary sclerosing cholangitis is a presumed immune-mediated liver disease of young men associated with significant morbidity and mortality. However, there is no proven medical treatment available for it. Further studies are needed for better understanding of the pathophysiology of the disease and for development of an optimal therapeutic strategy for patients with PSC to improve health related quality of life and halt progression of disease, thereby decreasing incidence of complications of advanced liver disease, and the need for transplantation.

## REFERENCES

- 1 Lee YM, Kaplan MM. Primary sclerosing cholangitis. *N Engl J Med* 1995; **332**: 924-933
- 2 Talwalkar JA, Lindor KD. Primary sclerosing cholangitis. *Inflamm Bowel Dis* 2005; **11**: 62-72
- 3 LaRusso NF, Shneider BL, Black D, Gores GJ, James SP, Doo E, Hoofnagle JH. Primary sclerosing cholangitis: summary of a workshop. *Hepatology* 2006; **44**: 746-764
- 4 Gautam M, Cheruvattath R, Balan V. Recurrence of autoimmune liver disease after liver transplantation: a systematic review. *Liver Transpl* 2006; **12**: 1813-1824
- 5 Farrant JM, Hayllar KM, Wilkinson ML, Karani J, Portmann BC, Westaby D, Williams R. Natural history and prognostic variables in primary sclerosing cholangitis. *Gastroenterology* 1991; **100**: 1710-1717
- 6 Chapman RW, Arborgh BA, Rhodes JM, Summerfield JA, Dick R, Scheuer PJ, Sherlock S. Primary sclerosing cholangitis: a review of its clinical features, cholangiography, and hepatic histology. *Gut* 1980; **21**: 870-877
- 7 Wiesner RH, Grambsch PM, Dickson ER, Ludwig J, MacCarty RL, Hunter EB, Fleming TR, Fisher LD, Beaver SJ, LaRusso NF. Primary sclerosing cholangitis: natural history, prognostic factors and survival analysis. *Hepatology* 1989; **10**: 430-436
- 8 Wiesner RH, LaRusso NF. Clinicopathologic features of the syndrome of primary sclerosing cholangitis. *Gastroenterology* 1980; **79**: 200-206
- 9 Broome U, Olsson R, Loof L, Bodemar G, Hultcrantz R, Danielsson A, Prytz H, Sandberg-Gertzen H, Wallerstedt S, Lindberg G. Natural history and prognostic factors in 305 Swedish patients with primary sclerosing cholangitis. *Gut* 1996; **38**: 610-615
- 10 Okolicsanyi L, Fabris L, Viaggi S, Carulli N, Podda M, Ricci G. Primary sclerosing cholangitis: clinical presentation, natural history and prognostic variables: an Italian multicentre study. The Italian PSC Study Group. *Eur J Gastroenterol Hepatol* 1996; **8**: 685-691
- 11 Broome U, Bergquist A. Primary sclerosing cholangitis, inflammatory bowel disease, and colon cancer. *Semin Liver Dis* 2006; **26**: 31-41
- 12 Rasmussen HH, Fallingborg JF, Mortensen PB, Vyberg M, Tage-Jensen U, Rasmussen SN. Hepatobiliary dysfunction and primary sclerosing cholangitis in patients with Crohn's disease. *Scand J Gastroenterol* 1997; **32**: 604-610
- 13 Fausa O, Schrumpf E, Elgjo K. Relationship of inflammatory bowel disease and primary sclerosing cholangitis. *Semin Liver Dis* 1991; **11**: 31-39
- 14 Rabinovitz M, Gavalier JS, Schade RR, Dindzans VJ, Chien MC, Van Thiel DH. Does primary sclerosing cholangitis occurring in association with inflammatory bowel disease differ from that occurring in the absence of inflammatory bowel disease? A study of sixty-six subjects. *Hepatology* 1990; **11**: 7-11
- 15 Loftus EV Jr, Harewood GC, Loftus CG, Tremaine WJ, Harmsen WS, Zinsmeister AR, Jewell DA, Sandborn WJ. PSC-IBD: a unique form of inflammatory bowel disease associated with primary sclerosing cholangitis. *Gut* 2005; **54**: 91-96
- 16 Saarinen S, Olerup O, Broome U. Increased frequency of autoimmune diseases in patients with primary sclerosing cholangitis. *Am J Gastroenterol* 2000; **95**: 3195-3199
- 17 Kaplan GG, Laupland KB, Butzner D, Urbanski SJ, Lee SS. The burden of large and small duct primary sclerosing cholangitis in adults and children: a population-based analysis. *Am J Gastroenterol* 2007; **102**: 1042-1049
- 18 Bergquist A, Said K, Broome U. Changes over a 20-year period in the clinical presentation of primary sclerosing cholangitis in Sweden. *Scand J Gastroenterol* 2007; **42**: 88-93
- 19 Charatcharoenwitthaya P, Lindor KD. Primary sclerosing cholangitis: diagnosis and management. *Curr Gastroenterol Rep* 2006; **8**: 75-82
- 20 Bjornsson E, Angulo P. Cholangiocarcinoma in young individuals with and without primary sclerosing cholangitis. *Am J Gastroenterol* 2007; **102**: 1677-1682
- 21 Younossi ZM, Boparai N, Price LL, Kiwi ML, McCormick M, Guyatt G. Health-related quality of life in chronic liver disease: the impact of type and severity of disease. *Am J Gastroenterol* 2001; **96**: 2199-2205
- 22 Kim WR, Lindor KD, Malinchoc M, Petz JL, Jorgensen R, Dickson ER. Reliability and validity of the NIDDK-QA instrument in the assessment of quality of life in ambulatory patients with cholestatic liver disease. *Hepatology* 2000; **32**: 924-929
- 23 Cullen SN, Chapman RW. Review article: current management of primary sclerosing cholangitis. *Aliment Pharmacol Ther* 2005; **21**: 933-948
- 24 Dickson ER, Murtaugh PA, Wiesner RH, Grambsch PM, Fleming TR, Ludwig J, LaRusso NF, Malinchoc M, Chapman RW, Kaplan MM. Primary sclerosing cholangitis: refinement and validation of survival models. *Gastroenterology* 1992; **103**: 1893-1901
- 25 Kim WR, Therneau TM, Wiesner RH, Poterucha JJ, Benson JT, Malinchoc M, LaRusso NF, Lindor KD, Dickson ER. A revised natural history model for primary sclerosing cholangitis. *Mayo Clin Proc* 2000; **75**: 688-694
- 26 Kamath PS, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, D'Amico G, Dickson ER, Kim WR. A model to predict survival in patients with end-stage liver disease. *Hepatology* 2001; **33**: 464-470
- 27 Chapman RW, Cottone M, Selby WS, Shepherd HA, Sherlock S, Jewell DP. Serum autoantibodies, ulcerative colitis and primary sclerosing cholangitis. *Gut* 1986; **27**: 86-91
- 28 Mulder AH, Horst G, Haagsma EB, Limburg PC, Kleibeuker JH, Kallenberg CG. Prevalence and characterization of neutrophil cytoplasmic antibodies in autoimmune liver diseases. *Hepatology* 1993; **17**: 411-417
- 29 Bansi D, Chapman R, Fleming K. Antineutrophil cytoplasmic antibodies in chronic liver diseases: prevalence, titre, specificity and IgG subclass. *J Hepatol* 1996; **24**: 581-586
- 30 Chapman RW. The enigma of anti-neutrophil antibodies in ulcerative colitis primary sclerosing cholangitis: important genetic marker or epiphenomenon? *Hepatology* 1995; **21**: 1473-1474
- 31 Wiesner RH. Current concepts in primary sclerosing cholangitis. *Mayo Clin Proc* 1994; **69**: 969-982
- 32 Bilbao MK, Dotter CT, Lee TG, Katon RM. Complications of endoscopic retrograde cholangiopancreatography (ERCP). A study of 10,000 cases. *Gastroenterology* 1976; **70**: 314-320
- 33 Freeman ML, Nelson DB, Sherman S, Haber GB, Herman ME, Dorsher PJ, Moore JP, Fennerty MB, Ryan ME, Shaw MJ, Lande JD, Pheley AM. Complications of endoscopic biliary sphincterotomy. *N Engl J Med* 1996; **335**: 909-918
- 34 Talwalkar JA, Angulo P, Johnson CD, Petersen BT, Lindor KD. Cost-minimization analysis of MRC versus ERCP for the diagnosis of primary sclerosing cholangitis. *Hepatology*



- 2004; **40**: 39-45
- 35 **Mehta SN**, Reinhold C, Barkun AN. Magnetic resonance cholangiopancreatography. *Gastrointest Endosc Clin N Am* 1997; **7**: 247-270
  - 36 **Fulcher AS**, Turner MA, Franklin KJ, Shiffman ML, Sterling RK, Luketic VA, Sanyal AJ. Primary sclerosing cholangitis: evaluation with MR cholangiography-a case-control study. *Radiology* 2000; **215**: 71-80
  - 37 **Moff SL**, Kamel IR, Eustace J, Lawler LP, Kantsevov S, Kalloo AN, Thuluvath PJ. Diagnosis of primary sclerosing cholangitis: a blinded comparative study using magnetic resonance cholangiography and endoscopic retrograde cholangiography. *Gastrointest Endosc* 2006; **64**: 219-223
  - 38 **Petrovic BD**, Nikolaidis P, Hammond NA, Martin JA, Petrovic PV, Desai PM, Miller FH. Correlation Between Findings on MRCP and Gadolinium-Enhanced MR of the Liver and a Survival Model for Primary Sclerosing Cholangitis. *Dig Dis Sci* 2007; **52**: 3499-3506
  - 39 **Angulo P**, Pearce DH, Johnson CD, Henry JJ, LaRusso NF, Petersen BT, Lindor KD. Magnetic resonance cholangiography in patients with biliary disease: its role in primary sclerosing cholangitis. *J Hepatol* 2000; **33**: 520-527
  - 40 **Ludwig J**. Surgical pathology of the syndrome of primary sclerosing cholangitis. *Am J Surg Pathol* 1989; **13** Suppl 1: 43-49
  - 41 **Scheuer PJ**. Ludwig Symposium on biliary disorders--part II. Pathologic features and evolution of primary biliary cirrhosis and primary sclerosing cholangitis. *Mayo Clin Proc* 1998; **73**: 179-183
  - 42 **Burak KW**, Angulo P, Lindor KD. Is there a role for liver biopsy in primary sclerosing cholangitis? *Am J Gastroenterol* 2003; **98**: 1155-1158
  - 43 **Gossard AA**, Angulo P, Lindor KD. Secondary sclerosing cholangitis: a comparison to primary sclerosing cholangitis. *Am J Gastroenterol* 2005; **100**: 1330-1333
  - 44 **Ludwig J**, Barham SS, LaRusso NF, Elveback LR, Wiesner RH, McCall JT. Morphologic features of chronic hepatitis associated with primary sclerosing cholangitis and chronic ulcerative colitis. *Hepatology* 1981; **1**: 632-640
  - 45 **Angulo P**, Lindor KD. Primary sclerosing cholangitis. *Hepatology* 1999; **30**: 325-332
  - 46 **Bergquist A**, Glaumann H, Stal P, Wang GS, Broome U. Biliary dysplasia, cell proliferation and nuclear DNA-fragmentation in primary sclerosing cholangitis with and without cholangiocarcinoma. *J Intern Med* 2001; **249**: 69-75
  - 47 **Fleming KA**, Boberg KM, Glaumann H, Bergquist A, Smith D, Clausen OP. Biliary dysplasia as a marker of cholangiocarcinoma in primary sclerosing cholangitis. *J Hepatol* 2001; **34**: 360-365
  - 48 **Bjornsson E**, Chapman RW. Sclerosing cholangitis. *Curr Opin Gastroenterol* 2003; **19**: 270-275
  - 49 **Kim WR**, Ludwig J, Lindor KD. Variant forms of cholestatic diseases involving small bile ducts in adults. *Am J Gastroenterol* 2000; **95**: 1130-1138
  - 50 **Bjornsson E**, Boberg KM, Cullen S, Fleming K, Clausen OP, Fausa O, Schrumpf E, Chapman RW. Patients with small duct primary sclerosing cholangitis have a favourable long term prognosis. *Gut* 2002; **51**: 731-735
  - 51 **Boberg KM**, Schrumpf E, Fausa O, Elgjo K, Kolmannskog F, Haaland T, Holter E. Hepatobiliary disease in ulcerative colitis. An analysis of 18 patients with hepatobiliary lesions classified as small-duct primary sclerosing cholangitis. *Scand J Gastroenterol* 1994; **29**: 744-752
  - 52 **Angulo P**, Maor-Kendler Y, Lindor KD. Small-duct primary sclerosing cholangitis: a long-term follow-up study. *Hepatology* 2002; **35**: 1494-1500
  - 53 **van Buuren HR**, van Hoogstraten HJE, Terkivatan T, Schalm SW, Vleggaar FP. High prevalence of autoimmune hepatitis among patients with primary sclerosing cholangitis. *J Hepatol* 2000; **33**: 543-548
  - 54 **Bjornsson E**, Chari ST, Smyrk TC, Lindor K. Immunoglobulin G4 associated cholangitis: description of an emerging clinical entity based on review of the literature. *Hepatology* 2007; **45**: 1547-1554
  - 55 **Chari ST**, Smyrk TC, Levy MJ, Topazian MD, Takahashi N, Zhang L, Clain JE, Pearson RK, Petersen BT, Vege SS, Farnell MB. Diagnosis of autoimmune pancreatitis: the Mayo Clinic experience. *Clin Gastroenterol Hepatol* 2006; **4**: 1010-1016; quiz 934
  - 56 **Okazaki K**, Uchida K, Chiba T. Recent concept of autoimmune-related pancreatitis. *J Gastroenterol* 2001; **36**: 293-302
  - 57 **Kamisawa T**, Egawa N, Nakajima H, Tsuruta K, Okamoto A. Extrapaneatic lesions in autoimmune pancreatitis. *J Clin Gastroenterol* 2005; **39**: 904-907
  - 58 **Zen Y**, Harada K, Sasaki M, Sato Y, Tsuneyama K, Haratake J, Kurumaya H, Katayanagi K, Masuda S, Niwa H, Morimoto H, Miwa A, Uchiyama A, Portmann BC, Nakanuma Y. IgG4-related sclerosing cholangitis with and without hepatic inflammatory pseudotumor, and sclerosing pancreatitis-associated sclerosing cholangitis: do they belong to a spectrum of sclerosing pancreatitis? *Am J Surg Pathol* 2004; **28**: 1193-1203
  - 59 **Mendes FD**, Jorgensen R, Keach J, Katzmman JA, Smyrk T, Donlinger J, Chari S, Lindor KD. Elevated serum IgG4 concentration in patients with primary sclerosing cholangitis. *Am J Gastroenterol* 2006; **101**: 2070-2075
  - 60 **LaRusso NF**, Wiesner RH, Ludwig J, MacCarty RL, Beaver SJ, Zinsmeister AR. Prospective trial of penicillamine in primary sclerosing cholangitis. *Gastroenterology* 1988; **95**: 1036-1042
  - 61 **Knox TA**, Kaplan MM. A double-blind controlled trial of oral-pulse methotrexate therapy in the treatment of primary sclerosing cholangitis. *Gastroenterology* 1994; **106**: 494-499
  - 62 **Angulo P**, Batts KP, Jorgensen RA, LaRusso NA, Lindor KD. Oral budesonide in the treatment of primary sclerosing cholangitis. *Am J Gastroenterol* 2000; **95**: 2333-2337
  - 63 **Olsson R**, Broome U, Danielsson A, Hagerstrand I, Jarnerot G, Loof L, Prytz H, Ryden BO, Wallerstedt S. Colchicine treatment of primary sclerosing cholangitis. *Gastroenterology* 1995; **108**: 1199-1203
  - 64 **Duchini A**, Younossi ZM, Saven A, Bordin GM, Knowles HJ, Pockros PJ. An open-label pilot trial of cladribine (2-chlorodeoxyadenosine) in patients with primary sclerosing cholangitis. *J Clin Gastroenterol* 2000; **31**: 292-296
  - 65 **Sandborn WJ**, Wiesner RH, Tremaine WJ, Larusso NF. Ulcerative colitis disease activity following treatment of associated primary sclerosing cholangitis with cyclosporin. *Gut* 1993; **34**: 242-246
  - 66 **Talwalkar JA**, Angulo P, Keach JC, Petz JL, Jorgensen RA, Lindor KD. Mycophenolate mofetil for the treatment of primary sclerosing cholangitis. *Am J Gastroenterol* 2005; **100**: 308-312
  - 67 **Sterling RK**, Salvatori JJ, Luketic VA, Sanyal AJ, Fulcher AS, Stravitz RT, Contos MJ, Mills AS, Shiffman ML. A prospective, randomized-controlled pilot study of ursodeoxycholic acid combined with mycophenolate mofetil in the treatment of primary sclerosing cholangitis. *Aliment Pharmacol Ther* 2004; **20**: 943-949
  - 68 **Epstein MP**, Kaplan MM. A pilot study of etanercept in the treatment of primary sclerosing cholangitis. *Dig Dis Sci* 2004; **49**: 1-4
  - 69 **Angulo P**, Bharucha AE, Jorgensen RA, DeSotel CK, Sandborn WJ, Larusso NF, Lindor KD. Oral nicotine in treatment of primary sclerosing cholangitis: a pilot study. *Dig Dis Sci* 1999; **44**: 602-607
  - 70 **Vleggaar FP**, van Buuren HR, van Berge Henegouwen GP, Hop WC, van Erpecum KJ. No beneficial effects of transdermal nicotine in patients with primary sclerosing cholangitis: results of a randomized double-blind placebo-controlled cross-over study. *Eur J Gastroenterol Hepatol* 2001; **13**: 171-175
  - 71 **Angulo P**. Silymarin in the treatment of primary sclerosing cholangitis: a pilot study. *Gastroenterology* 2001; **120** (suppl 1):

- A353
- 72 **Angulo P**, MacCarty RL, Sylvestre PB, Jorgensen RA, Wiesner RH, LaRusso NA, Lindor KD. Pirfenidone in the treatment of primary sclerosing cholangitis. *Dig Dis Sci* 2002; **47**: 157-61
  - 73 **Bharucha AE**, Jorgensen R, Lichtman SN, LaRusso NF, Lindor KD. A pilot study of pentoxifylline for the treatment of primary sclerosing cholangitis. *Am J Gastroenterol* 2000; **95**: 2338-2342
  - 74 **Van Thiel DH**, Carroll P, Abu-Elmagd K, Rodriguez-Rilo H, Irish W, McMichael J, Starzl TE. Tacrolimus (FK 506), a treatment for primary sclerosing cholangitis: results of an open-label preliminary trial. *Am J Gastroenterol* 1995; **90**: 455-459
  - 75 **Talwalkar JA**, Gossard AA, Keach JC, Jorgensen RA, Petz JL, Lindor RN. Tacrolimus for the treatment of primary sclerosing cholangitis. *Liver Int* 2007; **27**: 451-453
  - 76 **Beuers U**, Spengler U, Kruis W, Aydemir U, Wiebecke B, Heldwein W, Weinzierl M, Pape GR, Sauerbruch T, Paumgartner G. Ursodeoxycholic acid for treatment of primary sclerosing cholangitis: a placebo-controlled trial. *Hepatology* 1992; **16**: 707-714
  - 77 **Lindor KD**. Ursodiol for primary sclerosing cholangitis. Mayo Primary Sclerosing Cholangitis-Ursodeoxycholic Acid Study Group. *N Engl J Med* 1997; **336**: 691-695
  - 78 **O'Brien CB**, Senior JR, Arora-Mirchandani R, Batta AK, Salen G. Ursodeoxycholic acid for the treatment of primary sclerosing cholangitis: a 30-month pilot study. *Hepatology* 1991; **14**: 838-847
  - 79 **Stiehl A**, Walker S, Stiehl L, Rudolph G, Hofmann WJ, Theilmann L. Effect of ursodeoxycholic acid on liver and bile duct disease in primary sclerosing cholangitis. A 3-year pilot study with a placebo-controlled study period. *J Hepatol* 1994; **20**: 57-64
  - 80 **De Maria N**, Colantoni A, Rosenbloom E, Van Thiel DH. Ursodeoxycholic acid does not improve the clinical course of primary sclerosing cholangitis over a 2-year period. *Hepatogastroenterology* 1996; **43**: 1472-1479
  - 81 **van Hoogstraten HJ**, Wolfhagen FH, van de Meeberg PC, Kuiper H, Nix GA, Bex MC, Hoek AC, van Houte DP, Rijk MC, Salemans JM, Scherpenisse J, Schrijver M, Smit AM, Spoelstra P, Stadhouders PH, Tan TG, Hop WC, ten Kate FJ, vanBerge-Henegouwen GP, Schalm SW, van Buuren HR. Ursodeoxycholic acid therapy for primary sclerosing cholangitis: results of a 2-year randomized controlled trial to evaluate single versus multiple daily doses. *J Hepatol* 1998; **29**: 417-423
  - 82 **Mitchell SA**, Bansi DS, Hunt N, Von Bergmann K, Fleming KA, Chapman RW. A preliminary trial of high-dose ursodeoxycholic acid in primary sclerosing cholangitis. *Gastroenterology* 2001; **121**: 900-907
  - 83 **Harnois DM**, Angulo P, Jorgensen RA, Larusso NF, Lindor KD. High-dose ursodeoxycholic acid as a therapy for patients with primary sclerosing cholangitis. *Am J Gastroenterol* 2001; **96**: 1558-1562
  - 84 **Okolicsanyi L**, Groppo M, Floreani A, Morselli-Labate AM, Rusticali AG, Battocchia A, Colombo M, Galatola G, Gasbarrini G, Podda M, Ricci G, Rosina F, Zuin M. Treatment of primary sclerosing cholangitis with low-dose ursodeoxycholic acid: results of a retrospective Italian multicentre survey. *Dig Liver Dis* 2003; **35**: 325-331
  - 85 **Olsson R**, Boberg KM, de Muckadell OS, Lindgren S, Hultcrantz R, Folvik G, Bell H, Gangsoy-Kristiansen M, Matre J, Rydning A, Wikman O, Danielsson A, Sandberg-Gertzen H, Ung KA, Eriksson A, Loof L, Prytz H, Marschall HU, Broome U. High-dose ursodeoxycholic acid in primary sclerosing cholangitis: a 5-year multicenter, randomized, controlled study. *Gastroenterology* 2005; **129**: 1464-1472
  - 86 **Hoofnagle JH**. Primary sclerosing cholangitis. *Hepatology* 2005; **41**: 955
  - 87 **Fong DG**, Lindor KD. Future directions in the medical treatment of primary sclerosing cholangitis: the need for combination drug therapy. *Am J Gastroenterol* 2000; **95**: 1861-1862
  - 88 **Lindor KD**, Wiesner RH, Colwell LJ, Steiner B, Beaver S, LaRusso NF. The combination of prednisone and colchicine in patients with primary sclerosing cholangitis. *Am J Gastroenterol* 1991; **86**: 57-61
  - 89 **Lindor KD**, Jorgensen RA, Anderson ML, Gores GJ, Hofmann AF, LaRusso NF. Ursodeoxycholic acid and methotrexate for primary sclerosing cholangitis: a pilot study. *Am J Gastroenterol* 1996; **91**: 511-515
  - 90 **van Hoogstraten HJ**, Vleggaar FP, Boland GJ, van Steenberg W, Griffioen P, Hop WC, van Hattum J, van Berge Henegouwen GP, Schalm SW, van Buuren HR. Budesonide or prednisone in combination with ursodeoxycholic acid in primary sclerosing cholangitis: a randomized double-blind pilot study. Belgian-Dutch PSC Study Group. *Am J Gastroenterol* 2000; **95**: 2015-2022
  - 91 **Schramm C**, Schirmacher P, Helmreich-Becker I, Gerken G, zum Buschenfelde KH, Lohse AW. Combined therapy with azathioprine, prednisolone, and ursodiol in patients with primary sclerosing cholangitis. A case series. *Ann Intern Med* 1999; **131**: 943-946
  - 92 **Farkkila M**, Karvonen AL, Nurmi H, Nuutinen H, Taavitsainen M, Pikkarainen P, Karkkainen P. Metronidazole and ursodeoxycholic acid for primary sclerosing cholangitis: a randomized placebo-controlled trial. *Hepatology* 2004; **40**: 1379-1386
  - 93 **Pall H**, Zaman MM, Andersson C, Freedman SD. Decreased peroxisome proliferator activated receptor alpha is associated with bile duct injury in cystic fibrosis transmembrane conductance regulator-/- mice. *J Pediatr Gastroenterol Nutr* 2006; **42**: 275-281
  - 94 **Fickert P**, Wagner M, Marschall HU, Fuchsbichler A, Zollner G, Tsybrovskyy O, Zatloukal K, Liu J, Waalkes MP, Cover C, Denk H, Hofmann AF, Jaeschke H, Trauner M. 24-norUrsodeoxycholic acid is superior to ursodeoxycholic acid in the treatment of sclerosing cholangitis in Mdr2 (Abcb4) knockout mice. *Gastroenterology* 2006; **130**: 465-481
  - 95 **Kaya M**, Petersen BT, Angulo P, Baron TH, Andrews JC, Gostout CJ, Lindor KD. Balloon dilation compared to stenting of dominant strictures in primary sclerosing cholangitis. *Am J Gastroenterol* 2001; **96**: 1059-1066
  - 96 **Linder S**, Soderlund C. Endoscopic therapy in primary sclerosing cholangitis: outcome of treatment and risk of cancer. *Hepatogastroenterology* 2001; **48**: 387-392
  - 97 **Stiehl A**, Rudolph G, Sauer P, Benz C, Stremmel W, Walker S, Theilmann L. Efficacy of ursodeoxycholic acid treatment and endoscopic dilation of major duct stenoses in primary sclerosing cholangitis. An 8-year prospective study. *J Hepatol* 1997; **26**: 560-566
  - 98 **Baluyut AR**, Sherman S, Lehman GA, Hoen H, Chalasani N. Impact of endoscopic therapy on the survival of patients with primary sclerosing cholangitis. *Gastrointest Endosc* 2001; **53**: 308-312
  - 99 **McEntee G**, Wiesner RH, Rosen C, Cooper J, Wahlstrom E. A comparative study of patients undergoing liver transplantation for primary sclerosing cholangitis and primary biliary cirrhosis. *Transplant Proc* 1991; **23**: 1563-1564
  - 100 **Muiesan P**, Shanmugam RP, Devlin J, Rela M, Heaton ND, Saxena R, Portmann B, Tan KC, Williams R. Orthotopic liver transplantation for primary sclerosing cholangitis. *Transplant Proc* 1994; **26**: 3574-3576
  - 101 **Farges O**, Malassagne B, Sebag M, Bismuth H. Primary sclerosing cholangitis: liver transplantation or biliary surgery. *Surgery* 1995; **117**: 146-155
  - 102 **Narumi S**, Roberts JP, Emond JC, Lake J, Ascher NL. Liver transplantation for sclerosing cholangitis. *Hepatology* 1995; **22**: 451-457
  - 103 **Ahrendt SA**, Pitt HA, Kalloo AN, Venbrux AC, Klein AS, Herlong HF, Coleman J, Lillemoe KD, Cameron JL. Primary sclerosing cholangitis: resect, dilate, or transplant? *Ann Surg* 1998; **227**: 412-423

- 104 **Nashan B**, Schlitt HJ, Tusch G, Oldhafer KJ, Ringe B, Wagner S, Pichlmayr R. Biliary malignancies in primary sclerosing cholangitis: timing for liver transplantation. *Hepatology* 1996; **23**: 1105-1111
- 105 **Roberts MS**, Angus DC, Bryce CL, Valenta Z, Weissfeld L. Survival after liver transplantation in the United States: a disease-specific analysis of the UNOS database. *Liver Transpl* 2004; **10**: 886-897
- 106 **Merion RM**. When is a patient too well and when is a patient too sick for a liver transplant? *Liver Transpl* 2004; **10**: S69-S73
- 107 **Khettry U**, Keaveny A, Goldar-Najafi A, Lewis WD, Pomfret EA, Pomposelli JJ, Jenkins RL, Gordon FD. Liver transplantation for primary sclerosing cholangitis: a long-term clinicopathologic study. *Hum Pathol* 2003; **34**: 1127-1136
- 108 **Brandsaeter B**, Schrumpf E, Clausen OP, Abildgaard A, Hafsahl G, Bjoro K. Recurrent sclerosing cholangitis or ischemic bile duct lesions--a diagnostic challenge? *Liver Transpl* 2004; **10**: 1073-1074
- 109 **Gordon F**. Recurrent primary sclerosing cholangitis: Clinical diagnosis and long-term management issues. *Liver Transpl* 2006; **12**: S73-S75
- 110 **Tamura S**, Sugawara Y, Kaneko J, Matsui Y, Togashi J, Makuuchi M. Recurrence of primary sclerosing cholangitis after living donor liver transplantation. *Liver Int* 2007; **27**: 86-94
- 111 **Jones EA**, Bergasa NV. The pruritus of cholestasis. *Hepatology* 1999; **29**: 1003-1006
- 112 **Polter DE**, Gruhl V, Eigenbrodt EH, Combes B. Beneficial effect of cholestyramine in sclerosing cholangitis. *Gastroenterology* 1980; **79**: 326-333
- 113 **Tabibian N**. Rifampin as antipruritic agent in primary sclerosing cholangitis. *Am J Gastroenterol* 1989; **84**: 340
- 114 **Wolfhagen FH**, Sternieri E, Hop WC, Vitale G, Bertolotti M, Van Buuren HR. Oral naltrexone treatment for cholestatic pruritus: a double-blind, placebo-controlled study. *Gastroenterology* 1997; **113**: 1264-1269
- 115 **Bergasa NV**, Alling DW, Talbot TL, Swain MG, Yurdaydin C, Turner ML, Schmitt JM, Walker EC, Jones EA. Effects of naloxone infusions in patients with the pruritus of cholestasis. A double-blind, randomized, controlled trial. *Ann Intern Med* 1995; **123**: 161-167
- 116 **Jones EA**, Molenaar HA, Oosting J. Ondansetron and pruritus in chronic liver disease: a controlled study. *Hepatogastroenterology* 2007; **54**: 1196-1199
- 117 **Mayo MJ**, Handem I, Saldana S, Jacobe H, Getachew Y, Rush AJ. Sertraline as a first-line treatment for cholestatic pruritus. *Hepatology* 2007; **45**: 666-674
- 118 **Poupon RE**, Chretien Y, Chazouilleres O, Poupon R, Chwalow J. Quality of life in patients with primary biliary cirrhosis. *Hepatology* 2004; **40**: 489-494
- 119 **Angulo P**, Therneau TM, Jorgensen A, DeSotel CK, Egan KS, Dickson ER, Hay JE, Lindor KD. Bone disease in patients with primary sclerosing cholangitis: prevalence, severity and prediction of progression. *J Hepatol* 1998; **29**: 729-735
- 120 **Campbell MS**, Lichtenstein GR, Rhim AD, Pazianas M, Faust T. Severity of liver disease does not predict osteopenia or low bone mineral density in primary sclerosing cholangitis. *Liver Int* 2005; **25**: 311-316
- 121 **Porayko MK**, Wiesner RH, Hay JE, Krom RA, Dickson ER, Beaver S, Schwerman L. Bone disease in liver transplant recipients: incidence, timing, and risk factors. *Transplant Proc* 1991; **23**: 1462-1465
- 122 **Brandt DJ**, MacCarty RL, Charboneau JW, LaRusso NF, Wiesner RH, Ludwig J. Gallbladder disease in patients with primary sclerosing cholangitis. *AJR Am J Roentgenol* 1988; **150**: 571-574
- 123 **Leung UC**, Wong PY, Roberts RH, Koea JB. Gall bladder polyps in sclerosing cholangitis: does the 1-cm rule apply? *ANZ J Surg* 2007; **77**: 355-357
- 124 **Stiehl A**, Rudolph G, Kloters-Plachky P, Sauer P, Walker S. Development of dominant bile duct stenoses in patients with primary sclerosing cholangitis treated with ursodeoxycholic acid: outcome after endoscopic treatment. *J Hepatol* 2002; **36**: 151-156
- 125 **Stiehl A**, Rost D. Endoscopic treatment of dominant stenoses in patients with primary sclerosing cholangitis. *Clin Rev Allergy Immunol* 2005; **28**: 159-165
- 126 **Hirota WK**, Petersen K, Baron TH, Goldstein JL, Jacobson BC, Leighton JA, Mallory JS, Waring JP, Fanelli RD, Wheeler-Harborough J, Faigel DO. Guidelines for antibiotic prophylaxis for GI endoscopy. *Gastrointest Endosc* 2003; **58**: 475-482
- 127 **Bjornsson ES**, Kilander AF, Olsson RG. Bile duct bacterial isolates in primary sclerosing cholangitis and certain other forms of cholestasis--a study of bile cultures from ERCP. *Hepatogastroenterology* 2000; **47**: 1504-1508
- 128 **Lee YM**, Kaplan MM. Management of primary sclerosing cholangitis. *Am J Gastroenterol* 2002; **97**: 528-534
- 129 **Pohl J**, Ring A, Stremmel W, Stiehl A. The role of dominant stenoses in bacterial infections of bile ducts in primary sclerosing cholangitis. *Eur J Gastroenterol Hepatol* 2006; **18**: 69-74
- 130 **Burak K**, Angulo P, Pasha TM, Egan K, Petz J, Lindor KD. Incidence and risk factors for cholangiocarcinoma in primary sclerosing cholangitis. *Am J Gastroenterol* 2004; **99**: 523-526
- 131 **Harnois DM**, Gores GJ, Ludwig J, Steers JL, LaRusso NF, Wiesner RH. Are patients with cirrhotic stage primary sclerosing cholangitis at risk for the development of hepatocellular cancer? *J Hepatol* 1997; **27**: 512-516
- 132 **Wee A**, Ludwig J, Coffey RJ Jr, LaRusso NF, Wiesner RH. Hepatobiliary carcinoma associated with primary sclerosing cholangitis and chronic ulcerative colitis. *Hum Pathol* 1985; **16**: 719-726
- 133 **Kaya M**, de Groen PC, Angulo P, Nagorney DM, Gunderson LL, Gores GJ, Haddock MG, Lindor KD. Treatment of cholangiocarcinoma complicating primary sclerosing cholangitis: the Mayo Clinic experience. *Am J Gastroenterol* 2001; **96**: 1164-1169
- 134 **Boberg KM**, Bergquist A, Mitchell S, Pares A, Rosina F, Broome U, Chapman R, Fausa O, Egeland T, Rocca G, Schrumpf E. Cholangiocarcinoma in primary sclerosing cholangitis: risk factors and clinical presentation. *Scand J Gastroenterol* 2002; **37**: 1205-1211
- 135 **Ahrendt SA**, Pitt HA, Nakeeb A, Klein AS, Lillemoe KD, Kalloo AN, Cameron JL. Diagnosis and management of cholangiocarcinoma in primary sclerosing cholangitis. *J Gastrointest Surg* 1999; **3**: 357-367; discussion 367-368
- 136 **Bergquist A**, Ekblom A, Olsson R, Kornfeldt D, Loof L, Danielsson A, Hultcrantz R, Lindgren S, Prytz H, Sandberg-Gertzén H, Almer S, Granath F, Broome U. Hepatic and extrahepatic malignancies in primary sclerosing cholangitis. *J Hepatol* 2002; **36**: 321-327
- 137 **Ramage JK**, Donaghy A, Farrant JM, Iorns R, Williams R. Serum tumor markers for the diagnosis of cholangiocarcinoma in primary sclerosing cholangitis. *Gastroenterology* 1995; **108**: 865-869
- 138 **Hultcrantz R**, Olsson R, Danielsson A, Järnerot G, Loof L, Ryden BO, Wahren B, Broome U. A 3-year prospective study on serum tumor markers used for detecting cholangiocarcinoma in patients with primary sclerosing cholangitis. *J Hepatol* 1999; **30**: 669-673
- 139 **Levy C**, Lymp J, Angulo P, Gores GJ, Larusso N, Lindor KD. The value of serum CA 19-9 in predicting cholangiocarcinomas in patients with primary sclerosing cholangitis. *Dig Dis Sci* 2005; **50**: 1734-1740
- 140 **Feverly J**, Verslype C, Lai G, Aerts R, Van Steenberghe W. Incidence, diagnosis, and therapy of cholangiocarcinoma in patients with primary sclerosing cholangitis. *Dig Dis Sci* 2007; **52**: 3123-3135
- 141 **Brandsaeter B**, Isoniemi H, Broome U, Olausson M, Backman L, Hansen B, Schrumpf E, Oksanen A, Ericzon BG,

- Hockerstedt K, Makisalo H, Kirkegaard P, Friman S, Bjoro K. Liver transplantation for primary sclerosing cholangitis; predictors and consequences of hepatobiliary malignancy. *J Hepatol* 2004; **40**: 815-822
- 142 **Ghali P**, Marotta PJ, Yoshida EM, Bain VG, Marleau D, Peltekian K, Metrakos P, Deschenes M. Liver transplantation for incidental cholangiocarcinoma: analysis of the Canadian experience. *Liver Transpl* 2005; **11**: 1412-1416
- 143 **Robles R**, Figueras J, Turrion VS, Margarit C, Moya A, Varo E, Calleja J, Valdivieso A, Valdecasas JC, Lopez P, Gomez M, de Vicente E, Loinaz C, Santoyo J, Fleitas M, Bernardos A, Llado L, Ramirez P, Bueno FS, Jaurrieta E, Parrilla P. Spanish experience in liver transplantation for hilar and peripheral cholangiocarcinoma. *Ann Surg* 2004; **239**: 265-271
- 144 **Meyer CG**, Penn I, James L. Liver transplantation for cholangiocarcinoma: results in 207 patients. *Transplantation* 2000; **69**: 1633-1637
- 145 **Rea DJ**, Heimbach JK, Rosen CB, Haddock MG, Alberts SR, Kremers WK, Gores GJ, Nagorney DM. Liver transplantation with neoadjuvant chemoradiation is more effective than resection for hilar cholangiocarcinoma. *Ann Surg* 2005; **242**: 451-458; discussion 458-461
- 146 **Sudan D**, DeRoover A, Chinnakotla S, Fox I, Shaw B Jr, McCashland T, Sorrell M, Tempero M, Langnas A. Radiochemotherapy and transplantation allow long-term survival for nonresectable hilar cholangiocarcinoma. *Am J Transplant* 2002; **2**: 774-779
- 147 **Heimbach JK**, Gores GJ, Nagorney DM, Rosen CB. Liver transplantation for perihilar cholangiocarcinoma after aggressive neoadjuvant therapy: a new paradigm for liver and biliary malignancies? *Surgery* 2006; **140**: 331-334
- 148 **Rudolph G**, Kloeters-Plachky P, Rost D, Stiehl A. The incidence of cholangiocarcinoma in primary sclerosing cholangitis after long-time treatment with ursodeoxycholic acid. *Eur J Gastroenterol Hepatol* 2007; **19**: 487-491
- 149 **Broome U**, Chapman RW. Ulcerative colitis: sclerosing cholangitis today, cancer tomorrow? *Gut* 1997; **41**: 571-572
- 150 **Jayaram H**, Satsangi J, Chapman RW. Increased colorectal neoplasia in chronic ulcerative colitis complicated by primary sclerosing cholangitis: fact or fiction? *Gut* 2001; **48**: 430-434
- 151 **Broome U**, Lofberg R, Veress B, Eriksson LS. Primary sclerosing cholangitis and ulcerative colitis: evidence for increased neoplastic potential. *Hepatology* 1995; **22**: 1404-1408
- 152 **Broome U**, Lindberg G, Lofberg R. Primary sclerosing cholangitis in ulcerative colitis--a risk factor for the development of dysplasia and DNA aneuploidy? *Gastroenterology* 1992; **102**: 1877-1880
- 153 **Kornfeld D**, Ekblom A, Ihre T. Is there an excess risk for colorectal cancer in patients with ulcerative colitis and concomitant primary sclerosing cholangitis? A population based study. *Gut* 1997; **41**: 522-525
- 154 **Loftus EV Jr**, Sandborn WJ, Tremaine WJ, Mahoney DW, Zinsmeister AR, Offord KP, Melton LJ 3rd. Risk of colorectal neoplasia in patients with primary sclerosing cholangitis. *Gastroenterology* 1996; **110**: 432-440
- 155 **Nuako KW**, Ahlquist DA, Sandborn WJ, Mahoney DW, Siems DM, Zinsmeister AR. Primary sclerosing cholangitis and colorectal carcinoma in patients with chronic ulcerative colitis: a case-control study. *Cancer* 1998; **82**: 822-826
- 156 **Loftus EV Jr**, Aguilar HI, Sandborn WJ, Tremaine WJ, Krom RA, Zinsmeister AR, Graziadei IW, Wiesner RH. Risk of colorectal neoplasia in patients with primary sclerosing cholangitis and ulcerative colitis following orthotopic liver transplantation. *Hepatology* 1998; **27**: 685-690
- 157 **Tung BY**, Emond MJ, Haggitt RC, Bronner MP, Kimmey MB, Kowdley KV, Brentnall TA. Ursodiol use is associated with lower prevalence of colonic neoplasia in patients with ulcerative colitis and primary sclerosing cholangitis. *Ann Intern Med* 2001; **134**: 89-95
- 158 **Pardi DS**, Loftus EV Jr, Kremers WK, Keach J, Lindor KD. Ursodeoxycholic acid as a chemopreventive agent in patients with ulcerative colitis and primary sclerosing cholangitis. *Gastroenterology* 2003; **124**: 889-893
- 159 **Mir-Madjlessi SH**, Farmer RG, Sivak MV Jr. Bile duct carcinoma in patients with ulcerative colitis. Relationship to sclerosing cholangitis: report of six cases and review of the literature. *Dig Dis Sci* 1987; **32**: 145-154
- 160 **Lewis JT**, Talwalkar JA, Rosen CB, Smyrk TC, Abraham SC. Prevalence and risk factors for gallbladder neoplasia in patients with primary sclerosing cholangitis: evidence for a metaplasia-dysplasia-carcinoma sequence. *Am J Surg Pathol* 2007; **31**: 907-913
- 161 **Bergquist A**, Glaumann H, Persson B, Broome U. Risk factors and clinical presentation of hepatobiliary carcinoma in patients with primary sclerosing cholangitis: a case-control study. *Hepatology* 1998; **27**: 311-316
- 162 **Gossard AA**, Lindor KD. Pregnancy in a patient with primary sclerosing cholangitis. *J Clin Gastroenterol* 2002; **35**: 353-355
- 163 **Christensen KL**, Andersen BN, Vilstrup H. Primary sclerosing cholangitis with itching treated during pregnancy with ursodeoxycholic acid. *Ugeskr Laeger* 1997; **159**: 7151-7153
- 164 **Janczewska I**, Olsson R, Hultcrantz R, Broome U. Pregnancy in patients with primary sclerosing cholangitis. *Liver* 1996; **16**: 326-330

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## TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, Series Editors

# Etiopathogenesis of primary sclerosing cholangitis

Roger Chapman, Sue Cullen

Roger Chapman, Sue Cullen, Department of Gastroenterology, John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom

Correspondence to: Sue Cullen, MD, Department of Gastroenterology, John Radcliffe Hospital, Headington, Oxford OX3 9DU, United Kingdom. [sue.cullen@buckshosp.nhs.uk](mailto:sue.cullen@buckshosp.nhs.uk)  
Telephone: +44-1865-228756 Fax: +44-1865-751100

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## Abstract

Primary sclerosing cholangitis (PSC) is a chronic cholestatic liver disease of unknown etiology but lymphocytic portal tract infiltration is suggestive of an immune-mediated basis for this disease. Associations with inflammatory bowel disease (IBD) especially ulcerative colitis (UC), and with particular autoimmune diseases, as well as the genetic associations further suggest PSC may be an immune-mediated disease. The immunogenetics of PSC have been the subject of active research and several HLA and non-HLA associated genes have been implicated in the development of the disease. Lymphocytes derived from the inflamed gut may enter the liver *via* the enterohepatic circulation to cause hepatic disease. PSC may be triggered in genetically susceptible individuals by infections or toxins entering the portal circulation through a permeable colon and hence evoking an abnormal immune response.

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**Key words:** Autoantibody; Immunogenetics; Biliary epithelial cells; T cell receptor; Lymphocytes

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## INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic disease of the intra and/or extrahepatic bile ducts. It is characterized by a concentric obliterative fibrosis

that leads to bile duct strictures (Figure 1). In many, this in turn progresses to biliary cirrhosis and hepatic failure. Approximately one third of patients will develop cholangiocarcinoma<sup>[1]</sup>. PSC is frequently associated with inflammatory bowel disease (IBD) usually ulcerative colitis (UC) and those with Crohn's have disease predominantly affecting the colon. Approximately three quarters of the Northern European population with PSC have concomitant IBD particularly extensive UC<sup>[2]</sup>. 4.0%-7.5% of patients with UC have PSC<sup>[3]</sup>.

The term "secondary sclerosing cholangitis (SSC)" is used for a disease with similar clinical features to PSC but where a direct causative agent for the pathological process is known. Such agents include choledocholithiasis with intraductal stones, surgical damage to bile ducts, ischaemia from hepatic artery occlusion, infections, and chemical agents such as drugs. Table 1 comprises a full list of possible causes of SSC with a section also showing the conditions which can mimic sclerosing cholangitis on cholangiography. There is little good data on the natural history of SSC and very little information regarding the immunological processes occurring during the progression of SSC is known although liver biopsies often show similar changes to those of PSC with ductopenia and patchy inflammation. The remainder of this chapter will concentrate on the etiopathogenesis of PSC.

The etiology and pathogenesis of PSC remain very poorly understood. The insidious onset of the disease makes the identification of an aetiological agent very unlikely. As the disease is associated with autoantibodies and HLA haplotypes as well as being closely related to IBD it would appear to be immune mediated. An autoimmune mediated destructive process is also suggested by lymphocytic infiltration into areas of portal damage.

PSC is not however a classical autoimmune disease, as it occurs with a 2:1 male predominance compared with the female predominance found in classical autoimmune diseases such as primary biliary cirrhosis (PBC) and autoimmune hepatitis (AIH). Moreover PSC does not have the characteristic response to immunosuppressive treatment as seen in classical autoimmune disease (Table 2).

Circumstantial evidence that PSC may be immune mediated comes from the independent association of PSC with a number of autoimmune diseases. 119 patients with PSC were studied by Saarinen *et al*<sup>[4]</sup>. Each



**Figure 1** Cholangiogram showing beading and dilatation of the intra and extra hepatic bile ducts- the diagnostic features of primary sclerosing cholangitis (PSC).

**Table 1** Causes and mimics of secondary sclerosing cholangitis (SSC)

	SSC
Causes	Surgical trauma to bile ducts Ischaemic injury eg after transplantation Hepatic arterial chemotherapy eg floxuridine Intraductal gallstones <sup>[3]</sup> Viral or bacterial infection eg CMV or cryptosporidiosis Caustic injury eg formalin treatment of hydatid disease Congenital abnormalities eg cystic fibrosis
Conditions mimicking sclerosing cholangitis on imaging	Malignancy eg metastatic carcinoma Hypereosinophilic syndrome Choledochal cyst

**Table 2** The features of primary sclerosing cholangitis compared with classical autoimmune disease

Characteristic	Classical autoimmune disease	Immune-mediated inflammatory disease (such as IBD, psoriasis)	Primary sclerosing cholangitis
Age	Children and adults	Children and adults	Children and adults
Sex	Female predominance	No gender predilection	Male predominance
Autoantigens	Yes	No	No
Autoantibodies	Yes (pathogenic)	Yes (markers)	Yes (probably markers)
Associated autoimmune disease	Yes	Yes	Yes
HLA associations (class I and II)	Yes	Yes	Yes
Response to immunosuppression	Usually good	Often good	Good in children Poor in adults

PSC patient with IBD was matched to an IBD patient without PSC; 24% of the PSC patients had one or more autoimmune disorders outside the liver and colon compared with only 9% in the IBD group without PSC. Nine patients in the PSC group had 2 or more autoimmune diseases compared with only 2 in the IBD group. Diabetes mellitus and thyroid diseases were the most common in both groups. It is noteworthy that associated autoimmune disease did not seem to influence the outcome or clinical presentation of PSC<sup>[4]</sup>.

Simultaneous or sequential occurrence of PSC and AIH has been described in both adult and pediatric populations<sup>[5]</sup>. The reported prevalence of this overlap syndrome is variable from 8%-54% and depends on the age of the study population, the type of scoring system used for diagnosis and the completeness of the analysed data.

In general, sclerosing cholangitis in children is characterized by more pronounced autoimmune features with a clinical overlap with AIH. This condition “autoimmune sclerosing cholangitis in childhood” has been addressed elsewhere in this issue (see Miele Verghani).

## AUTOANTIBODIES

Atypical anti-neutrophil cytoplasmic antibodies (ANCA) are present in the serum of up to 88% patients with PSC (33%-88%)<sup>[5]</sup>. They are however not specific for PSC and are found in UC (60%-87%), and AIH (50%-96%)<sup>[6]</sup>. These ANCA are distinct from perinuclear-staining

antineutrophil cytoplasmic antibody (p-ANCA) found in microscopic polyangiitis and cytoplasmic-staining antineutrophil cytoplasmic antibody c-ANCA in Wegener's granulomatosis.

Immunoblotting showed reactivity in 92% of IBD or hepatobiliary disease patients with an atypical p-ANCA to a myeloid specific nuclear protein with a molecular mass of 50 kDa<sup>[7]</sup>. The target antigen in PSC for these atypical ANCA is probably a neutrophil nuclear envelope protein, viz tubulin-beta isotype 5<sup>[8]</sup>. Terjung and colleagues have suggested that the term p-ANNA is therefore more appropriate as the recognised antigen is not cytoplasmic but originating in the nuclear membrane<sup>[7]</sup>.

The importance of these autoantibodies in the development of PSC is unknown. Titres of ANCA correlate with disease activity in the systemic vasculitides, whereas in contrast there is a poor correlation between ANCA and clinical parameters in PSC<sup>[9-11]</sup>. Titres of ANCA remain unchanged after a transplant in PSC and after a colectomy in UC. Current evidence suggests that they are unlikely to play a role in the pathogenesis of PSC.

A high proportion of non-specific autoantibodies in addition to p-ANNA are found in patients with PSC (Table 3). They are of unclear relevance and unhelpful in diagnosis. These include anti nuclear antibodies (20%-67%), antimitochondrial antibodies (< 10%) and antithyroperoxidase antibodies (7%-16%)<sup>[5]</sup>. Anticardiolipin antibodies were found in 66% of PSC patients compared to 4% controls by Angulo but no

**Table 3** Autoantibody prevalence in primary sclerosing cholangitis

Antibody	Prevalence (%)
Anti-nuclear antibody (ANA)	7-77
Anti-smooth muscle antibody (ASMA)	13-20
Anti-endothelial cell antibody (AECA)	35
Anti-cardiolipin antibody	4-66
Thyroxperoxidase	7-16
Thyroglobulin	4
Rheumatoid factor	15

Anti-mitochondrial antibody is only rarely detected in PSC (< 10%). This is useful in differentiating PSC from primary biliary cirrhosis (PBC). Data taken from Angulo *et al*<sup>[12]</sup>.

resultant associations with thrombotic disease were demonstrated<sup>[12]</sup>.

Significantly more PSC patients have autoantibodies to surface antigens expressed on biliary epithelial cells (BEC) than patients with PBC, AIH or normal controls. These induce increased expression of CD44 on the BEC and increased production of IL-6 by BEC<sup>[13]</sup>. Anti-BEC autoantibodies may be both IgM and IgG. IL-6 induces BEC proliferation *in vitro* and suppresses BEC apoptosis, and it is increased in the bile in cholangitis and in the serum in cholangiocarcinoma. Persistent IL-6 production may be in part, responsible for the bile duct changes seen in PSC.

Antibodies to the baker's yeast, *Saccharomyces cerevisiae* (ASCA) have been reported in IBD especially active Crohn's disease. ASCA are not autoantibodies but there does seem to be some genetic predisposition to their presence. ASCA has also been seen in autoimmune liver disease including PSC but no conclusions can be drawn from their presence<sup>[14]</sup>.

## IMMUNOGENETICS

PSC is not attributable to one gene locus and is a non-Mendelian (complex) disorder. A number of associations have been made with HLA haplotypes as well as a number of other genes. There is controversy as to whether there is a primary susceptibility allele but PSC is probably acquired through inheriting a combination of genetic polymorphisms that act together to cause susceptibility to disease. The genetics of PSC is still the subject of active research.

### Major histocompatibility complex (MHC) genes in PSC

The MHC gene on the short arm of chromosome 6 encodes HLA molecules. Case control association studies have identified various HLA molecules and other immunoregulatory genes as determinants of disease susceptibility and progression in PSC. HLA molecules are highly polymorphic and have a central role in the T cell response. Class I molecules encode HLA A, B and Cw and class II encode the DR, DQ and DP families. The Class III region encodes a number of peptides which are active in the immune response including genes for TNF $\alpha$  and TNF $\beta$ , complement proteins C4, C2

**Table 4** Key HLA haplotypes in PSC<sup>[27]</sup>

	HLA haplotypes	Odds ratio
3 HLA haplotypes associated with an increased risk	B8-MICA*008-TNFA*2-DRB3*0101-DRB1*0301-DQB1*0201	2.69
	DRB3*0101-DRB1*1301-DQA1*0103-DQB1*0603	3.8
	MICA*008-DRB5*0101-DRB1*1501-DQA1*0102-DQB1*0602	1.52
	DRB4*0103-DRB4*0401-DQA1*03-DQB1*0302	0.26
3 HLA haplotypes associated with reduced risk (protective)	DRB4*0103-DRB1*0701-DQA1*0201-DQB1*0303	0.15
	MICA*002	0.12

and Bf and MHC class I chain-related (MICA) and MICB genes encoding the MHC class I chain related molecules  $\alpha$  and  $\beta$ . Normal biliary cells express HLA class I and not class II. HLA-DR, DQ and DP are aberrantly expressed on target cells in PSC.

There is an increased frequency of HLA B8 and DR3 (HLA DRB1\*0301) in PSC compared with healthy controls as first described in 1982 and then confirmed in other studies<sup>[15-17]</sup>. A later study by Donaldson showed a secondary association with DR2 in DR3 negative patients<sup>[18]</sup>. An increase in HLA-DR6 has also been observed in PSC patients<sup>[19,20]</sup>. HLA B8 and DR3 are in linkage disequilibrium. The HLA B8, DR3 haplotype is also associated with several organ specific autoimmune diseases including lupoid chronic active hepatitis, type I diabetes mellitus, myasthenia gravis and thyrotoxicosis. There is no difference in class II typing between PSC patients with and without autoimmune diseases outside the liver and colon suggesting association of PSC with autoimmune disease is not secondary to HLA but rather a primary phenomenon<sup>[4]</sup>.

HLA DR4 is less common in PSC than in control populations and the significance of this is disputed<sup>[20]</sup>. Studies have suggested that although it has a protective effect against PSC development, when present it is associated with poor prognosis and possibly cholangiocarcinoma<sup>[19,21]</sup>.

In rheumatoid arthritis (RA) more severe disease has also been seen with certain DR4 alleles. Gow described the association of RA and PSC in 4 cases<sup>[22]</sup>. In three, the liver disease was unusually progressive, proceeding to cirrhosis in 14, 18 and 48 mo from diagnosis. It has been suggested therefore that RA in association with PSC may be a marker of patients at high risk of progression to cirrhosis. PSC also needs to be considered in all RA patients with cholestatic liver tests. The DR3, DR2 heterozygote has been shown to be associated with an increased risk of death or liver transplant and a DQ6 encoding haplotype in DR3, DR2 negative individuals was associated with a reduced risk<sup>[19]</sup>.

Molecular genotyping has identified 6 haplotypes that encode for peptides involved in the immune response in PSC (Table 4)<sup>[23]</sup>.

The finding of multiple haplotypes associated with PSC indicates a complex relationship with the MHC. Susceptibility appears to involve either a combination

of *DR*, *DQ* and *MHC class I chain-like (MIC)* alleles or perhaps *MIC* alone. There is controversy concerning which allele or alleles within each haplotype may form the primary association.

*MICA* genes are a group of polymorphic genes on chromosome 6. They are localised in the class I region between *HLA-B* and *TNFA*. *MICA* molecules are stress and heat shock inducible and are expressed in non-diseased liver and on thymic and gastrointestinal epithelia. *MICA* has been identified as a ligand for  $\gamma\delta$  T cells, natural killer (NK) (CD56+) cells and cells expressing the NKG2D activatory receptor. Increased numbers of both  $\gamma\delta$  and NK cells have been documented in PSC livers<sup>[24,25]</sup>.

An association between the *MICA\*008* allele and PSC has been demonstrated by Norris *et al*<sup>[26]</sup> (which is due to an increased frequency of patients with 2 copies of this allele (i.e. homozygous). *MICA\*008* is the main allele carrying the *MICA5.1* microsatellite allele. PSC has been found to be significantly associated with both the *MICA5.1* and the *MICB24* (*MICB* microsatellite) markers. The association was lost when stratified for *DR3* or *B8* positive and negative individuals. However, *B8* and *DR3* were associated with PSC only in the presence of these markers<sup>[27]</sup>.

*MICA\*002* has a strong negative association with disease and is the functional opposite of *MICA\*008*. The *MICA\*002* allele carries the *MICA9* microsatellite allele which is also therefore less common in PSC patients compared with controls as this allele has been shown to be protective. One copy of the *MICA\*002* allele prevents PSC in most cases and so the resistant allele may be dominant<sup>[26,27]</sup>.

Bernal first concluded that genetic susceptibility to PSC might be determined by polymorphism within the *TNF* genes<sup>[28]</sup>. The *TNF- $\alpha$*  gene is located in the class III HLA region between the *HLA-B* and *DRB3* loci<sup>[29]</sup>. Increased frequency of the rare allele -308A (termed *TNF2*) of the *TNF* gene promotor has been reported in autoimmune disorders that include RA, systemic lupus erythematosus and coeliac disease. Individuals with this allele may produce high levels of *TNF- $\alpha$* . *TNF2* is in linkage disequilibrium with the extended *HLA-B8-DR3-DQ2* haplotype. The G to A substitution at position -308 in the *TNF- $\alpha$*  promotor has been shown by Mitchell *et al* to be associated with susceptibility to PSC, but this was secondary to the association with the *B8-DR3* haplotype<sup>[30]</sup>.

### Non-MHC genes in PSC

HLA haplotypes do not account for all of the susceptibility to develop PSC and genes outside the HLA region may also have a role in disease pathogenesis. Studies of non-MHC genes have failed to show an association between PSC and cytokine genes including *IL-1 $\beta$* , *IL-1RN* and *IL-10*<sup>[30,31]</sup>. The *CD95 (FAS)* gene (*TNFRSF6*), the gene encoding *CCR-5*, genes encoding *CTLA4* and the *Nod2* gene have also been examined in PSC. Karlsen *et al* have shown that genetic polymorphisms conferring susceptibility to IBD are not found in PSC/IBD patients.

viz *CARD15*, *TLR-4*, *CARD4*, *SLC22A4*, *SLC22A5*, *DLG5* and *MDR1*<sup>[32]</sup>. The chemokine receptor-5 (*CCR5*) data are contradictory. *CCR5-Delta32* is a 32 base pair deletion associated with significant reduction in cell surface expression of the receptor. Melum *et al* showed no association of *CCR5-Delta32* with susceptibility or resistance to PSC contradicting earlier reports suggesting an association<sup>[33,34]</sup>. Cytotoxic T lymphocyte antigen-4 (*CTLA-4*) is expressed on activated T lymphocytes. It is a cell surface molecule that binds to the ligand CD80 (*B.7*) on antigen presenting cells. A *CTLA-4* gene polymorphism is described in several autoimmune diseases but in PSC this remains in question. The most recent and largest study was unable to demonstrate any effect in PSC<sup>[35]</sup>.

PSC progression is related to periportal and septal fibrosis and this is associated with excess production and reduced degradation of extracellular matrix. This is regulated by a series of metalloproteinases (MMPs) and their naturally occurring inhibitors. There is a common polymorphism in the promotor sequence of the *stromelysin (MMP3)* gene with either a 5A or 6A repeat. The 5A allele is associated with increased transcription of stromelysin compared to the 6A variant. Satsangi *et al* in Oxford have found an association between the carriage rate of the 5A allele and susceptibility to PSC. 5A homozygosity was associated with development of portal hypertension<sup>[36]</sup>. This may suggest the *MMP3* 5A allele as a marker for fibrosis.

Wienke *et al* could not confirm the association of the *MMP-3 5A* allele with PSC and also found no general associations of the *MMP-1* promotor polymorphism among Norwegian patients<sup>[37]</sup>. Patients with PSC who also had UC were found however to have an increased frequency of the *MMP-3* allele 5A compared with PSC patients without UC (60% compared to 45%). All patients with cholangiocarcinoma were found to be carriers of the *MMP-1* allele 1G compared with 72% of those with PSC who did not have cholangiocarcinoma.

*Intracellular adhesion molecule-1 (ICAM-1, CD54)* gene polymorphisms have been implicated in the susceptibility to a number of inflammatory conditions, including IBD. In PSC, studies have found that patients with advanced disease express ICAM on proliferating bile ductules and interlobular bile ducts. Increased soluble ICAM levels have been found in the serum of patients with PSC probably indicating activation of the immune system and inflammatory responses<sup>[38]</sup>. Yang *et al* have shown recently that, in British patients, the *ICAM-1* polymorphism K469E is associated with PSC and may be a protective allele. This association is independent of the coexistence of IBD. There is no relationship between the *ICAM-1* genotype and the rate of PSC progression<sup>[39]</sup>. These results were not confirmed in a Scandinavian population<sup>[40]</sup>.

## CELLULAR IMMUNE ABNORMALITIES IN PSC

There is a T cell predominant portal infiltrate in PSC



although the relative proportions and importance of the CD4 and CD8 cells are not known. CD4 cells are seen more commonly in the portal tracts and CD8 cells predominate in areas of interface hepatitis<sup>[41]</sup>. The cell infiltrate may change as the disease progresses. These cells are functional and are likely to be involved in the pathogenesis of disease. In the peripheral circulation there does appear to be a fall in CD8 cells as the disease progresses. This only occurs late in disease so is unlikely to be significant in disease pathogenesis<sup>[41-44]</sup>.

Bo and colleagues showed that cell proliferation and function of liver derived T lymphocytes is impaired in PSC patients compared with liver derived T cells obtained from normal controls or patients with other autoimmune liver diseases<sup>[45]</sup>. They believe this is due to exposure to high levels of TNF *in vivo* and this exposure may be chronic.

Previously relatively high levels of TNF- $\alpha$  have been seen in T cell lines from liver biopsies in patients with different stages of PSC while decreased levels were observed in PBC patients. Therefore increased levels of TNF- $\alpha$  are present in PSC patients whether the disease be early or late stage<sup>[45-46]</sup>.

### T cells in PSC

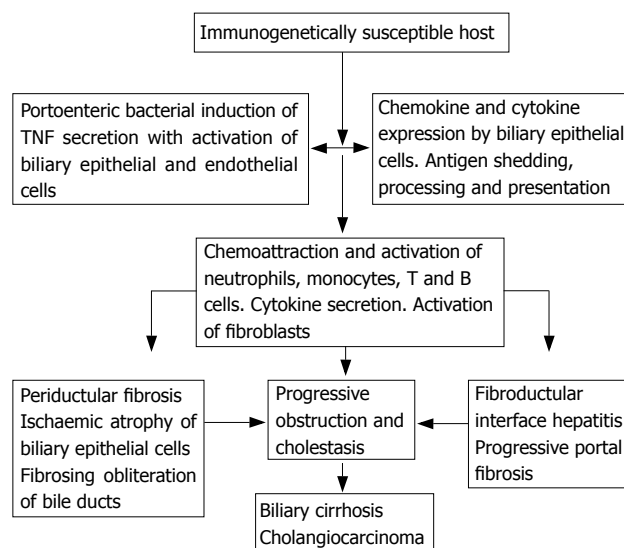
PSC is characterized by a prominent T cell infiltrate in areas of portal damage. The T cell receptor (TCR) determines the specificity of T cells. It consists of two disulphide linked polypeptides,  $\alpha$  and  $\beta$ . An alternative receptor, namely  $\gamma\delta$  has been identified. The predominant cell type is still  $\alpha\beta$  and the significance of T cells with  $\gamma\delta$  in PSC is not known<sup>[42]</sup>. TCR genes show genetic diversity but the *V $\alpha\beta$*  gene segment of the TCR can play a dominant role in recognition of certain peptide-MHC complexes. Expanded T cell populations using restricted sets of TCR *V* gene segments have been identified in areas of inflammation in the tissues affected in other immunopathic diseases such as RA and Sjogren's disease<sup>[47]</sup>. Broome reported the preferential expression in liver tissue of the V $\beta$ 3 region of the T cell receptor in PSC patients compared with liver tissue from PBC patients and healthy controls but no differences were seen in peripheral blood T cells<sup>[48]</sup>. This may indicate the presence of a specific antigen in the liver in PSC patients capable of driving the T cell production with this V $\beta$ 3 segment.

Oligoclonal T cell receptors that proliferate in culture with enterocytes and are cytotoxic to enterocyte cell lines were reported in PSC but this study is unconfirmed<sup>[49]</sup>. There are to date no studies of regulatory T cells (T regs) in PSC patients.

In summary, the available data do not as yet allow for any useful hypothesis on the T-cell contribution to the lesions of PSC.

### BEC

BEC appear to act as the target for the immune response in PSC and are also an active participant in the immune reaction. They express a number of cytokines, enzymes, intracellular adhesion molecules (ICAM-1) and HLA



**Figure 2** Vierling's hypothesis of the pathogenesis of primary sclerosing cholangitis<sup>[72]</sup>.

molecules. Normal BEC express only HLA class I and not class II whereas there is aberrant expression of class II molecules on BEC in PSC<sup>[50-52]</sup>, and also in PBC. Functionally important autoantibodies have been found to antigens on BEC in PSC. These induce BECs to produce IL-6 and increased expression of CD44. BEC however seem to lack the co stimulatory molecules necessary to activate T cells and unstimulated BEC inhibit T cell activation and this casts doubt upon the theory that BECs can act as antigen presenting cells<sup>[53,54]</sup>.

However, it has become clear that cholangiocytes rather than being passive targets may play primary roles in the pathogenesis of peribiliary inflammation and periductular fibrosis in PSC<sup>[54,55]</sup>. Stimulation by proinflammatory cytokines induces cholangiocyte secretion of multiple chemokines, cytokines, and growth factors that immunomodulate inflammation and fibrogenesis<sup>[55]</sup>. The chemoattracted T cells include a population of PSC-specific T cells primed in the gut.

### BACTERIA IN PSC

The association between PSC and IBD led to Vierling's hypothesis that colonic bacteria enter the portal circulation through a leaky mucosa in IBD thereby causing PSC (Figure 2)<sup>[55]</sup>.

Bacterial antigens may act as molecular mimics in genetically susceptible people and cause an immune reaction responsible for initiating PSC. The bacteria are able to get through gut walls made permeable by colitis or in theory by any infective episode of acute infective or inflammatory colitis. Chemokines and cytokines are then released from Kupffer cells in the liver attracting macrophages/monocytes, lymphocytes, activated neutrophils and fibroblasts to the portal tracts. Vierling further suggested that the concentric fibrosis resulting could cause atrophy of the BEC secondary to ischaemia. The bile duct loss would lead to progressive cholestasis, further fibrosis and secondary biliary

cirrhosis. This does not explain however why there are fewer PSC patients with Crohn's colitis as compared with UC and why there can be an associated stricturing of the pancreatic duct.

Portal bacteremia has been described in UC patients undergoing colectomy<sup>[56]</sup>. A study looking at explanted livers showed higher bacterial positivity rates in bile and bile ducts in PSC patients compared with PBC patients, and  $\alpha$ -haemolytic streptococci accounted for 46% of the bacterial strains found. Bile duct cannulation at endoscopic retrograde cholangiopancreatography (ERCP) could have accounted for this bacterial presence<sup>[57]</sup>. The study went on therefore to compare patients with PSC who had undergone ERCP to those who had not, in order to evaluate the potential role of these bacteria in the etiopathogenesis of PSC. Positive cultures were obtained from 3 of the naïve PSC patients and from 6 of the PSC patients with previous ERCP.  $\alpha$ -haemolytic streptococci were again the commonest bacteria seen. As most naïve PSC patients were found to have negative bacterial cultures this bacteria is unlikely to play a primary role in etiopathogenesis but may be involved in disease progression<sup>[58]</sup>.

Recent molecular studies have shown an increased prevalence of *H. pylori* and other non-gastric *Helicobacter* species in cholestatic liver diseases compared with healthy controls and noncholestatic liver disease. In PSC positivity was significantly but weakly associated with UC<sup>[59]</sup>.

Ponsioen *et al* have suggested an association between PSC and previous Chlamydia infection after the finding of an increase in seroprevalence of Chlamydia anti-lipopolysaccharide (LPS) antibodies in PSC patients, although no Chlamydia antibodies were found in liver tissue and thus the significance is unclear<sup>[60]</sup>.

Among animal models, none has yet have been developed showing all the features of PSC, although a rat model in which there is small bowel bacterial overgrowth has shown hepatic injury somewhat similar to that seen in human PSC<sup>[61,62]</sup>.

Abnormal accumulation of lipopolysaccharide (a bacterial endotoxin), presumably derived from portal blood, in the biliary epithelium has been shown in a rat model with a self-filling blind intestinal loop, and therefore may be involved in the pathogenesis of bile duct injury associated with intestinal injury<sup>[63]</sup>. Are these studies very persuasive?

## LYMPHOCYTE HOMING

PSC is strongly linked to IBD but it also runs a course independent from the bowel disease illustrated by the fact that the disease can develop many years after colectomy. Grant *et al* hypothesized that T lymphocytes generated in the gut during active inflammation persist as long-lived memory cells and undergo enterohepatic circulation and can then trigger an inflammatory response in the liver when activated by an appropriate stimulus. The nature of the stimulus remains unclear; possibilities include hepatic expression of the original priming antigen or possibly mediation solely by the

aberrant expression of gut specific adhesion molecules and chemokines<sup>[64]</sup>.

There is overlapping expression of many molecules between the gut and liver including the two potential addressins vascular adhesion protein-1 (VAP-1) and mucosal addressin cell adhesion molecule-1 (MAdCAM-1). VAP-1 expression on liver endothelium is normally far stronger than that seen on mucosal vessels. In IBD gut expression is greatly increased, suggesting that lymphocytes from the liver may be able to enter the inflamed gut using VAP-1. MAdCAM-1 endothelial expression was thought to be restricted to the gut but has been recently seen on portal endothelium in inflammatory liver disease (including PSC) associated with IBD. Mucosal lymphocytes express  $\alpha_4\beta_7$ , which allows adhesion to hepatic MAdCAM-1 suggesting it may play a role in lymphocyte recruitment<sup>[65-67]</sup>. They propose that memory lymphocyte cells recirculate between liver and gut using either/both MAdCAM-1 and VAP-1<sup>[65]</sup>.

The chemokine CCL21 activates lymphocyte adhesion to MAdCAM-1 dependent on  $\alpha_4\beta_7$ . CCL21, thought to only exist in secondary lymphoid tissue is upregulated in portal associated lymphoid tissue in PSC and plays an important role in recruiting lymphocytes. Expression of the gut-associated chemokine CCL25 (thymus-expressed chemokine (TECK)) has also been shown in PSC liver sinusoidal endothelium but was absent in liver in AIH/PBC. A significant population of CCR9+ mucosal lymphocytes (capable of binding CCL25) has been detected infiltrating PSC liver tissue compared with controls and matched peripheral blood, thus supporting the hypothesis of a T cell enterohepatic recirculation. CCR9 lymphocytes co-express the gut homing integrin  $\alpha_4\beta_7$ . Therefore CCL25 recruits CCR9+ lymphocytes to the liver in PSC by triggering adhesion to MAdCAM-1<sup>[66-67]</sup>. MAdCAM-1 and CCL25 are upregulated to the liver in inflammatory liver diseases whereas previously they were thought to be restricted to the gut. Conversely VAP-1, normally expressed in the liver, is up regulated in the gut in IBD<sup>[68]</sup>.

However this does not explain why PSC is associated more with UC than Crohn's disease, as it would be predicted that just as many memory T cells are produced in Crohn's disease as in UC.

## Hepatobiliary transporters in PSC

Defects in the hepatobiliary transport system have been shown to be the cause of a number hereditary cholestatic disorders eg progressive familial intrahepatic cholestasis and BSEP (bile salt export pump)<sup>[69]</sup>. This system is responsible for the hepatocellular uptake and excretion of bile salts into bile canaliculi. Defects in the transport system can result in bile duct injury.

Knockout mice for the *Mdr2 (Abcb4)* gene, which corresponds to human *MDR3/ABCB4*, spontaneously develop sclerosing cholangitis with features similar to human PSC<sup>[70]</sup>. A non-functional multidrug resistance 3 (MDR3) protein leads to the formation of a "toxic" bile with increased concentration of free, non-micellar bile acids which cause BEC injury, pericholangitis, periductal fibrosis and, eventually, sclerosing cholangitis. Studies in

Table 5 Comparison of PSC and AIP-SC

	PSC	AIP-SC
Gender	M:F = 2:1	Probably some male predominance <sup>[81,85,87]</sup>
Clinical presentation	Usually insidious. Sometimes with obstructive jaundice secondary to cholangiocarcinoma.	Mild abdo/Back pain Sometimes with short history of obstructive jaundice due to CBD stricture
Associated inflammatory bowel disease	Yes	No
Cholangiographic findings	Diffuse changes throughout intra- and extrahepatic bile ducts. Abnormalities in pancreatic duct common.	Pancreatic duct strictures or narrowing. Often stricture of distal 1/3 of common bile duct. Intrahepatic duct changes less common.
Blood chemistry data	Often cholestatic but bilirubin usually near normal.	May be cholestatic. Bilirubin often high
Autoantibodies	Atypical pANCA plus range of others	Antibodies to carbonic anhydrase II plus range of others <sup>[80,81,84]</sup>
Immunoglobulins	IgG4 levels normal	IgG4 levels usually elevated <sup>[82]</sup>
Histology	Absence of plasma cells positive for IgG4 on immunostaining	IgG4 positive plasma cells present in bile ducts and portal tracts <sup>[79]</sup>
Liver biopsy staging	Range of Ludwig staging including higher stages eg III or IV	Ludwig staging usually only I or II <sup>[86]</sup>
Treatment	Ursodeoxycholic acid ± biliary drainage for dominant strictures	Systemic steroid therapy usually leads to complete resolution of symptoms and signs of disease. Occasionally patients relapse and require longer courses of steroids

Table 6 Evidence for the influence of immune mechanisms on the aetiology of PSC

	Evidence for the influence of immune mechanisms
Humoral immunity	Increased circulating immune complexes Elevated immunoglobulin levels (IgG and IgM) Low titres of non-organ specific autoantibodies (ANA and SMA) High titres of antineutrophil nuclear antibody (ANNA)
Cell mediated immunity	Decreased levels of circulating peripheral CD8+ve T cells Portal T cell and NK cell infiltrate Increased activated and memory T cells Restricted T cell receptor repertoire (Vβ3) Aberrant expression of HLA-DR on BEC Coexpression of costimulatory molecules and HLA-DR on BECs Abnormal expression of adhesion molecules on biliary epithelial cells Abnormal expression of chemokine ligands on biliary epithelial cells
Immune effector mechanisms	Enhanced cytokine expression in the liver
Immunogenetic mechanisms	HLA associations

PSC patients, however, did not find MDR3 variations<sup>[71]</sup>. Similarly, the role of the cystic fibrosis transmembrane conductance regulator (CFTR) remains controversial<sup>[72-74]</sup>. The potential role of other hepatobiliary transporters eg BSEP, AE2 in the pathogenesis of PSC remains to be explored. As defects in these systems are known to cause bile duct injury and cholangitis, they are excellent candidates for further investigation.

The nuclear receptor SXR is a nuclear bile acid receptor which plays an important role in endogenous bile acid homeostasis and cholesterol synthesis. A recent study of PSC patients has shown that functional SXR gene variants modify the disease progression and affect survival<sup>[75]</sup>.

### Autoimmune pancreatitis (IgG4 associated sclerosing cholangitis)

Sarles *et al*<sup>[76]</sup> in 1961 provided the first description of what was later identified as autoimmune pancreatitis, an increasingly recognised benign inflammatory disease of the pancreas<sup>[77]</sup>. Abnormalities and sclerosing changes in both the intra- and extra hepatic bile ducts are well recognised in AIP (see pp this issue), and can cause diagnostic confusion with PSC. Correct diagnosis is important as AIP responds well to corticosteroid therapy and tends to have a significantly better outcome than PSC<sup>[71-81]</sup>. The association of AIP and sclerosing changes in the bile ducts has been termed AIP-SC<sup>[81-85]</sup>. Diagnostic criteria for AIP have been proposed and developed by the Japan Pancreas society<sup>[86]</sup>. These criteria consist of the finding of a diffuse narrowing of the pancreatic duct on imaging studies, and either a laboratory finding of an abnormally elevated serum gamma globulin, IgG, or more particularly IgG4 or the presence of autoantibodies or classical histopathological features of the disease ie fibrotic changes with a lymphocyte and, characteristically, plasma cell infiltration. The differences between the two conditions are summarised in Table 5.

## CONCLUSION

Immune mechanisms play an important role in the pathogenesis of PSC, although it remains unclear whether it is a classical autoimmune disease (Tables 2 and 6). There are strong MHC genetic associations including HLA molecules and the MIC molecules. HLA haplotypes however do not account for all the genetic susceptibility in the development of PSC and there is uncertainty about the importance of genes outside this region. Bacterial antigens may act as molecular mimics in hosts who are genetically susceptible and therefore cause an immune reaction leading to PSC initiation.



Lymphocytes may move from the inflamed gut in IBD *via* the enterohepatic circulation and cause inflammation of the liver when activated by a specific stimulus such as bacterially derived antigens.

## REFERENCES

- 1 **Worthington J**, Cullen S, Chapman R. Immunopathogenesis of primary sclerosing cholangitis. *Clin Rev Allergy Immunol* 2005; **28**: 93-103
- 2 **Aadland E**, Schrumpf E, Fausa O, Elgjo K, Heilo A, Aakhus T, Gjone E. Primary sclerosing cholangitis: a long-term follow-up study. *Scand J Gastroenterol* 1987; **22**: 655-664
- 3 **Broome U**, Bergquist A. Primary sclerosing cholangitis, inflammatory bowel disease, and colon cancer. *Semin Liver Dis* 2006; **26**: 31-41
- 4 **Saarinen S**, Olerup O, Broome U. Increased frequency of autoimmune diseases in patients with primary sclerosing cholangitis. *Am J Gastroenterol* 2000; **95**: 3195-3199
- 5 **Levy C**, Lindor KD. Primary sclerosing cholangitis: epidemiology, natural history, and prognosis. *Semin Liver Dis* 2006; **26**: 22-30
- 6 **Terjung B**, Worman HJ. Anti-neutrophil antibodies in primary sclerosing cholangitis. *Best Pract Res Clin Gastroenterol* 2001; **15**: 629-642
- 7 **Terjung B**, Spengler U, Sauerbruch T, Worman HJ. "Atypical p-ANCA" in IBD and hepatobiliary disorders react with a 50-kilodalton nuclear envelope protein of neutrophils and myeloid cell lines. *Gastroenterology* 2000; **119**: 310-322
- 8 **Terjung B**, Muennich M, Gottwein J, Soehne J. Identification of myeloid-specific tubulin-beta isotype 5 as target antigen of antineutrophil cytoplasmic antibodies in autoimmune liver disease. *Hepatology* 2005; **42**: 288A
- 9 **Mulder AH**, Horst G, Haagsma EB, Limburg PC, Kleibeuker JH, Kallenberg CG. Prevalence and characterization of neutrophil cytoplasmic antibodies in autoimmune liver diseases. *Hepatology* 1993; **17**: 411-417
- 10 **Bansi DS**, Bauducci M, Bergqvist A, Boberg K, Broome U, Chapman R, Fleming K, Jorgensen R, Lindor K, Rosina F, Schrumpf E. Detection of antineutrophil cytoplasmic antibodies in primary sclerosing cholangitis: a comparison of the alkaline phosphatase and immunofluorescent techniques. *Eur J Gastroenterol Hepatol* 1997; **9**: 575-580
- 11 **Pokorny CS**, Norton ID, McCaughan GW, Selby WS. Antineutrophil cytoplasmic antibody: a prognostic indicator in primary sclerosing cholangitis. *J Gastroenterol Hepatol* 1994; **9**: 40-44
- 12 **Angulo P**, Peter JB, Gershwin ME, DeSotel CK, Shoenfeld Y, Ahmed AE, Lindor KD. Serum autoantibodies in patients with primary sclerosing cholangitis. *J Hepatol* 2000; **32**: 182-187
- 13 **Xu B**, Broome U, Ericzon BG, Sumitran-Holgersson S. High frequency of autoantibodies in patients with primary sclerosing cholangitis that bind biliary epithelial cells and induce expression of CD44 and production of interleukin 6. *Gut* 2002; **51**: 120-127
- 14 **Muratori P**, Muratori L, Guidi M, Maccariello S, Pappas G, Ferrari R, Gionchetti P, Campieri M, Bianchi FB. Anti-Saccharomyces cerevisiae antibodies (ASCA) and autoimmune liver diseases. *Clin Exp Immunol* 2003; **132**: 473-476
- 15 **Chapman RW**, Varghese Z, Gaul R, Patel G, Kokinin N, Sherlock S. Association of primary sclerosing cholangitis with HLA-B8. *Gut* 1983; **24**: 38-41
- 16 **Shepherd HA**, Selby WS, Chapman RW, Nolan D, Barbatis C, McGee JO, Jewell DP. Ulcerative colitis and persistent liver dysfunction. *Q J Med* 1983; **52**: 503-513
- 17 **Schrumpf E**, Fausa O, Forre O, Dobloug JH, Ritland S, Thorsby E. HLA antigens and immunoregulatory T cells in ulcerative colitis associated with hepatobiliary disease. *Scand J Gastroenterol* 1982; **17**: 187-191
- 18 **Donaldson PT**, Farrant JM, Wilkinson ML, Hayllar K, Portmann BC, Williams R. Dual association of HLA DR2 and DR3 with primary sclerosing cholangitis. *Hepatology* 1991; **13**: 129-133
- 19 **Farrant JM**, Doherty DG, Donaldson PT, Vaughan RW, Hayllar KM, Welsh KI, Eddleston AL, Williams R. Amino acid substitutions at position 38 of the DR beta polypeptide confer susceptibility to and protection from primary sclerosing cholangitis. *Hepatology* 1992; **16**: 390-395
- 20 **Spurkland A**, Saarinen S, Boberg KM, Mitchell S, Broome U, Caballeria L, Ciusani E, Chapman R, Ercilla G, Fausa O, Knutsen I, Pares A, Rosina F, Olerup O, Thorsby E, Schrumpf E. HLA class II haplotypes in primary sclerosing cholangitis patients from five European populations. *Tissue Antigens* 1999; **53**: 459-469
- 21 **Mehal WZ**, Lo YM, Wordsworth BP, Neuberger JM, Hubscher SC, Fleming KA, Chapman RW. HLA DR4 is a marker for rapid disease progression in primary sclerosing cholangitis. *Gastroenterology* 1994; **106**: 160-167
- 22 **Gow PJ**, Fleming KA, Chapman RW. Primary sclerosing cholangitis associated with rheumatoid arthritis and HLA DR4: is the association a marker of patients with progressive liver disease? *J Hepatol* 2001; **34**: 631-635
- 23 **Donaldson PT**. Genetics of liver disease: immunogenetics and disease pathogenesis. *Gut* 2004; **53**: 599-608
- 24 **Hata K**, Van Thiel DH, Herberman RB, Whiteside TL. Phenotypic and functional characteristics of lymphocytes isolated from liver biopsy specimens from patients with active liver disease. *Hepatology* 1992; **15**: 816-823
- 25 **Martins EB**, Graham AK, Chapman RW, Fleming KA. Elevation of gamma delta T lymphocytes in peripheral blood and livers of patients with primary sclerosing cholangitis and other autoimmune liver diseases. *Hepatology* 1996; **23**: 988-993
- 26 **Norris S**, Kondeatis E, Collins R, Satsangi J, Clare M, Chapman R, Stephens H, Harrison P, Vaughan R, Donaldson P. Mapping MHC-encoded susceptibility and resistance in primary sclerosing cholangitis: the role of MICA polymorphism. *Gastroenterology* 2001; **120**: 1475-1482
- 27 **Wiencke K**, Spurkland A, Schrumpf E, Boberg KM. Primary sclerosing cholangitis is associated to an extended B8-DR3 haplotype including particular MICA and MICB alleles. *Hepatology* 2001; **34**: 625-630
- 28 **Bernal W**, Moloney M, Underhill J, Donaldson PT. Association of tumor necrosis factor polymorphism with primary sclerosing cholangitis. *J Hepatol* 1999; **30**: 237-241
- 29 **Donaldson PT**, Norris S. Immunogenetics in PSC. *Best Pract Res Clin Gastroenterol* 2001; **15**: 611-627
- 30 **Mitchell SA**, Grove J, Spurkland A, Boberg KM, Fleming KA, Day CP, Schrumpf E, Chapman RW. Association of the tumour necrosis factor alpha -308 but not the interleukin 10 -627 promoter polymorphism with genetic susceptibility to primary sclerosing cholangitis. *Gut* 2001; **49**: 288-294
- 31 **Donaldson PT**, Norris S, Constantini PK, Bernal W, Harrison P, Williams R. The interleukin-1 and interleukin-10 gene polymorphisms in primary sclerosing cholangitis: no associations with disease susceptibility/resistance. *J Hepatol* 2000; **32**: 882-886
- 32 **Karlsen TH**, Hampe J, Wiencke K, Schrumpf E, Thorsby E, Lie BA, Broome U, Schreiber S, Boberg KM. Genetic polymorphisms associated with inflammatory bowel disease do not confer risk for primary sclerosing cholangitis. *Am J Gastroenterol* 2007; **102**: 115-121
- 33 **Eri R**, Jonsson JR, Pandeya N, Purdie DM, Clouston AD, Martin N, Duffy D, Powell EE, Fawcett J, Florin TH, Radford-Smith GL. CCR5-Delta32 mutation is strongly associated with primary sclerosing cholangitis. *Genes Immun* 2004; **5**: 444-450
- 34 **Melum E**, Karlsen TH, Broome U, Thorsby E, Schrumpf E, Boberg KM, Lie BA. The 32-base pair deletion of the



- chemokine receptor 5 gene (CCR5-Delta32) is not associated with primary sclerosing cholangitis in 363 Scandinavian patients. *Tissue Antigens* 2006; **68**: 78-81
- 35 **Wiencke K**, Boberg KM, Donaldson P, Harbo H, Ling V, Schruppf E, Spurkland A. No major effect of the CD28/CTLA4/ICOS gene region on susceptibility to primary sclerosing cholangitis. *Scand J Gastroenterol* 2006; **41**: 586-591
  - 36 **Satsangi J**, Chapman RW, Haldar N, Donaldson P, Mitchell S, Simmons J, Norris S, Marshall SE, Bell JL, Jewell DP, Welsh KI. A functional polymorphism of the stromelysin gene (MMP-3) influences susceptibility to primary sclerosing cholangitis. *Gastroenterology* 2001; **121**: 124-130
  - 37 **Wiencke K**, Louka AS, Spurkland A, Vatn M, Schruppf E, Boberg KM. Association of matrix metalloproteinase-1 and -3 promoter polymorphisms with clinical subsets of Norwegian primary sclerosing cholangitis patients. *J Hepatol* 2004; **41**: 209-214
  - 38 **Bloom S**, Fleming K, Chapman R. Adhesion molecule expression in primary sclerosing cholangitis and primary biliary cirrhosis. *Gut* 1995; **36**: 604-609
  - 39 **Yang X**, Cullen SN, Li JH, Chapman RW, Jewell DP. Susceptibility to primary sclerosing cholangitis is associated with polymorphisms of intercellular adhesion molecule-1. *J Hepatol* 2004; **40**: 375-379
  - 40 **Bowlus CL**, Karlens TH, Broome U, Thorsby E, Vatn M, Schruppf E, Lie BA, Boberg KM. Analysis of MAdCAM-1 and ICAM-1 polymorphisms in 365 Scandinavian patients with primary sclerosing cholangitis. *J Hepatol* 2006; **45**: 704-710
  - 41 **Hashimoto E**, Lindor KD, Homburger HA, Dickson ER, Czaja AJ, Wiesner RH, Ludwig J. Immunohistochemical characterization of hepatic lymphocytes in primary biliary cirrhosis in comparison with primary sclerosing cholangitis and autoimmune chronic active hepatitis. *Mayo Clin Proc* 1993; **68**: 1049-1055
  - 42 **Lindor KD**, Wiesner RH, Katzmman JA, LaRusso NF, Beaver SJ. Lymphocyte subsets in primary sclerosing cholangitis. *Dig Dis Sci* 1987; **32**: 720-725
  - 43 **Whiteside TL**, Lasky S, Si L, Van Thiel DH. Immunologic analysis of mononuclear cells in liver tissues and blood of patients with primary sclerosing cholangitis. *Hepatology* 1985; **5**: 468-474
  - 44 **Snook JA**, Chapman RW, Sachdev GK, Heryet A, Kelly PM, Fleming KA, Jewell DP. Peripheral blood and portal tract lymphocyte populations in primary sclerosing cholangitis. *J Hepatol* 1989; **9**: 36-41
  - 45 **Bo X**, Broome U, Remberger M, Sumitran-Holgersson S. Tumour necrosis factor alpha impairs function of liver derived T lymphocytes and natural killer cells in patients with primary sclerosing cholangitis. *Gut* 2001; **49**: 131-141
  - 46 **Spengler U**, Moller A, Jung MC, Messer G, Zachoval R, Hoffmann RM, Eisenburg J, Paumgartner G, Riethmuller G, Weiss EH. T lymphocytes from patients with primary biliary cirrhosis produce reduced amounts of lymphotoxin, tumor necrosis factor and interferon-gamma upon mitogen stimulation. *J Hepatol* 1992; **15**: 129-135
  - 47 **Imberti L**, Sottini A, Primi D. T cell repertoire and autoimmune diseases. *Immunol Res* 1993; **12**: 149-167
  - 48 **Broome U**, Grunewald J, Scheynius A, Olerup O, Hultcrantz R. Preferential V beta3 usage by hepatic T lymphocytes in patients with primary sclerosing cholangitis. *J Hepatol* 1997; **26**: 527-534
  - 49 **Probert CS**, Christ AD, Saubermann LJ, Turner JR, Chott A, Carr-Locke D, Balk SP, Blumberg RS. Analysis of human common bile duct-associated T cells: evidence for oligoclonality, T cell clonal persistence, and epithelial cell recognition. *J Immunol* 1997; **158**: 1941-1948
  - 50 **Broome U**, Glaumann H, Hultcrantz R, Forsum U. Distribution of HLA-DR, HLA-DP, HLA-DQ antigens in liver tissue from patients with primary sclerosing cholangitis. *Scand J Gastroenterol* 1990; **25**: 54-58
  - 51 **Chapman RW**, Kelly PM, Heryet A, Jewell DP, Fleming KA. Expression of HLA-DR antigens on bile duct epithelium in primary sclerosing cholangitis. *Gut* 1988; **29**: 422-427
  - 52 **Van den Oord JJ**, Sciort R, Desmet VJ. Expression of MHC products by normal and abnormal bile duct epithelium. *J Hepatol* 1986; **3**: 310-317
  - 53 **Cruickshank SM**, Southgate J, Selby PJ, Trejdosiewicz LK. Inhibition of T cell activation by normal human biliary epithelial cells. *J Hepatol* 1999; **31**: 1026-1033
  - 54 **Leon MP**, Bassendine MF, Wilson JL, Ali S, Thick M, Kirby JA. Immunogenicity of biliary epithelium: investigation of antigen presentation to CD4+ T cells. *Hepatology* 1996; **24**: 561-567
  - 55 **O'Mahony CA**, Vierling JM. Etiopathogenesis of primary sclerosing cholangitis. *Semin Liver Dis* 2006; **26**: 3-21
  - 56 **Brooke BN**, Dykes PW, Walker FC. A study of liver disorder in ulcerative colitis. *Postgrad Med J* 1961; **37**: 245-251
  - 57 **Olsson R**, Bjornsson E, Backman L, Friman S, Hockerstedt K, Kaijser B, Olausson M. Bile duct bacterial isolates in primary sclerosing cholangitis: a study of explanted livers. *J Hepatol* 1998; **28**: 426-432
  - 58 **Bjornsson ES**, Kilander AF, Olsson RG. Bile duct bacterial isolates in primary sclerosing cholangitis and certain other forms of cholestasis--a study of bile cultures from ERCP. *Hepatogastroenterology* 2000; **47**: 1504-1508
  - 59 **Nilsson HO**, Taneera J, Castedal M, Glatz E, Olsson R, Wadstrom T. Identification of Helicobacter pylori and other Helicobacter species by PCR, hybridization, and partial DNA sequencing in human liver samples from patients with primary sclerosing cholangitis or primary biliary cirrhosis. *J Clin Microbiol* 2000; **38**: 1072-1076
  - 60 **Ponsioen CY**, Defoer J, Ten Kate FJ, Weverling GJ, Tytgat GN, Pannekoek Y, Wertheim-Dillen PM. A survey of infectious agents as risk factors for primary sclerosing cholangitis: are Chlamydia species involved? *Eur J Gastroenterol Hepatol* 2002; **14**: 641-648
  - 61 **Lichtman SN**, Sartor RB, Keku J, Schwab JH. Hepatic inflammation in rats with experimental small intestinal bacterial overgrowth. *Gastroenterology* 1990; **98**: 414-423
  - 62 **Vierling JM**. Animal models for primary sclerosing cholangitis. *Best Pract Res Clin Gastroenterol* 2001; **15**: 591-610
  - 63 **Koga H**, Sakisaka S, Yoshitake M, Harada M, Kumemura H, Hanada S, Taniguchi E, Kawaguchi T, Kumashiro R, Sata M. Abnormal accumulation in lipopolysaccharide in biliary epithelial cells of rats with self-filling blind loop. *Int J Mol Med* 2002; **9**: 621-626
  - 64 **Grant AJ**, Lalor PF, Salmi M, Jalkanen S, Adams DH. Homing of mucosal lymphocytes to the liver in the pathogenesis of hepatic complications of inflammatory bowel disease. *Lancet* 2002; **359**: 150-157
  - 65 **Eksteen B**, Grant AJ, Miles A, Curbishley SM, Lalor PF, Hubscher SG, Briskin M, Salmon M, Adams DH. Hepatic endothelial CCL25 mediates the recruitment of CCR9+ gut-homing lymphocytes to the liver in primary sclerosing cholangitis. *J Exp Med* 2004; **200**: 1511-1517
  - 66 **Grant AJ**, Lalor PF, Hubscher SG, Briskin M, Adams DH. MAdCAM-1 expressed in chronic inflammatory liver disease supports mucosal lymphocyte adhesion to hepatic endothelium (MAdCAM-1 in chronic inflammatory liver disease). *Hepatology* 2001; **33**: 1065-1072
  - 67 **Eksteen B**, Miles AE, Grant AJ, Adams DH. Lymphocyte homing in the pathogenesis of extra-intestinal manifestations of inflammatory bowel disease. *Clin Med* 2004; **4**: 173-180
  - 68 **Eksteen B**, Curbishley SM, Lai WK, Adams DH. Liver dendritic cells in primary sclerosing cholangitis (PSC) are unable to imprint mucosal adhesion molecules in primed lymphocytes without exogenous retinoic acid. *J Hepatol* 2006; **44**: S10 (Abstract)
  - 69 **Strautnieks SS**, Bull LN, Knisely AS, Kocoshis SA, Dahl N, Arnell H, Sokal E, Dahan K, Childs S, Ling V, Tanner MS, Kagalwalla AF, Nemeth A, Pawlowska J, Baker A, Mieli-Vergani G, Freimer NB, Gardiner RM, Thompson RJ. A

- gene encoding a liver-specific ABC transporter is mutated in progressive familial intrahepatic cholestasis. *Nat Genet* 1998; **20**: 233-238
- 70 **Fickert P**, Fuchsbichler A, Wagner M, Zollner G, Kaser A, Tilg H, Krause R, Lammert F, Langner C, Zatloukal K, Marschall HU, Denk H, Trauner M. Regurgitation of bile acids from leaky bile ducts causes sclerosing cholangitis in Mdr2 (Abcb4) knockout mice. *Gastroenterology* 2004; **127**: 261-274
- 71 **Pauli-Magnus C**, Kerb R, Fattinger K, Lang T, Anwald B, Kullak-Ublick GA, Beuers U, Meier PJ. BSEP and MDR3 haplotype structure in healthy Caucasians, primary biliary cirrhosis and primary sclerosing cholangitis. *Hepatology* 2004; **39**: 779-791
- 72 **Gallegos-Orozco JF**, E Yurk C, Wang N, Rakela J, Charlton MR, Cutting GR, Balan V. Lack of association of common cystic fibrosis transmembrane conductance regulator gene mutations with primary sclerosing cholangitis. *Am J Gastroenterol* 2005; **100**: 874-878
- 73 **Sheth S**, Shea JC, Bishop MD, Chopra S, Regan MM, Malmberg E, Walker C, Ricci R, Tsui LC, Durie PR, Zielenski J, Freedman SD. Increased prevalence of CFTR mutations and variants and decreased chloride secretion in primary sclerosing cholangitis. *Hum Genet* 2003; **113**: 286-292
- 74 **Girodon E**, Sternberg D, Chazouilleres O, Cazeneuve C, Huot D, Calmus Y, Poupon R, Goossens M, Housset C. Cystic fibrosis transmembrane conductance regulator (CFTR) gene defects in patients with primary sclerosing cholangitis. *J Hepatol* 2002; **37**: 192-197
- 75 **Karlsen TH**, Lie BA, Frey Frosli K, Thorsby E, Broome U, Schrumpf E, Boberg KM. Polymorphisms in the steroid and xenobiotic receptor gene influence survival in primary sclerosing cholangitis. *Gastroenterology* 2006; **131**: 781-787
- 76 **Sarles H**, Sarles JC, Muratore R, Guieu C. Chronic inflammatory sclerosis of the pancreas--an autonomous pancreatic disease? *Am J Dig Dis* 1961; **6**: 688-698
- 77 **Erkelens GW**, Vleggaar FP, Lesterhuis W, van Buuren HR, van der Werf SD. Sclerosing pancreato-cholangitis responsive to steroid therapy. *Lancet* 1999; **354**: 43-44
- 78 **Kim KP**, Kim M, Lee YJ, Song MH, Park DH, Lee SS, Seo DW, Lee SK, Min YI, Song DE, Yu ES. [Clinical characteristics of 17 cases of autoimmune chronic pancreatitis] *Korean J Gastroenterol* 2004; **43**: 112-119
- 79 **Uehara T**, Hamano H, Kawa S, Sano K, Honda T, Ota H. Distinct clinicopathological entity 'autoimmune pancreatitis-associated sclerosing cholangitis'. *Pathol Int* 2005; **55**: 405-411
- 80 **Ito T**, Nakano I, Koyanagi S, Miyahara T, Migita Y, Ogoshi K, Sakai H, Matsunaga S, Yasuda O, Sumii T, Nawata H. Autoimmune pancreatitis as a new clinical entity. Three cases of autoimmune pancreatitis with effective steroid therapy. *Dig Dis Sci* 1997; **42**: 1458-1468
- 81 **Okazaki K**. Autoimmune pancreatitis: etiology, pathogenesis, clinical findings and treatment. The Japanese experience. *JOP* 2005; **6** Suppl 1: 89-96
- 82 **Hamano H**, Kawa S, Horiuchi A, Unno H, Furuya N, Akamatsu T, Fukushima M, Nikaide T, Nakayama K, Usuda N, Kiyosawa K. High serum IgG4 concentrations in patients with sclerosing pancreatitis. *N Engl J Med* 2001; **344**: 732-738
- 83 **Okazaki K**, Uchida K, Chiba T. Recent concept of autoimmune-related pancreatitis. *J Gastroenterol* 2001; **36**: 293-302
- 84 **Uchida K**, Okazaki K, Konishi Y, Ohana M, Takakuwa H, Hajiro K, Chiba T. Clinical analysis of autoimmune-related pancreatitis. *Am J Gastroenterol* 2000; **95**: 2788-2794
- 85 **Zamboni G**, Luttges J, Capelli P, Frulloni L, Cavallini G, Pederzoli P, Leins A, Longnecker D, Kloppel G. Histopathological features of diagnostic and clinical relevance in autoimmune pancreatitis: a study on 53 resection specimens and 9 biopsy specimens. *Virchows Arch* 2004; **445**: 552-563
- 86 **Nakazawa T**, Ohara H, Sano H, Ando T, Aoki S, Kobayashi S, Okamoto T, Nomura T, Joh T, Itoh M. Clinical differences between primary sclerosing cholangitis and sclerosing cholangitis with autoimmune pancreatitis. *Pancreas* 2005; **30**: 20-25
- 87 **Kamisawa T**, Yoshiike M, Egawa N, Nakajima H, Tsuruta K, Okamoto A. Treating patients with autoimmune pancreatitis: results from a long-term follow-up study. *Pancreatol* 2005; **5**(2-3): 234-238; discussion 8-40.

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## TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, Series Editors

# Autoimmune paediatric liver disease

Giorgina Mieli-Vergani, Diego Vergani

Giorgina Mieli-Vergani, Diego Vergani, Institute of Liver Studies, King's College London School of Medicine at King's College Hospital, London SE5 9RS, United Kingdom

Correspondence to: Giorgina Mieli-Vergani, Professor, Paediatric Liver Centre, Variety Club Children's Hospital, King's College Hospital, Denmark Hill, London SE5 9RS, United Kingdom. [giorgina.vergani@kcl.ac.uk](mailto:giorgina.vergani@kcl.ac.uk)

Telephone: +44-20-32994643-3357 Fax: +44-20-32994224

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## Abstract

Liver disorders with a likely autoimmune pathogenesis in childhood include autoimmune hepatitis (AIH), autoimmune sclerosing cholangitis (ASC), and *de novo* AIH after liver transplantation. AIH is divided into two subtypes according to seropositivity for smooth muscle and/or antinuclear antibody (SMA/ANA, type 1) or liver kidney microsomal antibody (LKM1, type 2). There is a female predominance in both. LKM1 positive patients tend to present more acutely, at a younger age, and commonly have partial IgA deficiency, while duration of symptoms before diagnosis, clinical signs, family history of autoimmunity, presence of associated autoimmune disorders, response to treatment, and long-term prognosis are similar in both groups. The most common type of paediatric sclerosing cholangitis is ASC. The clinical, biochemical, immunological, and histological presentation of ASC is often indistinguishable from that of AIH type 1. In both, there are high IgG, non-organ specific autoantibodies, and interface hepatitis. Diagnosis is made by cholangiography. Children with ASC respond to immunosuppression satisfactorily and similarly to AIH in respect to remission and relapse rates, times to normalization of biochemical parameters, and decreased inflammatory activity on follow up liver biopsies. However, the cholangiopathy can progress. There may be evolution from AIH to ASC over the years, despite treatment. *De novo* AIH after liver transplantation affects patients not transplanted for autoimmune disorders and is strikingly reminiscent of classical AIH, including elevated titres of serum antibodies, hypergammaglobulinaemia, and histological findings of interface hepatitis, bridging fibrosis, and collapse. Like classical AIH, it responds to treatment with prednisolone and azathioprine. *De novo* AIH post

liver transplantation may derive from interference by calcineurin inhibitors with the intrathymic physiological mechanisms of T-cell maturation and selection. Whether this condition is a distinct entity or a form of atypical rejection in individuals susceptible to the development of autoimmune phenomena is unclear. Whatever its etiology, the recognition of this potentially life-threatening syndrome is important since its management differs from that of standard anti-rejection therapy.

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**Key words:** Autoimmune hepatitis; Autoimmune sclerosing cholangitis; Liver transplant; Children

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## INTRODUCTION

Autoimmune liver disorders of childhood are inflammatory liver diseases characterized histologically by a dense mononuclear cell infiltrate in the portal tract and serologically by the presence of non-organ and liver specific autoantibodies and increased levels of transaminases and IgG, in the absence of a known etiology. They usually respond to immunosuppressive treatment, which should be instituted as soon as diagnosis is made. In children, as well as in young adults, autoimmune hepatitis (AIH) often presents acutely and has a more aggressive course than in older patients. The previously accepted requirement of 6 mo duration of symptoms before diagnosis can be made has been abandoned. In children, there are two liver disorders in which the liver damage is likely to arise from an autoimmune attack: classical AIH and AIH/sclerosing cholangitis overlap syndrome (autoimmune sclerosing cholangitis, ASC). A possible autoimmune pathogenesis has also been postulated for the so called post liver transplantation "*de novo* AIH", a condition originally described in children and later confirmed in adults. According to data collected at the Kings College Hospital tertiary center, there is an increase in

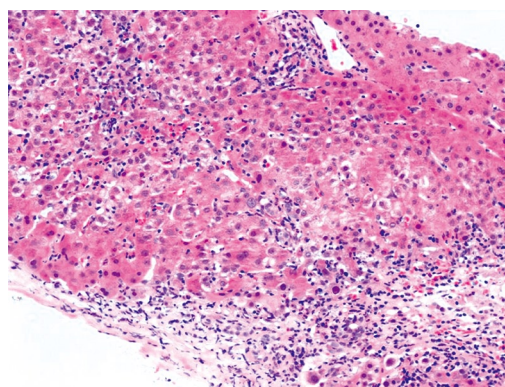
the yearly prevalence of AIH and ASC in childhood, although referral bias may play a role. Thus, in the 1990s these conditions were diagnosed in 2.3% of about 400 children older than 4 mo referred during one year, while in the 2000s their incidence increased to 12%.

## AUTOIMMUNE HEPATITIS (AIH)

### Clinical features

Two types of childhood AIH are recognized: AIH type 1 is characterized by the presence of smooth muscle (SMA) and/or antinuclear (ANA) antibodies; AIH type 2 is positive for anti liver kidney microsomal type 1 (anti-LKM-1) antibody<sup>[1]</sup>. Type 1 AIH represents two thirds of the cases and is a disease of children and adults, while type 2 AIH is mainly described in children. Severity of disease is similar in the two types of AIH<sup>[1]</sup>. In both, there is a predominance of girls (75%-80%). Anti-LKM-1 positive patients are younger and have a greater tendency to present with acute liver failure, but the duration of symptoms before diagnosis and the frequency of hepatosplenomegaly are similar in both groups. Both have a high frequency of associated autoimmune disorders (about 20%) and a family history of autoimmune disease (40%). Associated autoimmune disorders include thyroiditis, inflammatory bowel disease, vitiligo, insulin-dependent diabetes, nephrotic syndrome in both types<sup>[1]</sup>. Type 2 AIH can be associated to autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED), an autosomal recessive genetic disorder in which the liver disease is reportedly present in about 20% of the cases<sup>[2]</sup>.

There are three clinical patterns of disease onset<sup>[1]</sup>: (1) in at least 40% of patients, the presentation is indistinguishable from that of an acute viral hepatitis (non-specific symptoms of malaise, nausea/vomiting, anorexia, and abdominal pain, followed by jaundice, dark urine, and pale stools), some children, particularly anti-LKM-1 positive, develop acute hepatic failure with grade II to IV encephalopathy 2-8 wk from onset of symptoms; (2) in 25%-40% of patients, the onset is insidious, with an illness characterized by progressive fatigue, relapsing jaundice, headache, anorexia, and weight loss, lasting from several months and even years before diagnosis; (3) in about 10% of patients, there is no history of jaundice, and the diagnosis follows presentation with complications of portal hypertension, such as splenomegaly, hematemesis from esophageal varices, bleeding diathesis, chronic diarrhea, and weight loss. The mode of presentation of AIH in childhood is therefore variable, and the disease should be suspected and excluded in all children presenting with symptoms and signs of prolonged or severe liver disease. The course of the disease can be fluctuating, with flares and spontaneous remissions, a pattern which may result in delayed referral and diagnosis. The majority of children, however, even those presenting with acute hepatitis, on physical examination reveal clinical signs of an underlying chronic liver disease, i.e. cutaneous stigmata (spider nevi, palmar erythema, leukonychia, striae),



**Figure 1** Portal and periportal lymphocyte and plasma cell infiltrate, extending to and disrupting the parenchymal limiting plate (interface hepatitis). Swollen hepatocytes, pyknotic necroses, and acinar inflammation are present. HE staining (Picture kindly provided by Dr. Alberto Quaglia).

firm liver and splenomegaly; at ultrasound the liver parenchyma is often nodular and heterogeneous.

### Diagnosis and laboratory findings

Diagnosis of AIH is based on a series of positive and negative criteria defined by the International AIH Group (IAIHG)<sup>[3,4]</sup>. Though these criteria have been produced mainly for research purposes, they have been validated also in the clinical practice. Liver biopsy is necessary to establish the diagnosis of AIH, the typical histological picture include: a dense mononuclear and plasma cell infiltration of the portal areas, which expands into the liver lobule; destruction of the hepatocytes at the periphery of the lobule with erosion of the limiting plate ("interface hepatitis"); connective tissue collapse resulting from hepatocyte death and expanding from the portal area into the lobule ("bridging collapse"); hepatic regeneration with "rosette" formation (Figure 1). In addition to the typical histology, other positive criteria include elevated serum transaminase and IgG/gammaglobulin levels, and presence of ANA, SMA, or anti-LKM-1. Negative criteria relevant to the paediatric age are evidence of infection with hepatitis B or C virus, Wilson disease, and drug or alcohol consumption.

**Autoantibodies:** A key criterion for the diagnosis of AIH is the detection of ANA, SMA, and anti-LKM-1 by indirect immunofluorescence. Autoantibody detection not only assists in the diagnosis but also allows, as mentioned above, differentiation of AIH in type 1 and type 2. ANA and SMA and anti-LKM-1 are practically mutually exclusive<sup>[5]</sup>; in those rare instances when they are present simultaneously, the child is classified as having AIH type 2. Recognition and interpretation of the immunofluorescence patterns is not always straightforward<sup>[5]</sup>. The operator dependency of the technique and the relative rarity of AIH explain the non-infrequent occurrence of errors in reporting, particularly of less frequent specificities such as anti-LKM-1. Problems do exist between laboratory reporting and clinical interpretation of the results that are partly dependent on clinicians' unfamiliarity with the disease spectrum of AIH, but also partly dependent on



insufficient standardization of the tests. This problem is being addressed by the autoimmune serology committee of the IAIHG<sup>[5]</sup>.

The basic technique for the routine testing of autoantibodies relevant to AIH is indirect immunofluorescence on a freshly prepared rodent substrate that should include kidney, liver, and stomach to allow the detection of ANA, SMA, anti-LKM-1 as well as anti liver cytosol type 1 (anti-LC-1, see below), but also of anti-mitochondrial antibody (AMA), the serological hallmark of primary biliary cirrhosis, a disease affecting almost exclusively adults. Since a high proportion of healthy adults may show ANA or SMA reactivity at the conventional starting serum dilution of 1/10, the arbitrary dilution of 1/40 is considered clinically significant by the IAIHG in the adult population. In contrast, in healthy children autoantibody reactivity is infrequent, so that titers of 1/20 for ANA and SMA and 1/10 for anti-LKM-1 are clinically relevant. Hence, the laboratory should report any level of positivity from 1/10, and the attending physician should interpret the result within the clinical context and the age of the patient.

ANA is detectable as a nuclear staining in kidney, stomach, and liver. Its pattern can be homogeneous, or coarsely or finely speckled. In most cases of AIH, but not in all, the pattern is homogeneous. For a clearer and easier definition of the nuclear pattern, HEp2 cells that have prominent nuclei can be used. These cells, however, should not be used for screening purposes, because nuclear reactivity to HEp2 cells is frequent at low serum dilution (1/40) in the normal population<sup>[6]</sup>. ANA reactivity is not specific to AIH, being detectable in chronic viral hepatitis B and especially C, though at lower titre, and in non-hepatic autoimmune disorders.

SMA is detected on kidney, stomach, and liver. On the renal substrate, it is possible to visualize a V (vessels), G (glomeruli), and T (tubules) staining<sup>[7]</sup>. VG and VGT patterns are the most frequently detected in AIH<sup>[8]</sup>. The VGT pattern corresponds to the so called "F actin" or microfilament (MF) pattern observed using cultured fibroblasts as substrate. Though "anti-actin" reactivity is present in the majority of patients with AIH type 1, some 20% of SMA positive AIH type 1 patients do not have the F-actin/VGT pattern<sup>[8]</sup>. The absence, therefore, of anti-actin SMA does not exclude the diagnosis of AIH<sup>[8]</sup>. As for ANA, SMA can be found in chronic viral hepatitis B or C and extrahepatic autoimmune disorders.

Anti-LKM-1 stains brightly the liver cell cytoplasm and the P3 portion of the renal tubules, but does not stain gastric parietal cells. Anti-LKM-1 is often confused with AMA, since both autoantibodies stain liver and kidney, though AMA stains the liver more faintly and the renal tubules more diffusely with an accentuation of the small distal ones and, in contrast to anti-LKM-1, it also stains the gastric parietal cells. In the context of childhood AIH, patients reported to be AMA positive are almost invariably positive for anti-LKM-1, since AMA positive AIH in children is extremely rare<sup>[9]</sup> and PBC even rarer, only two cases having been documented histologically and immunoserologically, both being

teenage girls<sup>[10]</sup>. The identification of the molecular targets of anti-LKM-1, i.e. cytochrome P4502D6, and of AMA, i.e. enzymes of the 2-oxo-acid dehydrogenase complexes, has led to the establishment of commercial immunoassays based on the use of the recombinant or purified antigens<sup>[11]</sup> that can resolve any doubts remaining after immunofluorescence examination. Anti-LKM-1 is highly specific for AIH type 2, being found outside this condition in a small proportion (~5%) of patients chronically infected by the hepatitis C virus, that usually possess the human leukocyte antigen (HLA) allele *DRB1\*07*.

Other autoantibodies less commonly tested but of diagnostic importance in paediatric AIH include those to liver cytosol type 1 (LC-1), anti-neutrophil cytoplasm (ANCA) and soluble liver antigen (SLA). Anti-LC-1, which can be present on its own, but frequently occurs in association with anti-LKM-1, is an additional marker for AIH type 2 and targets formimino-transferase cyclodeaminase (F<sub>1</sub>TCDF)<sup>[12]</sup>. In AIH type 1, as well as in inflammatory bowel disease and sclerosing cholangitis, ANCA is frequently found and targets a peripheral nuclear perinuclear antigen (hence the suggested name of pANNA, i.e. peripheral anti nuclear neutrophil antibody). pANNA is virtually absent in type 2 AIH<sup>[7]</sup>. Anti-SLA that was originally described as the hallmark of a third type of AIH<sup>[13]</sup>, is also found in some 50% of paediatric patients with type 1 and type 2 AIH, where it defines a more severe course<sup>[14]</sup>.

There are a small proportion of children with AIH without detectable autoantibodies. The prevalence and the clinical characteristics of this rare seronegative form of AIH, which responds to immunosuppression similarly to the seropositive forms, remain to be defined.

**Comparison between type 1 and type 2 AIH:** Clinical, laboratory and histological features of type 1 and 2 AIH are summarized in Table 1. In Northern Europe, type 1 AIH is associated with the presence of human leukocyte antigen (HLA) *DRB1\*03*<sup>[11,11]</sup>, while type 2 AIH is associated with the presence of *DRB1\*07* and, less frequently, with *DRB1\*03*<sup>[15]</sup>. In South America, the HLA *DRB1\*1301* allele is reported to predispose to paediatric AIH type 1 and is also associated with persistent infection with the endemic hepatitis A virus<sup>[16,17]</sup>. Interestingly, in Northern European children HLA *DRB1\*1301* is associated with autoimmune sclerosing cholangitis (see below). It is conceivable that some South American children diagnosed as having AIH type 1, but in whom routine cholangiograms were not performed, indeed had ASC.

Paediatric patients with AIH, whether anti-LKM-1 or ANA/SMA positive, have isolated partial deficiency of the HLA class III complement component C4, which is genetically determined<sup>[18]</sup>.

Anti-LKM-1-positive patients have higher levels of bilirubin and transaminases at presentation than those who are ANA/SMA positive and present significantly more frequently with fulminant hepatic failure<sup>[1]</sup>. Excluding children with the fulminant presentation, a

**Table 1** Clinical, laboratory, and histological features at presentation of autoimmune hepatitis type 1, autoimmune hepatitis type 2, and autoimmune sclerosing cholangitis<sup>[1,20]</sup>

	Type 1 AIH	Type 2 AIH	ASC
Median age in year	11	7	12
Females (%)	75	75	55
Mode of presentation (%)			
Acute hepatitis	47	40	37
Acute liver failure	3	25	0
Insidious onset	38	25	37
Complication of chronic liver disease	12	10	26
Associated autoimmune diseases (%)	22	20	48
Inflammatory bowel disease (%)	20	12	44
Family history of autoimmune disease (%)	43	40	37
Abnormal cholangiogram (%)	0	0	100
ANA/SMA (%)	100	25	96
Anti LKM1 (%)	0	100	4
pANCA (%)	45	11	74
Anti SLA (%) <sup>1</sup>	58	58	41
Increased IgG level (%)	84	75	89
Partial IgA deficiency (%)	9	45	5
Low C4 level (%)	89	83	70
Increased frequency of HLA DR*0301	Yes	No <sup>2</sup>	No
Increased frequency of HLA DR*0701	No	Yes	No
Increased frequency of HLA DR*1301	No	No	Yes
Interface hepatitis (%)	66	72	35
Biliary features (%)	28	6	31
Cirrhosis (%)	69	38	15
Remission after immunosuppressive Treatment (%)	97	87	89

AIH: Autoimmune hepatitis; ASC: Autoimmune sclerosing cholangitis; ANA: Anti-nuclear antibodies; SMA: Anti-smooth muscle antibody; LKM1: Liver kidney microsomal type 1 antibody; pANCA: Perinuclear anti-neutrophil cytoplasmic antibody; SLA: Soluble liver antigen; C4: C4 component of complement; HLA: Human leukocyte antigen. <sup>1</sup>Measured by radioligand assay; <sup>2</sup>Increased in HLA DR\*0701 negative patients.

severely impaired hepatic synthetic function, as assessed by the presence of both prolonged prothrombin time and hypoalbuminemia, is more common in ANA/SMA-positive than in anti-LKM-1 positive patients. The vast majority of patients have increased levels of IgG, but some 20% do not<sup>[1]</sup>, indicating that normal IgG values do not exclude the diagnosis of AIH. Partial IgA deficiency is significantly more common in LKM1-positive than in ANA/SMA-positive patients<sup>[1,19]</sup>.

The severity of interface hepatitis at diagnosis is similar in both types, but cirrhosis on initial biopsy is more frequent in type 1 than in type 2 AIH, suggesting a more chronic course of disease in the former. Of note is that most patients already cirrhotic at diagnosis present with a clinical picture reminiscent of that of prolonged acute viral-like hepatitis. Multiacinar or panacinar collapse, which suggests an acute liver injury, is more frequently seen in type 2 AIH. The question as to whether the acute presentation in these patients represents a sudden deterioration of an underlying unrecognized chronic process or a genuinely acute liver damage remains open. Progression to cirrhosis during treatment is more frequent in type 1 AIH. As mentioned above, in both a more severe disease and a higher tendency to relapse is associated to the possession of antibodies to soluble liver antigen (SLA), which are

present in about half of the patients with AIH type 1 or 2 at diagnosis<sup>[11]</sup>.

**Differential diagnosis:** Since positive autoimmune serology can be present in conditions other than AIH, in particular ASC<sup>[20]</sup>, chronic hepatitis B<sup>[21]</sup> or C<sup>[22]</sup> virus infections, and Wilson disease<sup>[23]</sup>, all these disorders must be considered in the differential diagnosis and excluded. ASC, described below, shares the same serological profile as type 1 AIH, but has typical bile duct lesions on cholangiography. Up to 50% of children with hepatitis B and C are positive for ANA and/or SMA, usually at low titres<sup>[21,22]</sup>, and some 5% of patients with chronic hepatitis C have anti-LKM-1 antibodies. In these patients the histology can also mimic AIH, though usually the degree of inflammation is milder. Detection of the typical viral markers allows a correct diagnosis. ANA, and at times SMA, can be present in Wilson disease, in association with high IgG and an inflammatory liver histology, which can make the differential diagnosis with AIH type 1 difficult. Urinary, serum, and liver tissue copper studies and search for Kayser Fleischer rings should be performed in all cases.

**APECED:** APECED is a monogenic disorder<sup>[24,25]</sup> with a variable phenotype. About 20% of the cases develop AIH that resembles AIH type 2<sup>[2]</sup>. This condition, also known as autoimmune polyendocrine syndrome 1 is an autosomal recessive disorder caused by homozygous mutations in the *AIRE1* gene and characterized by a variety of organ-specific autoimmune diseases, the most common of which are hypoparathyroidism and primary adrenocortical failure, accompanied by chronic mucocutaneous candidiasis.

### Etiology and pathogenesis

The etiology of AIH is unknown, though both genetic and environmental factors are involved in its expression. Etiological hypotheses and possible mechanisms leading to the liver autoimmune attack are described under "Etiopathogenesis of AIH" in this issue.

### Management and prognosis

AIH is exquisitely responsive to immunosuppression. The rapidity and degree of response depends on the disease severity at presentation. All types of presentations, apart from fulminant hepatic failure with encephalopathy, respond to standard treatment with prednisolone with or without azathioprine.

Standard treatment for AIH consists of prednisolone 2 mg/kg per day (maximum 60 mg/d), which is gradually decreased over a period of 4 to 8 wk with progressive normalization of the transaminases, and then the patient is maintained on the minimal dose able to sustain normal transaminase levels, usually 2.5 mg/d or 5 mg/d depending on age<sup>[1,26]</sup>. During the first 6 to 8 wk of treatment, liver function tests should be checked weekly to allow a frequent fine-tuning, avoiding severe steroid side effects. If progressive normalization of the liver function tests is not obtained over this period of time

or if too high a dose of prednisolone is required to maintain normal transaminases, azathioprine is added at a starting dose of 0.5 mg/kg per day, which, in the absence of signs of toxicity, is increased up to a maximum of 2-2.5 mg/kg per day until biochemical control is achieved. Azathioprine is not recommended as first-line treatment because of its hepatotoxicity in severely jaundiced patients, but 85% of the patients will eventually require azathioprine addition. A preliminary report in a cohort of 30 children with AIH suggests that the measurements of the azathioprine metabolites 6-thioguanine and 6-methylmercaptopurine are useful in identifying drug toxicity and non-adherence and in achieving a level of 6-thioguanine considered therapeutic for inflammatory bowel disease<sup>[27]</sup>, though what is an ideal therapeutic level for AIH has not been determined. Although an 80% decrease of initial transaminase activity is obtained within 6 wk from starting treatment in most patients, complete normalization of liver function may take several months. In the King's series, normalization of serum transaminase activity occurred at median of 6 mo in ANA/SMA positive children and 9 mo in LKM-1 positive children<sup>[1]</sup>. Relapse while on treatment is common, occurring in about 40% of the patients and requiring a temporary increase of the steroid dose. An important role in relapse is played by non-adherence that is common, particularly in adolescents<sup>[28]</sup>. Moreover, the risk of relapse is higher if steroids are administered on an alternate-day schedule, often instituted in the belief that it has a less negative effect on the child's growth. Small daily doses are more effective in maintaining disease control and minimize the need for high-dose steroid pulses during relapses, with consequent more severe side effects<sup>[1]</sup>.

A question frequently asked by the parents of teenaged girls is whether treatment can be safely continued during pregnancy. Although the experience is limited, there does not appear to be adverse events for mother and baby<sup>[29]</sup>. In particular, no teratogenic effects have been described with azathioprine in humans, though for women concerned about its use, treatment with steroids alone can be used.

Cessation of treatment is considered if a liver biopsy shows minimal or no inflammatory changes after at least one year of normal liver function tests. However, it is advisable not to attempt to withdraw treatment within three years from diagnosis or during or immediately before puberty, when relapses are more common. The reasons for this are unclear, though an important role may be played by non-adherence, as mentioned above. In the Kings experience, successful long-term withdrawal of treatment was achieved in 20% of patients with AIH type 1, but in none with AIH type 2<sup>[1]</sup>.

In paediatrics, an important role in monitoring the response to treatment is the measurement of autoantibody titers and IgG levels, the fluctuation of which is correlated with disease activity<sup>[30]</sup>.

Despite the efficacy of standard immunosuppressive treatment, severe hepatic decompensation may develop even after many years of apparently good biochemical

control, leading to transplantation 10-15 years after diagnosis in 10% of the patients. In the Kings College Hospital series<sup>[1]</sup>, over 97% of the patients treated with standard immunosuppression were alive between 0.3 and 19 years (median 5 years) after diagnosis, including 8% after liver transplantation. Side effects of steroid treatment were mild, the only serious complication being psychosis during induction of remission in 4%, which resolved after prednisolone withdrawal. All patients developed a transient increase in appetite and mild cushingoid features during the first few weeks of treatment. After five years of treatment, 56% of the patients maintained the baseline centile for height or went up across a centile line, 38% dropped across one centile line, and only 6% dropped across two centile lines<sup>[31]</sup>. Moreover, it has recently been shown that long-term daily treatment with prednisolone in children with autoimmune liver disease does not affect their expected final adult height according to parental stature<sup>[32]</sup>.

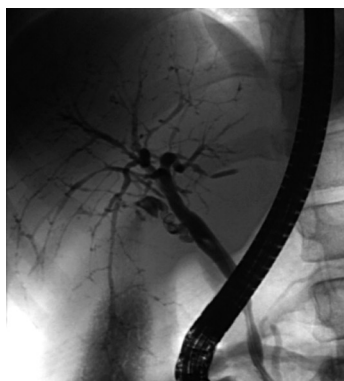
Sustained remission, achieved with prednisolone and azathioprine, can be maintained with azathioprine alone in some children with AIH type 1, akin to the experience in adults<sup>[33]</sup>, but not in AIH type 2.

In those patients (up to 10%) in whom standard immunosuppression is unable to induce stable remission or who are intolerant to azathioprine, mycophenolate mofetil at a dose of 20 mg/kg twice daily can be successfully used<sup>[31]</sup>. In case of persistent no response or of intolerance to mycophenolate mofetil (headache, diarrhea, nausea, dizziness, hair loss, or neutropenia), the use of calcineurin inhibitors (cyclosporine A or tacrolimus) should be considered.

Children who present with acute hepatic failure pose a particularly difficult therapeutic problem. If not encephalopathic, they usually benefit from conventional immunosuppressive therapy, but only one of the six children with acute liver failure and encephalopathy in the Kings series responded to immunosuppression and survived without transplantation<sup>[1]</sup>.

## AUTOIMMUNE SCLEROSING CHOLANGITIS

ASC has the same prevalence as AIH type 1 in childhood<sup>[18]</sup>. This has been shown in a prospective study conducted over a period of 16 years, in which all children with serological (i.e. positive autoantibodies, high IgG levels) and histological (i.e. interface hepatitis) features of autoimmune liver disease underwent a cholangiogram at the time of presentation. Approximately 50% of these patients had alterations of the bile ducts characteristic of sclerosing cholangitis, though generally less advanced than those observed in adult primary sclerosing cholangitis (Figure 2). A quarter of the children with ASC, despite abnormal cholangiograms, had no histological features suggesting bile duct involvement and the diagnosis of sclerosing cholangitis was only possible because of the cholangiographic studies. Virtually all patients were seropositive for



**Figure 2** Retrograde cholangiogram of a child with autoimmune sclerosing cholangitis showing widespread bile duct strictures and dilatations (Picture kindly provided by Dr. Maria Sellars).

**Table 2** Laboratory parameters at presentation in children with autoimmune hepatitis and autoimmune sclerosing cholangitis<sup>[20]</sup>

	AIH	ASC
Bilirubin (nv < 20 micromol/L)	35 (4-306)	20 (4-179)
Albumin (nv > 35 g/L)	35 (25-47)	39 (27-54)
AST (nv < 50 IU/L)	333 (24-4830)	102 (18-1215)
INR (< 1.2)	1.2 (0.96-2.5)	1.1 (0.9-1.6)
GGT (nv < 50 IU/L)	76 (29-383)	129 (13-948)
AP (nv < 350 IU/L)	356 (131-878)	303 (104-1710)

AST: Aspartate aminotransferase; INR: International normalized prothrombin ratio; GGT: Gamma glutamyl transpeptidase; AP: Alkaline phosphatase; nv: Normal values.

ANA and/or SMA. Fifty-five percent were girls, and the mode of presentation was similar to that of typical AIH. Inflammatory bowel disease was present in about 45% of children with ASC compared to about 20% of those with AIH, and 90% of children with ASC had greatly increased serum IgG levels. At the time of presentation, standard liver function tests did not help in discriminating between AIH and ASC (Table 2), though the alkaline phosphatase/aspartate aminotransferase ratio was significantly higher in ASC. pANNA were present in 74% of patients with ASC compared to 45% of patients with AIH type 1 and 11% of those with AIH type 2. Susceptibility to ASC in children is conferred by the presence of HLA *DRB1\*1301*<sup>[32]</sup>. Clinical, laboratory, and histological features of type 1 and 2 AIH and ASC are compared in Table 1.

Children with ASC respond to the same immuno-suppressive schedule described above for AIH<sup>[18]</sup>, liver test abnormalities resolving within a few months after starting treatment in most patients. Steroids and azathioprine, however, though beneficial in abating the parenchymal inflammatory lesions, appear to be less effective in controlling the bile duct disease. Following favorable reports in adult primary sclerosing cholangitis<sup>[33,34]</sup>, ursodeoxycholic acid is added at the dose of 20-30 mg/kg per day, though there is no information as to whether it is helpful in arresting the progression of ASC. Akin to AIH, measurement of autoantibody titers and IgG levels is useful in monitoring disease activity and response to treatment<sup>[20]</sup>. The medium-term prognosis is good, with a reported 7-year survival of 100%, though 15% of the patients required liver transplantation during this period

of follow-up<sup>[18]</sup>. Evolution from AIH to ASC has been documented suggesting that AIH and ASC are part of the same pathogenic process<sup>[18]</sup>.

The prospective study conducted at Kings College Hospital shows that in childhood ASC and AIH have a similar prevalence<sup>[20]</sup>. It also shows that ASC is more frequent than sclerosing cholangitis without autoimmune features<sup>[20]</sup>, autoantibody negative sclerosing cholangitis having been observed in only 9 children referred over the 16-year study period<sup>[20]</sup>.

Whether childhood ASC and adult PSC belong to the same disease spectrum remains to be established, since no prospective study in a large patient cohort has investigated at the time of presentation the presence of bile duct damage in adults with features of autoimmune liver disease. Interestingly, in a retrospective study, a high proportion of adult patients originally diagnosed as having AIH type 1 were found to have sclerosing cholangitis on magnetic resonance cholangiography<sup>[34]</sup>. The long term follow up of the Kings paediatric ASC series will provide important information about the possible links between ASC and PSC.

## DE NOVO AIH AFTER LIVER TRANSPLANTATION

In the late 1990s, it was observed that AIH can arise *de novo* after liver transplantation in children who had not been transplanted for autoimmune liver disease<sup>[35]</sup>. Characteristic of this condition is a histological picture of interface hepatitis and multilobular collapse associated with increased IgG levels and positive autoantibodies. These include ANA, SMA, and classical anti-LKM-1, but also atypical anti-LKM-1, staining the renal tubules but not the liver. After this original report, *de novo* AIH after liver transplantation has been confirmed by several studies both in adult and paediatric patients<sup>[36,37]</sup>. Importantly, treatment with prednisolone and azathioprine using the same schedule for classical AIH, concomitant with reduction of the calcineurin inhibitor dose, is highly effective in *de novo* AIH, leading to excellent graft and patient survival. It is of interest that these patients do not respond satisfactorily to standard anti-rejection treatment, making it essential to reach an early diagnosis to avoid graft loss.

Whether the liver damage observed in these patients is a form of rejection or the consequence of an "auto-immune" injury, possibly triggered by drugs or viral infection, remains to be established. The administration of cyclosporin A or tacrolimus to rodents after bone marrow transplantation can result in a "paradoxical" autoimmune syndrome in which the immunosuppressive drugs interfere with maturation of T lymphocytes and favor the emergence of autoaggressive T-cell clones<sup>[35-37]</sup>. This experience in animals may explain, in part, the development of this disorder in immunosuppressed children after liver transplantation.

The manifestations of the autoimmune condition in rodents vary in different strains and depend on genetic



factors possibly encoded by the major histocompatibility complex<sup>[35]</sup>. Analysis of the HLA phenotypes of the recipients and donors in the original report did not show an association between the development of autoimmune features, the presence of either HLA *DRB1\*03* or *-DRB1\*04*, or the degree of donor-recipient HLA mismatch<sup>[28]</sup>. Five of the seven patients, however, had received livers from donors with HLA markers known to be associated with susceptibility to AIH, including two with *DRB1\*04*, one with *DRB1\*03*, and two with both *DRB1\*03* and *DRB1\*04*<sup>[38]</sup>.

## REFERENCES

- Gregorio GV, Portmann B, Reid F, Donaldson PT, Doherty DG, McCartney M, Mowat AP, Vergani D, Mieli-Vergani G. Autoimmune hepatitis in childhood: a 20-year experience. *Hepatology* 1997; **25**: 541-547
- Ahonen P, Myllärniemi S, Sipilä I, Perheentupa J. Clinical variation of autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) in a series of 68 patients. *N Engl J Med* 1990; **322**: 1829-1836
- Johnson PJ, McFarlane IG. Meeting report: International Autoimmune Hepatitis Group. *Hepatology* 1993; **18**: 998-1005
- Alvarez F, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, Chapman RW, Cooksley WG, Czaja AJ, Desmet VJ, Donaldson PT, Eddleston AL, Fainboim L, Heathcote J, Homberg JC, Hoofnagle JH, Kakumu S, Krawitt EL, Mackay IR, MacSween RN, Maddrey WC, Manns MP, McFarlane IG, Meyer zum Büschenfelde KH, Zeniya M. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; **31**: 929-938
- Vergani D, Alvarez F, Bianchi FB, Cancado EL, Mackay IR, Manns MP, Nishioka M, Penner E. Liver autoimmune serology: a consensus statement from the committee for autoimmune serology of the International Autoimmune Hepatitis Group. *J Hepatol* 2004; **41**: 677-683
- Tan EM, Feltkamp TE, Smolen JS, Butcher B, Dawkins R, Fritzler MJ, Gordon T, Hardin JA, Kalder JR, Lahita RG, Maini RN, McDougal JS, Rothfield NF, Smeenk RJ, Takasaki Y, Wiik A, Wilson MR, Koziol JA. Range of antinuclear antibodies in "healthy" individuals. *Arthritis Rheum* 1997; **40**: 1601-1611
- Bottazzo GF, Florin-Christensen A, Fairfax A, Swana G, Doniach D, Groeschel-Stewart U. Classification of smooth muscle autoantibodies detected by immunofluorescence. *J Clin Pathol* 1976; **29**: 403-410
- Muratori P, Muratori L, Agostinelli D, Pappas G, Veronesi L, Granito A, Cassani F, Terlizzi P, Lenzi M, Bianchi FB. Smooth muscle antibodies and type 1 autoimmune hepatitis. *Autoimmunity* 2002; **35**: 497-500
- Gregorio GV, Portmann B, Mowat AP, Vergani D, Mieli-Vergani G. A 12-year-old girl with antimitochondrial antibody-positive autoimmune hepatitis. *J Hepatol* 1997; **27**: 751-754
- Dahlan Y, Smith L, Simmonds D, Jewell LD, Wanless I, Heathcote EJ, Bain VG. Pediatric-onset primary biliary cirrhosis. *Gastroenterology* 2003; **125**: 1476-1479
- Donaldson PT. Genetics in autoimmune hepatitis. *Semin Liver Dis* 2002; **22**: 353-364
- Lapierre P, Hajoui O, Homberg JC, Alvarez F. Formiminotransferase cyclodeaminase is an organ-specific autoantigen recognized by sera of patients with autoimmune hepatitis. *Gastroenterology* 1999; **116**: 643-649
- Manns M, Gerken G, Kyriatsoulis A, Staritz M, Meyer zum Büschenfelde KH. Characterisation of a new subgroup of autoimmune chronic active hepatitis by autoantibodies against a soluble liver antigen. *Lancet* 1987; **1**: 292-294
- Ma Y, Okamoto M, Thomas MG, Bogdanos DP, Lopes AR, Portmann B, Underhill J, Dürr R, Mieli-Vergani G, Vergani D. Antibodies to conformational epitopes of soluble liver antigen define a severe form of autoimmune liver disease. *Hepatology* 2002; **35**: 658-664
- Ma Y, Bogdanos DP, Hussain MJ, Underhill J, Bansal S, Longhi MS, Cheeseman P, Mieli-Vergani G, Vergani D. Polyclonal T-cell responses to cytochrome P450IID6 are associated with disease activity in autoimmune hepatitis type 2. *Gastroenterology* 2006; **130**: 868-882
- Fainboim L, Canero Velasco MC, Marcos CY, Ciocca M, Roy A, Theiler G, Capucchio M, Nuncifora S, Sala L, Zelazko M. Protracted, but not acute, hepatitis A virus infection is strongly associated with HLA-DRB\*1301, a marker for pediatric autoimmune hepatitis. *Hepatology* 2001; **33**: 1512-1517
- Pando M, Larriba J, Fernandez GC, Fainboim H, Ciocca M, Ramonet M, Badia I, Daruich J, Findor J, Tanno H, Cañero-Velasco C, Fainboim L. Pediatric and adult forms of type I autoimmune hepatitis in Argentina: evidence for differential genetic predisposition. *Hepatology* 1999; **30**: 1374-1380
- Vergani D, Wells L, Larcher VF, Nasaruddin BA, Davies ET, Mieli-Vergani G, Mowat AP. Genetically determined low C4: a predisposing factor to autoimmune chronic active hepatitis. *Lancet* 1985; **2**: 294-298
- Homberg JC, Abuaf N, Bernard O, Islam S, Alvarez F, Khalil SH, Poupon R, Darnis F, Lévy VG, Gripon P. Chronic active hepatitis associated with antiliver/kidney microsome antibody type 1: a second type of "autoimmune" hepatitis. *Hepatology* 1987; **7**: 1333-1339
- Gregorio GV, Portmann B, Karani J, Harrison P, Donaldson PT, Vergani D, Mieli-Vergani G. Autoimmune hepatitis/sclerosing cholangitis overlap syndrome in childhood: a 16-year prospective study. *Hepatology* 2001; **33**: 544-553
- Gregorio GV, Jones H, Choudhuri K, Vegnente A, Bortolotti F, Mieli-Vergani G, Vergani D. Autoantibody prevalence in chronic hepatitis B virus infection: effect in interferon alfa. *Hepatology* 1996; **24**: 520-523
- Gregorio GV, Pensati P, Iorio R, Vegnente A, Mieli-Vergani G, Vergani D. Autoantibody prevalence in children with liver disease due to chronic hepatitis C virus (HCV) infection. *Clin Exp Immunol* 1998; **112**: 471-476
- Dhawan A, Taylor RM, Cheeseman P, De Silva P, Katsiyiannakis L, Mieli-Vergani G. Wilson's disease in children: 37-year experience and revised King's score for liver transplantation. *Liver Transpl* 2005; **11**: 441-448
- Liston A, Lesage S, Gray DH, Boyd RL, Goodnow CC. Genetic lesions in T-cell tolerance and thresholds for autoimmunity. *Immunol Rev* 2005; **204**: 87-101
- Simmonds MJ, Gough SC. Genetic insights into disease mechanisms of autoimmunity. *Br Med Bull* 2004; **71**: 93-113
- Mieli-Vergani G, Vergani D. Autoimmune hepatitis in children. *Clin Liver Dis* 2002; **6**: 623-634
- Rumbo C, Emerick KM, Emre S, Shneider BL. Azathioprine metabolite measurements in the treatment of autoimmune hepatitis in pediatric patients: a preliminary report. *J Pediatr Gastroenterol Nutr* 2002; **35**: 391-398
- Kerkar N, Annunziato RA, Foley L, Schmeidler J, Rumbo C, Emre S, Shneider B, Shemesh E. Prospective analysis of nonadherence in autoimmune hepatitis: a common problem. *J Pediatr Gastroenterol Nutr* 2006; **43**: 629-634
- Heneghan MA, Norris SM, O'Grady JG, Harrison PM, McFarlane IG. Management and outcome of pregnancy in autoimmune hepatitis. *Gut* 2001; **48**: 97-102
- Gregorio GV, McFarlane B, Bracken P, Vergani D, Mieli-Vergani G. Organ and non-organ specific autoantibody titres and IgG levels as markers of disease activity: a longitudinal study in childhood autoimmune liver disease. *Autoimmunity* 2002; **35**: 515-519
- Mieli-Vergani G, Bargiota K, Samyn M, Vergani D. Therapeutic aspects of autoimmune liver disease in children. In: Dienes HP, Leuschner U, Lohse AW, Manns MP, eds. Autoimmune Liver Diseases-Falk Symposium Dordrecht:

- Springer, 2005: 278-282
- 32 **Samaroo B**, Samyn M, Buchanan C, Mieli-Vergani G. Long-term daily oral treatment with prednisolone in children with autoimmune liver disease does not affect final adult height. *Hepatology* 2006; **44**: 438A
- 33 **Johnson PJ**, McFarlane IG, Williams R. Azathioprine for long-term maintenance of remission in autoimmune hepatitis. *N Engl J Med* 1995; **333**: 958-963
- 34 **Abdalian R**, Dhar P, Jhaveri K, Haider M, Guindi M, Heathcote EJ. Prevalence of sclerosing cholangitis in adults with autoimmune hepatitis: evaluating the role of routine magnetic resonance imaging. *Hepatology* 2008; **47**: 949-957
- 35 **Bucy PB**, Yan Xu X, Li J, Huang GQ. Cyclosporin A-induced autoimmune disease in mice. *J Immunol* 1993; **151**: 1039-1050
- 36 **Cooper MH**, Hartman GG, Starzl TE, Fung JJ. The induction of pseudo-graft-versus-host disease following syngeneic bone marrow transplantation using FK 506. *Transplant Proc* 1991; **23**: 3234-3235
- 37 **Hess AD**, Fischer AC, Horwitz LR, Laulis MK. Cyclosporine-induced autoimmunity: critical role of autoregulation in the prevention of major histocompatibility class II-dependent autoaggression. *Transplant Proc* 1993; **25**: 2811-2813
- 38 **Donaldson PT**, Doherty DG, Hayllar KM, McFarlane IG, Johnson PJ, Williams R. Susceptibility to autoimmune chronic active hepatitis: human leukocyte antigens DR4 and A1-B8-DR3 are independent risk factors. *Hepatology* 1991; **13**: 701-706

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## TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, Series Editors

# Overlap syndromes among autoimmune liver diseases

Christian Rust, Ulrich Beuers

Christian Rust, Department of Medicine II, Klinikum Grosshadern, University of Munich, Munich 81377, Germany  
Ulrich Beuers, Department of Gastroenterology & Hepatology, Academic Medical Center, University of Amsterdam, Amsterdam NL-1100 DE, The Netherlands

Correspondence to: Ulrich Beuers, MD, Professor of Gastroenterology & Hepatology, Department of Gastroenterology & Hepatology, Academic Medical Center, University of Amsterdam, PO Box 22700, Amsterdam NL-1100 DE, The Netherlands. [u.h.beuers@amc.uva.nl](mailto:u.h.beuers@amc.uva.nl)

Telephone: +31-20-5662422 Fax: +31-20-6917033

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## Abstract

The three major immune disorders of the liver are autoimmune hepatitis (AIH), primary biliary cirrhosis (PBC) and primary sclerosing cholangitis (PSC). Variant forms of these diseases are generally called overlap syndromes, although there has been no standardized definition. Patients with overlap syndromes present with both hepatitic and cholestatic serum liver tests and have histological features of AIH and PBC or PSC. The AIH-PBC overlap syndrome is the most common form, affecting almost 10% of adults with AIH or PBC. Single cases of AIH and autoimmune cholangitis (AMA-negative PBC) overlap syndrome have also been reported. The AIH-PSC overlap syndrome is predominantly found in children, adolescents and young adults with AIH or PSC. Interestingly, transitions from one autoimmune to another have also been reported in a minority of patients, especially transitions from PBC to AIH-PBC overlap syndrome. Overlap syndromes show a progressive course towards liver cirrhosis and liver failure without treatment. Therapy for overlap syndromes is empiric, since controlled trials are not available in these rare disorders. Anticholestatic therapy with ursodeoxycholic acid is usually combined with immunosuppressive therapy with corticosteroids and/or azathioprine in both AIH-PBC and AIH-PSC overlap syndromes. In end-stage disease, liver transplantation is the treatment of choice.

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**Key words:** Autoimmune hepatitis; Immunosuppressive agents; Primary biliary cirrhosis; Primary sclerosing cholangitis; Ursodeoxycholic acid

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## INTRODUCTION

The term “overlap syndrome” is used to describe variant forms of autoimmune hepatitis (AIH) which present with characteristics of AIH and primary biliary cirrhosis (PBC) or primary sclerosing cholangitis (PSC). Standardization of diagnostic criteria for overlap syndromes has not been achieved so far, since these disorders are uncommon. It remains unclear whether these overlap syndromes form distinct disease entities or are only variants of the major immune hepatopathies<sup>[1,2]</sup>.

Overlap syndromes should always be considered once an autoimmune liver disease has been diagnosed<sup>[1]</sup>. Patients with overlap syndromes usually present with nonspecific symptoms, including fatigue, arthralgias, and myalgias. A hepatitic biochemical profile typically coexists with cholestatic laboratory changes<sup>[3,4]</sup>. Interestingly, transitions from one to another autoimmune hepatopathy have also been reported and are discussed together with the overlap syndromes<sup>[5,6]</sup>. Overlap of autoimmune cholangitis (AMA-negative PBC) and AIH has been described anecdotically and is discussed together with AIH-PBC overlap syndromes. Although combined features of both PBC and PSC have been reported in single cases<sup>[7]</sup>, there is no clear evidence for the existence of an overlap of PBC and PSC.

It appears inappropriate to use the term overlap syndrome for coexistence of AIH and other chronic liver diseases like chronic hepatitis C. Autoantibodies are detected in up to 65% of patients with chronic hepatitis C and LKM1 antibodies, the hallmark of AIH type 2 was also observed in 7% of the patients with chronic hepatitis C<sup>[8]</sup>. Conversely, in patients with AIH and hypergammaglobulinemia, anti-HCV tests in the past turned out to be false positive in many cases<sup>[9]</sup>. Thus, the term overlap syndrome should not be used for patients with AIH and concomitant chronic hepatitis C.

This article is an extension of a recent review<sup>[10]</sup> and discusses current views and controversies on overlap syndromes. A case report is included to exemplify the typical features of an AIH-PBC overlap syndrome.

## DIAGNOSIS OF AUTOIMMUNE LIVER DISEASES

The diagnostic criteria of AIH, PBC and PSC are discussed in detail in this issue of the *World Journal of Gastroenterology* and are therefore just summarized briefly, since they are the basis for the diagnosis of the respective overlap syndrome.

### AIH

The diagnosis of AIH depends on several descriptive criteria which were summarized and updated by the International AIH Group (IAIHG) in 1999<sup>[11]</sup>. A definite diagnosis requires exclusion of other major causes of liver damage, including alcoholic, viral, drug- and toxin-induced, and hereditary liver disease. The scoring system includes characteristic laboratory features (hepatic serum liver tests, the presence of elevated serum IgG or  $\gamma$ -globulins and of serum autoantibodies), histocompatibility leucocyte antigen (HLA) associations, a portal mononuclear cell infiltration and interface hepatitis in the liver tissue and a positive treatment response to corticosteroids<sup>[11]</sup>.

### PBC

The diagnosis of PBC is based on a cholestatic serum enzyme pattern, serum antimitochondrial antibodies (AMA) and/or PBC-specific AMA-M2, and a compatible histology. Although elevated serum IgM is characteristic for patients with PBC, it is not regarded mandatory to establish the diagnosis<sup>[12,13]</sup>. PBC is frequently associated with other autoimmune disorders, like Sjögren's syndrome, Hashimoto thyroiditis, and celiac disease.

### PSC

PSC is a rare chronic cholestatic disease of the liver and bile ducts that is generally progressive and leads to end-stage liver disease. In contrast to PBC, twice as many men as women are affected. PSC is diagnosed most frequently in patients aged between 25 and 40 years<sup>[14]</sup>. Criteria for the diagnosis of PSC include cholestatic serum enzyme pattern, typical cholangiographic findings of bile duct stenoses and dilatations and histologic findings compatible with PSC showing mild to moderate portal infiltration<sup>[14,15]</sup>. Concomitant inflammatory bowel disease is found in 70%-90% of the patients and atypical perinuclear antineutrophil cytoplasmatic antibodies (pANCA) are detected in more than 70% of the patients<sup>[16]</sup>.

## AIH-PBC OVERLAP SYNDROME

PBC and AIH are the most frequent autoimmune hepatopathies with a prevalence of 25-40/100 000<sup>[17,18]</sup> and 17/100 000<sup>[19]</sup>, respectively, in recent epidemiologic studies in Europe and the United States and female gender predominates in both AIH (80%) and PBC (90%-95%). Serum liver tests typically show a hepatic pattern in AIH and a cholestatic pattern with marked elevation of aP and  $\gamma$ -GT, but mild elevation of

**Table 1** Diagnostic criteria of AIH-PBC overlap syndrome proposed by Chazouillères *et al* in 1998<sup>[5]</sup>

AIH (2 out of 3 criteria)

- (1) Alanine aminotransferase (ALT) levels  $> 5 \times$  ULN
- (2) Serum immunoglobulin G (IgG) levels  $> 2 \times$  ULN or a positive test for smooth muscle antibodies (ASMA)
- (3) Liver biopsy showing moderate or severe periportal or periportal lymphocytic piecemeal necrosis

PBC (2 out of 3 criteria)

- (1) Alkaline phosphatase (AP) levels  $> 2 \times$  or  $\gamma$ -glutamyltranspeptidase (GGT) levels  $> 5 \times$  ULN
- (2) Positive test for antimitochondrial antibodies (AMA)
- (3) Liver biopsy specimen showing florid bile duct lesions

ULN: Upper limit of normal value.

serum transaminases in PBC. While serum IgG is the predominant immunoglobulin elevated in AIH, serum IgM is elevated in most patients with PBC.

Patients presenting with clinical, biochemical, serological and histological features of both diseases have been reported since the 1970s<sup>[20,21]</sup>. Later, the term "overlap syndrome" was used to describe these conditions, although there was no common definition or uniformly accepted diagnostic criteria for this<sup>[22,23]</sup>. Two extended analyses provided evidence for AIH-PBC overlap in 8% of 199 patients with AIH ( $n = 162$ ) or PBC ( $n = 37$ )<sup>[1]</sup> and in 9% of 130 patients with PBC<sup>[5]</sup>. In the latter study, an AIH-PBC overlap syndrome was accepted when 2 or 3 criteria for PBC and AIH were fulfilled<sup>[5]</sup> (Table 1). Although these diagnostic criteria for an AIH-PBC overlap syndrome are not validated and their sensitivity has not been established, they provide a diagnostic template that can be consistently applied<sup>[4]</sup>.

In a comparative study, patients with AIH-PBC overlap syndrome presented with typical features of PBC (AMA-M2 positive, bile duct damage compatible with PBC), but a more hepatic picture than a cohort of PBC patients<sup>[24]</sup>. Patients with AIH-PBC overlap syndrome showed a predominant HLA type B8, DR3, or DR4 similar to AIH and a good response to corticosteroid treatment, and this was, therefore, named "PBC, hepatic form"<sup>[24]</sup>. Autoantibodies are generally believed to present a hallmark for the diagnosis of AIH, but up to 20% of patients with AIH present without antinuclear antibodies (ANA), anti-smooth muscle antibodies (ASMA), or antibodies against liver-kidney-microsomes (LKM) 1<sup>[11]</sup>. ANA represent the least specific serum autoantibodies for the diagnosis of chronic liver diseases and are also found in 30% of elderly healthy controls, 10% of pregnant women, and 30% of patients with malignancies<sup>[25]</sup>. Serum ANA in patients with PBC are not a marker of AIH-PBC overlap syndrome, but often found in PBC patients without further signs of AIH<sup>[26]</sup>. In contrast, ANA with a specific immunofluorescence pattern of multiple nuclear dots directed against Sp100 (5-10 dots) or Coilin p80 (2-6 dots) are rather specific although less sensitive for PBC<sup>[25]</sup>. In 3.9% of 233 patients with PBC, the presence of soluble liver antigen (SLA) autoantibodies was found to be a marker of AIH-PBC overlap syndrome with a good



response to immunosuppressive therapy<sup>[27]</sup>.

In addition to AIH-PBC overlap syndrome, some patients presented with typical features of PBC or AIH<sup>[5,28]</sup>. A well-defined series of 12 patients with PBC followed by AIH was described in 282 PBC patients<sup>[29]</sup>. The time interval between the diagnosis of PBC and the diagnosis of AIH varied from 6 mo to 13 years. Of importance, patients with multiple flares of hepatitis at the time of diagnosis of AIH had already developed cirrhosis on liver biopsy<sup>[29]</sup>. Remission was achieved in 80% of the patients who received additional immunosuppressive therapy. This case series emphasizes the possible role of AIH in the deterioration of liver function in PBC patients unless diagnosis is made early and steroid therapy is administered. This study suggested that these patients may have two coincident autoimmune diseases rather than a variant of PBC or AIH<sup>[29]</sup>. A recent retrospective analysis indicated that patients with AIH-PBC overlap syndrome might have worse clinical outcomes compared to patients with PBC alone<sup>[30]</sup>. However, this conclusion is somewhat controversial, since the treatment was not standardized and no difference was found when the diagnostic criteria proposed by Chazouilleres *et al*<sup>[5]</sup> were applied in this cohort of patients.

### Therapy

Randomized controlled trials are the best method to address therapeutic issues. However, the low prevalence of AIH-PBC overlap syndrome has made controlled therapeutic trials in these patients impossible so far. Thus, therapeutic recommendations still rely on the experience in the treatment of either AIH or PBC, and on retrospective, non-randomized studies with inherent limitations. It remains controversial if patients with AIH-PBC overlap syndrome require immunosuppressive treatment in addition to ursodeoxycholic acid (UDCA). In a strictly defined cohort of 16 patients with AIH-PBC overlap syndrome, the response to UDCA therapy (13-15 mg/kg daily) and the survival of the patients were similar to patients with classical PBC<sup>[31]</sup>. However, other groups reported that a combined therapy of UDCA and corticosteroids is required in most patients to obtain a complete biochemical response<sup>[5,24]</sup>. This question was addressed again recently in a retrospective study of 17 patients with AIH-PBC overlap syndrome<sup>[32]</sup>. In this study, patients received UDCA alone or UDCA in combination with immunosuppressors and were followed up for 7.5 years. In the patients treated with UDCA alone, biochemical response was observed in only 3 patients whereas 8 patients were non-responders and 50% of them showed increased fibrosis. All but one of the non-responders subsequently received combined therapy, and 85% of the patients achieved biochemical remission<sup>[32]</sup>. In the second group of patients who received combined therapy throughout the study, fibrosis did not progress and 67% achieved biochemical remission. Thus, it appears appropriate to start treatment with UDCA (13-15 mg/kg daily). However, if this therapy does not induce an adequate biochemical response in an appropriate time span (e.g. 3 mo) or in patients with predominantly

hepatic serum liver tests, a glucocorticosteroid should be added. Prednisone has been used at an initial dose of 0.5 mg/kg daily and should be progressively tapered once ALT levels show a response<sup>[32]</sup>. The role of other immuno-suppressants, e.g. azathioprine (1-1.5 mg/kg daily) in the long-term management of patients with AIH-PBC overlap syndrome is unclear, but its successful use in AIH makes azathioprine an attractive alternative to corticosteroids for long-term immunosuppressive therapy<sup>[3,32]</sup>. Budesonide, a synthetic corticosteroid with a high first pass metabolism that reduces its systemic side effects, is a promising treatment option for patients with AIH and has also been used in patients with AIH-PBC overlap syndrome with success<sup>[33,34]</sup>. For corticosteroid-resistant patients with AIH-PBC overlap syndrome, intermediate treatment with other immunosuppressants such as cyclosporine A has been considered<sup>[23]</sup>. Liver transplantation is regarded as the treatment of choice for end-stage disease.

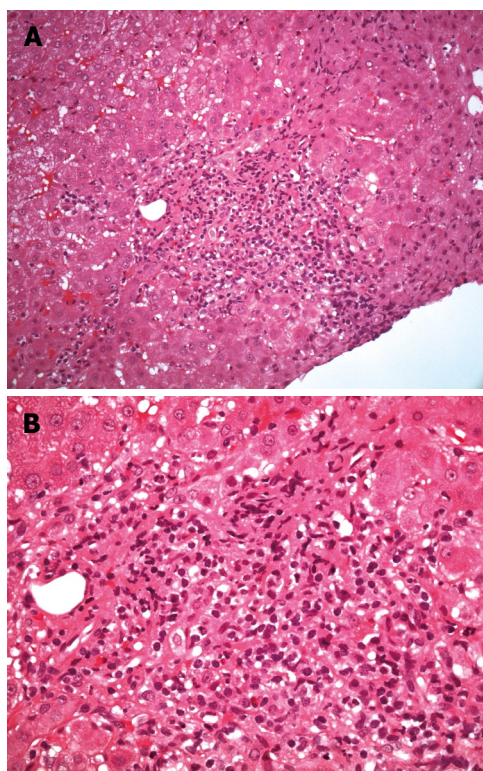
### A case report of AIH-PBC overlap syndrome

A 57-year-old woman presented to our outpatient clinic in May 2007 for evaluation of abnormal serum liver tests. She reported fatigue and slight pruritus, but was otherwise in good general health. In January 2007, elevated serum liver tests were detected for the first time when she went for a routine medical examination. At presentation, serum liver tests revealed elevated  $\gamma$ -GT ( $2 \times N$ ), elevated transaminases (AST  $2.5 \times N$ , ALT  $5.5 \times N$ ), and normal bilirubin. Serum AMA (1:3840) and AMA-M2, ASMA and SLA were positive, whereas ANA, LKM1 and ANCA were all negative. Her immunoglobulins showed elevated IgG (23.2 g/L) and IgM (3.3 g/L). Metabolic and viral liver diseases were ruled out. A liver biopsy disclosed an interface hepatitis and mild portal fibrosis without evidence of cirrhosis (Figure 1). AIH-PBC overlap syndrome was diagnosed and a combined therapy of UDCA (13-15 mg/kg daily) and budesonide (6 mg/d) was initiated. Two weeks later, her transaminases decreased by 50% and azathioprine (100 mg/d) was administered. After 3 mo of combined therapy, all serum liver tests were normal and budesonide was tapered to 3 mg/d. At the last follow-up visit in August 2007, the patient reported an improved general condition, and fatigue and pruritus disappeared.

## AUTOIMMUNE CHOLANGITIS (AIC)

### AIC-AIH OVERLAP SYNDROME

AIC shares many features with PBC and is therefore also called AMA-negative PBC. Like PBC, it is characterized by a female preponderance, a cholestatic serum enzyme pattern and florid bile duct lesions on histology and it slowly progresses to fibrosis and cirrhosis of the liver if left untreated<sup>[35]</sup>. Patients with AIC are AMA negative and often present with serum ANA and/or ASMA. Several studies support the view that AIC and PBC are variants of one single disease only differing in serum autoantibody pattern<sup>[36-38]</sup>. Twenty-two of 30 patients



**Figure 1** Overlap syndrome autoimmune hepatitis-primary biliary cirrhosis. A 57-year-old woman presented with elevated  $\gamma$ -GT ( $2 \times \text{ULN}$ ) and transaminases (AST  $2.5 \times \text{ULN}$ , ALT  $5.5 \times \text{ULN}$ ), and normal bilirubin. Serum AMA (1:3840), AMA-M2, ASMA and SLA were positive. Her immunoglobulins showed elevated IgG (23.2 g/L) and IgM (3.3 g/L). A liver biopsy disclosed an interface hepatitis and mild portal fibrosis without evidence of cirrhosis. **A:** HE,  $\times 20$ ; **B:** HE,  $\times 40$  (Courtesy of Prof. Dr. Müller-Höcker, Munich).

with AIC (AMA-negative PBC), but none of the 316 controls, were positive in a new AMA-M2 recombinant assay which detected autoantibodies directed against human E2 members of the 2-oxo acid dehydrogenase complex family<sup>[36]</sup>. In addition, immunohistochemical studies showed that PDC-E2 immunoreactivity was expressed on apical membranes of biliary epithelial cells not only in patients with PBC, but also in 7 of 9 patients with AIC<sup>[37]</sup>. Treatment response to UDCA (13-15 mg/kg daily) and outcome of liver transplantation in end-stage disease were also similar in patients with AIC and those with PBC<sup>[39,40]</sup>. Thus, these data indicate that a majority of AIC patients (when defined as AMA-negative PBC) suffer from “true” PBC.

Concomitant features of AIH and AIC have been reported in single cases. An AMA-negative woman presented with features of an AIH-AIC overlap syndrome based on the presence of hepatitic and cholestatic biochemical changes and interface hepatitis as well as bile duct lesions on histology. In analogy to the therapy of AIH-PBC overlap syndrome, this patient responded to a combined treatment with UDCA and immunosuppressors<sup>[41]</sup>.

## AIH-PSC OVERLAP SYNDROME

While AIH-PBC overlap syndrome is predominantly found among adults, AIH-PSC overlap syndromes have

mainly been described in children, adolescents and young adults<sup>[42-44]</sup>. Use of the modified AIH score led to the diagnosis of an overlap syndrome in 8% of 113 PSC patients and 1.4% of 211 PSC patients, respectively, when evaluated retrospectively<sup>[45,46]</sup>. However, diagnostic criteria are not defined for AIH-PSC overlap syndrome which makes comparability of these studies difficult. In a recently published prospective study, 41 consecutive patients who were diagnosed with PSC were evaluated for an AIH-PSC overlap syndrome<sup>[47]</sup>. The diagnosis of AIH-PSC overlap syndrome was established when the following criteria were met: (1) revised AIH score  $> 15$ ; (2) ANA or ASMA antibodies present in a titre of at least 1:40; and (3) liver histology with piecemeal necrosis, lymphocyte rosetting, moderate or severe periportal or periseptal inflammation<sup>[47]</sup>. By applying these criteria, 17% of the PSC patients were diagnosed with AIH-PSC overlap syndrome. Patients with AIH-PSC overlap syndrome were treated with UDCA (15-20 mg/kg daily), prednisolone (0.5 mg/kg daily, tapered to 10-15 mg/d) and 50-75 mg azathioprine with good biochemical response<sup>[47]</sup>. Of interest, the survival probability of the patients with AIH-PSC overlap syndrome was better than those with classical PSC as assessed by the Mayo score, a prognostic index.

The largest case series of AIH-PSC overlap syndromes in children and adolescents was published by colleagues from the Kings' College in London<sup>[44]</sup>. In this prospective study, a group of 55 children was followed up for 16 years who showed clinical, biochemical, and histological signs of AIH. In 27 of the 55 children, cholangiographic findings were typical of sclerosing cholangitis, whereas other signs and symptoms were characteristic of AIH. Therefore, the term “autoimmune sclerosing cholangitis” (ASC) was proposed for this AIH-PSC overlap syndrome. Patients with ASC more commonly suffered from inflammatory bowel disease and more often were positive for ANCA in serum than those with AIH. Serum transaminases tended to be higher in AIH, but serum alkaline phosphatase although mostly elevated in PSC was normal at several occasions in both diseases. Thus, AIH and ASC may belong to the same disease process and may overlap with PSC.

Increasing awareness for the AIH-PSC overlap syndrome has led to the observation that AIH and PSC may be sequential in their occurrence, and this has first been described in children<sup>[44]</sup>. More recently, a similar observation has been reported in a small case series of 6 adults (mean age 31 years; 4 male; 3 with ulcerative colitis) who developed biochemical and cholangiographic features of PSC after an average of 4.6 years of a diagnosis of AIH and became resistant to immunosuppressive therapy<sup>[6]</sup>. Thus, in patients with AIH who become cholestatic and/or resistant to immunosuppression, PSC should be considered and ruled out.

## Therapy

Ursodeoxycholic acid (UDCA) is widely administered in PSC due to its beneficial effects on serum liver tests,



histological features, prognostic surrogate markers, and development of colonic dysplasia associated with accompanying ulcerative colitis, although long-term efficacy of UDCA still remains unproven<sup>[48-52]</sup>. UDCA at higher doses (> 20 mg/kg daily) may be superior to standard doses for patients with PSC<sup>[53]</sup> and has also been used in the treatment of AIH-PSC overlap syndrome<sup>[47,52]</sup>. UDCA has been used in combination with immunosuppressive drugs in AIH-PSC overlap syndrome, and the long-term course was considered favorable<sup>[44,47]</sup>. Thus, UDCA in combination with an immunosuppressive regimen may be an adequate medical treatment for most patients with AIH-PSC overlap syndrome although data from controlled trials are lacking. Liver transplantation should be considered in late-stage diseases.

## REFERENCES

- 1 Czaja AJ. The variant forms of autoimmune hepatitis. *Ann Intern Med* 1996; **125**: 588-598
- 2 Poupon R. Autoimmune overlapping syndromes. *Clin Liver Dis* 2003; **7**: 865-878
- 3 Beuers U. Hepatic overlap syndromes. *J Hepatol* 2005; **42** Suppl: S93-S99
- 4 Ben-Ari Z, Czaja AJ. Autoimmune hepatitis and its variant syndromes. *Gut* 2001; **49**: 589-594
- 5 Chazouilleres O, Wendum D, Serfaty L, Montembault S, Rosmorduc O, Poupon R. Primary biliary cirrhosis-autoimmune hepatitis overlap syndrome: clinical features and response to therapy. *Hepatology* 1998; **28**: 296-301
- 6 Abdo AA, Bain VG, Kichian K, Lee SS. Evolution of autoimmune hepatitis to primary sclerosing cholangitis: A sequential syndrome. *Hepatology* 2002; **36**: 1393-1399
- 7 Burak KW, Urbanski SJ, Swain MG. A case of coexisting primary biliary cirrhosis and primary sclerosing cholangitis: a new overlap of autoimmune liver diseases. *Dig Dis Sci* 2001; **46**: 2043-2047
- 8 Pawlotsky JM, Ben Yahia M, Andre C, Voisin MC, Intrator L, Roudot-Thoraval F, Deforges L, Duvoux C, Zafrani ES, Duval J. Immunological disorders in C virus chronic active hepatitis: a prospective case-control study. *Hepatology* 1994; **19**: 841-848
- 9 Lunel F, Cacoub P. Treatment of autoimmune and extrahepatic manifestations of hepatitis C virus infection. *J Hepatol* 1999; **31** Suppl 1: 210-216
- 10 Beuers U, Rust C. Overlap syndromes. *Semin Liver Dis* 2005; **25**: 311-320
- 11 Alvarez F, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, Chapman RW, Cooksley WG, Czaja AJ, Desmet VJ, Donaldson PT, Eddleston AL, Fainboim L, Heathcote J, Homberg JC, Hoofnagle JH, Kakumu S, Krawitt EL, Mackay IR, MacSween RN, Maddrey WC, Manns MP, McFarlane IG, Meyer zum Buschenfelde KH, Zeniya M. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; **31**: 929-938
- 12 Prince MI, Chetwynd A, Craig WL, Metcalf JV, James OF. Asymptomatic primary biliary cirrhosis: clinical features, prognosis, and symptom progression in a large population based cohort. *Gut* 2004; **53**: 865-870
- 13 Vierling JM. Primary biliary cirrhosis and autoimmune cholangiopathy. *Clin Liver Dis* 2004; **8**: 177-194
- 14 Poupon R, Chazouilleres O, Poupon RE. Chronic cholestatic diseases. *J Hepatol* 2000; **32**: 129-140
- 15 LaRusso NF, Shneider BL, Black D, Gores GJ, James SP, Doo E, Hoofnagle JH. Primary sclerosing cholangitis: summary of a workshop. *Hepatology* 2006; **44**: 746-764
- 16 Lee YM, Kaplan MM. Primary sclerosing cholangitis. *N Engl J Med* 1995; **332**: 924-933
- 17 Kim WR, Lindor KD, Locke GR 3rd, Therneau TM, Homburger HA, Batts KP, Yawn BP, Petz JL, Melton LJ 3rd, Dickson ER. Epidemiology and natural history of primary biliary cirrhosis in a US community. *Gastroenterology* 2000; **119**: 1631-1636
- 18 Prince MI, James OF. The epidemiology of primary biliary cirrhosis. *Clin Liver Dis* 2003; **7**: 795-819
- 19 Boberg KM. Prevalence and epidemiology of autoimmune hepatitis. *Clin Liver Dis* 2002; **6**: 635-647
- 20 Kloppel G, Seifert G, Lindner H, Dammermann R, Sack HJ, Berg PA. Histopathological features in mixed types of chronic aggressive hepatitis and primary biliary cirrhosis. Correlations of liver histology with mitochondrial antibodies of different specificity. *Virchows Arch A Pathol Anat Histol* 1977; **373**: 143-160
- 21 Okuno T, Seto Y, Okanoue T, Takino T. Chronic active hepatitis with histological features of primary biliary cirrhosis. *Dig Dis Sci* 1987; **32**: 775-779
- 22 Davis PA, Leung P, Manns M, Kaplan M, Munoz SJ, Gorin FA, Dickson ER, Krawitt E, Coppel R, Gershwin ME. M4 and M9 antibodies in the overlap syndrome of primary biliary cirrhosis and chronic active hepatitis: epitopes or epiphenomena? *Hepatology* 1992; **16**: 1128-1136
- 23 Duclos-Vallee JC, Hadengue A, Ganne-Carrie N, Robin E, Degott C, Erlinger S. Primary biliary cirrhosis-autoimmune hepatitis overlap syndrome. Corticoreistance and effective treatment by cyclosporine A. *Dig Dis Sci* 1995; **40**: 1069-1073
- 24 Lohse AW, zum Buschenfelde KH, Franz B, Kanzler S, Gerken G, Dienes HP. Characterization of the overlap syndrome of primary biliary cirrhosis (PBC) and autoimmune hepatitis: evidence for it being a hepatic form of PBC in genetically susceptible individuals. *Hepatology* 1999; **29**: 1078-1084
- 25 Terjung B, Spengler U. Role of auto-antibodies for the diagnosis of chronic cholestatic liver diseases. *Clin Rev Allergy Immunol* 2005; **28**: 115-133
- 26 Invernizzi P, Selmi C, Ranftler C, Podda M, Wieserska-Gadek J. Antinuclear antibodies in primary biliary cirrhosis. *Semin Liver Dis* 2005; **25**: 298-310
- 27 Kanzler S, Bozkurt S, Herkel J, Galle PR, Dienes HP, Lohse AW. [Presence of SLA/LP autoantibodies in patients with primary biliary cirrhosis as a marker for secondary autoimmune hepatitis (overlap syndrome)] *Dtsch Med Wochenschr* 2001; **126**: 450-456
- 28 Colombato LA, Alvarez F, Cote J, Huet PM. Autoimmune cholangiopathy: the result of consecutive primary biliary cirrhosis and autoimmune hepatitis? *Gastroenterology* 1994; **107**: 1839-1843
- 29 Poupon R, Chazouilleres O, Corpechot C, Chretien Y. Development of autoimmune hepatitis in patients with typical primary biliary cirrhosis. *Hepatology* 2006; **44**: 85-90
- 30 Silveira MG, Talwalkar JA, Angulo P, Lindor KD. Overlap of autoimmune hepatitis and primary biliary cirrhosis: long-term outcomes. *Am J Gastroenterol* 2007; **102**: 1244-1250
- 31 Joshi S, Cauch-Dudek K, Wanless IR, Lindor KD, Jorgensen R, Batts K, Heathcote EJ. Primary biliary cirrhosis with additional features of autoimmune hepatitis: response to therapy with ursodeoxycholic acid. *Hepatology* 2002; **35**: 409-413
- 32 Chazouilleres O, Wendum D, Serfaty L, Rosmorduc O, Poupon R. Long term outcome and response to therapy of primary biliary cirrhosis-autoimmune hepatitis overlap syndrome. *J Hepatol* 2006; **44**: 400-406
- 33 Wiegand J, Schuler A, Kanzler S, Lohse A, Beuers U, Kreisel W, Spengler U, Koletzko S, Jansen PL, Hochhaus G, Mollmann HW, Prols M, Manns MP. Budesonide in previously untreated autoimmune hepatitis. *Liver Int* 2005; **25**: 927-934
- 34 Csepregi A, Rocken C, Treiber G, Malfertheiner P. Budesonide induces complete remission in autoimmune hepatitis. *World J Gastroenterol* 2006; **12**: 1362-1366
- 35 Heathcote J. Autoimmune cholangitis. *Gut* 1997; **40**: 440-442
- 36 Miyakawa H, Tanaka A, Kikuchi K, Matsushita M,

- Kitazawa E, Kawaguchi N, Fujikawa H, Gershwin ME. Detection of antimitochondrial autoantibodies in immunofluorescent AMA-negative patients with primary biliary cirrhosis using recombinant autoantigens. *Hepatology* 2001; **34**: 243-248
- 37 **Tsuneyama K**, Van De Water J, Van Thiel D, Coppel R, Ruebner B, Nakanuma Y, Dickson ER, Gershwin ME. Abnormal expression of PDC-E2 on the apical surface of biliary epithelial cells in patients with antimitochondrial antibody-negative primary biliary cirrhosis. *Hepatology* 1995; **22**: 1440-1446
- 38 **Ikuno N**, Scealy M, Davies JM, Whittingham SF, Omagari K, Mackay IR, Rowley MJ. A comparative study of antibody expressions in primary biliary cirrhosis and autoimmune cholangitis using phage display. *Hepatology* 2001; **34**: 478-486
- 39 **Kim WR**, Poterucha JJ, Jorgensen RA, Batts KP, Homburger HA, Dickson ER, Krom RA, Wiesner RH, Lindor KD. Does antimitochondrial antibody status affect response to treatment in patients with primary biliary cirrhosis? Outcomes of ursodeoxycholic acid therapy and liver transplantation. *Hepatology* 1997; **26**: 22-26
- 40 **Lacerda MA**, Ludwig J, Dickson ER, Jorgensen RA, Lindor KD. Antimitochondrial antibody-negative primary biliary cirrhosis. *Am J Gastroenterol* 1995; **90**: 247-249
- 41 **Li CP**, Tong MJ, Hwang SJ, Luo JC, Co RL, Tsay SH, Chang FY, Lee SD. Autoimmune cholangitis with features of autoimmune hepatitis: successful treatment with immunosuppressive agents and ursodeoxycholic acid. *J Gastroenterol Hepatol* 2000; **15**: 95-98
- 42 **McNair AN**, Moloney M, Portmann BC, Williams R, McFarlane IG. Autoimmune hepatitis overlapping with primary sclerosing cholangitis in five cases. *Am J Gastroenterol* 1998; **93**: 777-784
- 43 **van Buuren HR**, van Hoogstraten HJE, Terkivatan T, Schalm SW, Vleggaar FP. High prevalence of autoimmune hepatitis among patients with primary sclerosing cholangitis. *J Hepatol* 2000; **33**: 543-548
- 44 **Gregorio GV**, Portmann B, Karani J, Harrison P, Donaldson PT, Vergani D, Mieli-Vergani G. Autoimmune hepatitis/sclerosing cholangitis overlap syndrome in childhood: a 16-year prospective study. *Hepatology* 2001; **33**: 544-553
- 45 **Kaya M**, Angulo P, Lindor KD. Overlap of autoimmune hepatitis and primary sclerosing cholangitis: an evaluation of a modified scoring system. *J Hepatol* 2000; **33**: 537-542
- 46 **van Buuren HR**, van Hoogstraten HJE, Terkivatan T, Schalm SW, Vleggaar FP. High prevalence of autoimmune hepatitis among patients with primary sclerosing cholangitis. *J Hepatol* 2000; **33**: 543-548
- 47 **Floreani A**, Rizzotto ER, Ferrara F, Carderi I, Caroli D, Blasone L, Baldo V. Clinical course and outcome of autoimmune hepatitis/primary sclerosing cholangitis overlap syndrome. *Am J Gastroenterol* 2005; **100**: 1516-1522
- 48 **Rust C**, Beuers U. Medical treatment of primary biliary cirrhosis and primary sclerosing cholangitis. *Clin Rev Allergy Immunol* 2005; **28**: 135-145
- 49 **Beuers U**, Spengler U, Kruis W, Aydemir U, Wiebecke B, Heldwein W, Weinzierl M, Pape GR, Sauerbruch T, Paumgartner G. Ursodeoxycholic acid for treatment of primary sclerosing cholangitis: a placebo-controlled trial. *Hepatology* 1992; **16**: 707-714
- 50 **Stiehl A**, Walker S, Stiehl L, Rudolph G, Hofmann WJ, Theilmann L. Effect of ursodeoxycholic acid on liver and bile duct disease in primary sclerosing cholangitis. A 3-year pilot study with a placebo-controlled study period. *J Hepatol* 1994; **20**: 57-64
- 51 **Lindor KD**. Ursodiol for primary sclerosing cholangitis. Mayo Primary Sclerosing Cholangitis-Ursodeoxycholic Acid Study Group. *N Engl J Med* 1997; **336**: 691-695
- 52 **Mitchell SA**, Bansl DS, Hunt N, Von Bergmann K, Fleming KA, Chapman RW. A preliminary trial of high-dose ursodeoxycholic acid in primary sclerosing cholangitis. *Gastroenterology* 2001; **121**: 900-907
- 53 **Cullen SN**, Rust C, Flemming K, Edwards C, Beuers U, Chapman RW. High dose ursodeoxycholic acid for the treatment of primary sclerosing cholangitis is safe and effective. *J Hepatol* 2008; **48**: 792-800

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## TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, Series Editors

# Autoimmune liver serology: Current diagnostic and clinical challenges

Dimitrios P Bogdanos, Pietro Invernizzi, Ian R Mackay, Diego Vergani

Dimitrios P Bogdanos, Diego Vergani, Institute of Liver Studies, King's College London School of Medicine at King's College Hospital, Denmark Hill Campus, London SE5 9RS, United Kingdom

Pietro Invernizzi, Division of Internal Medicine and Liver Unit, San Paolo Hospital School of Medicine, University of Milan, Milan 20142, Italy

Ian R Mackay, Department of Biochemistry and Molecular Biology, Monash University, Clayton, Victoria 3800, Australia

Correspondence to: Diego Vergani, Professor, Institute of Liver Studies, King's College London School of Medicine at King's College Hospital, Denmark Hill Campus, London SE5 9RS, United Kingdom. [diego.vergani@kcl.ac.uk](mailto:diego.vergani@kcl.ac.uk)

Telephone: +44-20-32993305 Fax: +44-20-32993700

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technologies such as ELISAs and bead assays, become available to complement (or even compete with) traditional immunofluorescence procedures. We survey for the first time global trends in quality assurance impacting as it does on (1) manufacturers/purveyors of kits and reagents, (2) diagnostic service laboratories that fulfill clinicians' requirements, and (3) the end-user, the physician providing patient care, who must properly interpret test results in the overall clinical context.

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**Key words:** Autoantigen; Autoimmune hepatitis; Auto-antibody; Primary biliary cirrhosis; Primary sclerosing cholangitis; Liver disease

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## Abstract

Liver-related autoantibodies are crucial for the correct diagnosis and classification of autoimmune liver diseases (AiLD), namely autoimmune hepatitis types 1 and 2 (AIH-1 and 2), primary biliary cirrhosis (PBC), and the sclerosing cholangitis variants in adults and children. AIH-1 is specified by anti-nuclear antibody (ANA) and smooth muscle antibody (SMA). AIH-2 is specified by antibody to liver kidney microsomal antigen type-1 (anti-LKM1) and anti-liver cytosol type 1 (anti-LC1). SMA, ANA and anti-LKM antibodies can be present in de-novo AIH following liver transplantation. PBC is specified by antimitochondrial antibodies (AMA) reacting with enzymes of the 2-oxo-acid dehydrogenase complexes (chiefly pyruvate dehydrogenase complex E2 subunit) and disease-specific ANA mainly reacting with nuclear pore gp210 and nuclear body sp100. Sclerosing cholangitis presents as at least two variants, first the classical primary sclerosing cholangitis (PSC) mostly affecting adult men wherein the only (and non-specific) reactivity is an atypical perinuclear antineutrophil cytoplasmic antibody (p-ANCA), also termed perinuclear anti-neutrophil nuclear antibodies (p-ANNA) and second the childhood disease called autoimmune sclerosing cholangitis (ASC) with serological features resembling those of type 1 AIH. Liver diagnostic serology is a fast-expanding area of investigation as new purified and recombinant autoantigens, and automated

## INTRODUCTION

The presence of autoantibodies plays a central role in the diagnosis and classification of autoimmune liver diseases (AiLD)<sup>[1,2]</sup>, but their nature and significance remain challenging in regard to pathogenesis. Such antibodies discriminate between distinct subtypes of the AiLD and facilitate diagnosis of the overlap syndromes<sup>[3]</sup>. AiLD represent a broad range of disorders that can affect one or the other of the two cellular components, namely hepatocytes in autoimmune hepatitis (AIH), and cholangiocytes in primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and the autoimmune hepatitis/sclerosing cholangitis overlap syndrome of childhood, designated as autoimmune sclerosing cholangitis (ASC)<sup>[4]</sup>, and discussed elsewhere in this issue.

Antibody to nuclei (ANA) and/or to smooth muscle (SMA) characterizes type 1 AIH (AIH-1) and antibody to a liver kidney microsomal constituent (anti-LKM) defines patients with type 2 AIH (AIH-2)<sup>[5]</sup>. Usually the two patterns of serology are mutually exclusive, but in the rare cases in which they coexist, the disease features resemble those of AIH-2<sup>[6]</sup>. ASC is a third form of AiLD

which is similar clinically, histologically and serologically to AIH-1, but is associated with radiological changes of sclerosing cholangitis<sup>[7]</sup>. SMA, ANA and to a lesser extent anti-LKM can be found in post-transplantation *de novo* AIH<sup>[8]</sup>. The presence of anti-mitochondrial antibodies (AMA) with a specificity for the E2 subunit of the pyruvate complex (PDC-E2), and certain PBC-specific ANA, characterise PBC<sup>[1,9]</sup>. Perinuclear anti-neutrophil cytoplasmic antibody (p-ANCA) is the most frequent antibody reactivity in primary sclerosing cholangitis (PSC)<sup>[1,3]</sup>, but *per se* has low specificity for diagnosis.

## HISTORICAL NOTES ON AUTOIMMUNE LIVER SEROLOGY

The evolution of knowledge on AIH is discussed in another article in this issue. Here we provide a brief historical survey of the serological tests currently used by diagnostic laboratories.

### Anti-nuclear antibody (ANA)

Serum antibodies with specificity for cell nuclear antigens were first described by Miescher *et al* in 1954<sup>[10]</sup> following the discovery of the lupus erythematosus (LE) cell by Hargraves and colleagues<sup>[11]</sup> and the recognition that the LE cell phenomenon was related to a serum factor reacting with nuclear antigens, subsequently termed “antinuclear factor” (ANF), and later antinuclear antibody (ANA). Deoxyribonucleic acid (DNA) and deoxyribonucleoprotein (DNAP) were identified in 1957 as “ANF” target antigens<sup>[11,12]</sup> and it was further shown that antibodies responsible for the LE-cell phenomenon reacted with DNA and gave a “homogenous” pattern of nuclear staining by immunofluorescence<sup>[13]</sup>. In 1956 a positive test for LE cells in blood was reported in young women with a chronic liver disease then called chronic active hepatitis (CAH), leading to the designation of “lupoid hepatitis”, an early label for what is now known as AIH-1<sup>[14,15]</sup>. Testing for ANF/ANA by immunofluorescence (IFL) supplanted the cumbersome LE cell test in the early 1960s.

### Smooth-muscle autoantibody (SMA)

Antibodies binding to smooth muscle of rat stomach were initially detected in serum samples of patients with liver diseases by Johnson *et al*, in 1965<sup>[16]</sup>. The presence of SMA in patients with AiLD was confirmed by Whittingham *et al*<sup>[17]</sup>. Patients with non-AiLDs were reported as seronegative for SMA and, notably, also negative were patients with SLE. The antibody was often found in association with ANA, which was already a known marker of AIH, and tended to fade with steroid induced remission. Bottazzo *et al*<sup>[18]</sup> reported that the SMA staining arterial vessels (V), glomerular mesangium (G) and fibers surrounding the kidney tubules (T), responsible for the VGT pattern, was confined to an aggressive form of hepatitis now known to be AIH-1. The antigenic moiety mainly but not exclusively responsible for SMA activity in what in the 1970s was called CAH was identified as filamentous (F) actin<sup>[19-21]</sup>.

### Liver kidney microsomal antibody (anti-LKM)

Cytoplasmic antibodies in “CAH” were described in the laboratory of Deborah Doniach<sup>[22,23]</sup> whose group first used the expression anti-liver kidney microsomal (anti-LKM) antibodies<sup>[24]</sup>. “Microsomal” is something of a misnomer as “microsomes” are the *in vitro* equivalent of particles of the endoplasmic reticulum wherein the antigen is located. Other nosological entities in which anti-microsomal antibodies were evident included drug induced hepatitis, leading to the use of LKM1, LKM2, LKM3 to designate the different immunofluorescent patterns, which reflect the different targeted autoantigens<sup>[25]</sup>. The ability of anti-LKM1 antibodies to define a second serological type of AIH, i.e. AIH type 2, was proposed by Homberg *et al*<sup>[26]</sup>. Three groups independently identified cytochrome P450 IID6 (CYP2D6) as the molecular target of anti-LKM1 antibodies<sup>[27-29]</sup>; the group of Alvarez<sup>[27]</sup> was the first to publish its data in the form of a full-length paper.

As mentioned, other LKM antibody patterns were subsequently described. LKM2 antibodies were recognised in patients with hepatitis induced by tienilic acid<sup>[24]</sup>, a uricosuric diuretic withdrawn from clinical use in 1980 and Rizzetto’s group described LKM3 antibodies in a proportion of cases of chronic hepatitis D infected patients<sup>[30]</sup>. In contrast to anti-LKM1 and LKM2 antibodies, anti-LKM3 stained human exocrine pancreas and thyroid. Anti-LKM2 reacted with CYP2C9 and anti-LKM3 with uridine diphosphate glucuronosyl transferases (UGT)<sup>[25]</sup>. A fourth type of LKM antibodies recognising CYP1A2 and CYP2A6 has been described in patients with AIH associated with autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED)<sup>[31]</sup>. The IFL pattern of the antibody is indistinguishable from that of anti-LKM1. An anti-liver microsomal antibody (anti-LM) staining the centrolobular hepatocytes but not the kidney and which recognises CYP1A2 has been described in dihydralazine-induced hepatitis and in a few cases of AIH<sup>[32-34]</sup>.

### Liver cytosol antibody (anti-LC1)

Anti-LC1 were originally described in association with anti-LKM1, or in isolation, by Martini *et al* in patients with AIH-2<sup>[35]</sup>. Lenzi *et al* have also found anti-LC1 antibodies in 14% anti-LKM-1 antibody positive patients suffering from chronic hepatitis C virus infection<sup>[36]</sup>. The enzyme formiminotransferase cyclodeaminase (FTCD) has been identified as the molecular target of anti-LC1 antibodies<sup>[37,38]</sup>.

### Mitochondrial antibody (AMA)

The first indication that PBC could be an autoimmune disease was obtained in 1958 when the serum of a woman with PBC was found to contain high titres of complement-fixing antibodies directed to tissue homogenates<sup>[39]</sup>, that later, by absorption studies, were shown to be absorbed by a rat liver mitochondrial fraction<sup>[40]</sup>. A breakthrough for the clinical hepatologist was the observation in 1965 by Walker, Doniach,

Roitt and Sherlock that human tissue sections rich in mitochondria give a characteristic immunofluorescence pattern when they are incubated with sera from patients with PBC but not with controls which, in that study, included patients with extra-hepatic bile duct obstruction, drug induced cholestasis and viral hepatitis<sup>[41]</sup>. In 1967, Berg *et al*<sup>[42]</sup> demonstrated that PBC sera reacted *in vitro* with a trypsin-sensitive mitochondrial antigen that was named M2 antigen, in contrast to M1, the target of anti-cardiolipin antibody. Subsequently Berg developed a nomenclature based on the types of anti-mitochondrial reactivity that spanned M3-M9, but this is no longer used. The M2 antigen was located at the inner surface of the inner mitochondrial membrane of all mitochondria tested<sup>[42-45]</sup>. The target antigens of M2 were identified in the 1980s as components of the 2-oxo-acid dehydrogenase complexes, the predominant target being the E2 subunit of pyruvate dehydrogenase complex, as judged by molecular cloning<sup>[46,47]</sup>. PBC-specific AMA were later shown to recognise other enzymes of the 2-OADC, including the E2 subunits of branched chain oxoacid dehydrogenase complex (BCOADC), the oxoglutarate dehydrogenase complex (OGDC) and the PDC-E3 binding protein<sup>[1,48]</sup>.

#### **Antibodies against soluble liver antigen/liver-pancreas antigen**

Two autoantibodies, anti-soluble liver antigen (SLA) and anti-liver-pancreas (LP), both described in AIH by two independent German groups, have been shown to target the same antigen, hence the current name of anti-SLA/LP antibodies<sup>[49-51]</sup>. The LP antigen has first been reported by Berg's group in the supernatant of liver and pancreas homogenates<sup>[50]</sup>. The SLA antigen was described by Manns and colleagues in 1987 as a component of the supernatant of liver and kidney homogenates<sup>[49]</sup>. Anti-SLA antibodies detected by a competitive ELISA were then proposed as markers of a third type of severe AIH seronegative for the conventional AIH-1 autoantibodies<sup>[49]</sup>.

#### **Anti-asialoglycoprotein receptor antibodies**

Attempts to identify antigens specifically expressed on the hepatocyte surface which could serve as self targets in AiLD have led to the description of a crude liver extract preparation known as the liver specific protein (LSP) and its major component, the asialoglycoprotein receptor (ASGPR)<sup>[52,53]</sup>. ASGPR, also designated as hepatic lectin, is a type II transmembrane glycoprotein. It is the only known liver-specific autoantigen, and is constitutively expressed on the hepatocellular membrane.

### **RECOMMENDATIONS FOR AUTOANTIBODY DETECTION BY IMMUNOFLUORESCENCE (IFL)**

IFL is the main technique for the screening of autoantibodies diagnostically relevant to liver disease. The methodology is practically unchanged from that

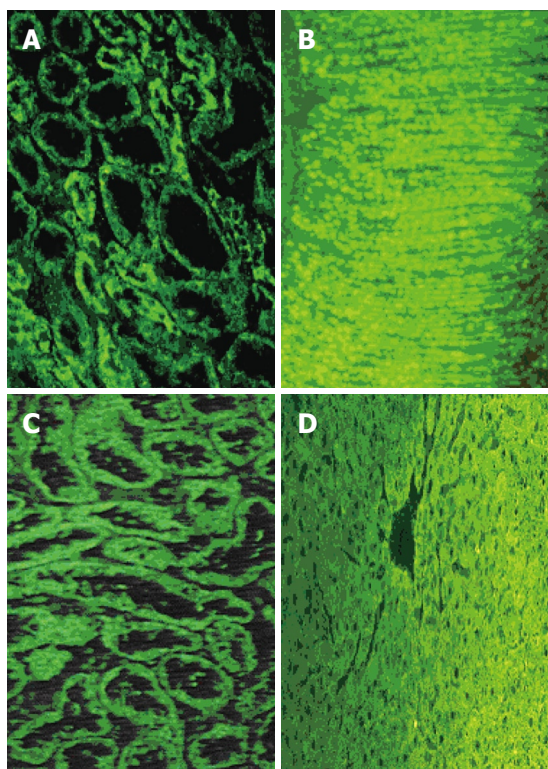
introduced by Weller and Coons in 1954<sup>[54]</sup>. It uses unfixed, air-dried, tissue sections which are incubated with a test serum potentially containing an antibody. After removing unbound serum by washing, a fluorochrome labelled second antibody, raised in animal and specific for human immunoglobulins, is applied to detect the first tissue-bound antibody<sup>[55]</sup>. Specific patterns can then be recognised using an ultraviolet microscope. A consensus statement in 2004 from the Committee for Autoimmune Serology of the International Autoimmune Hepatitis Group (IAIHG) provided guidelines on how to test for autoantibodies relevant to AIH and concluded that indirect IFL on fresh sections of multi-organ (liver, kidney, stomach) from rodents (usually rat) should be the first line screening<sup>[55]</sup>. The recommendations of the Committee include detailed guidelines for the preparation of substrate, application of the test serum samples, optimal dilution of samples and fluorochrome-labelled revealing agents, selection of controls and identification of diagnostically relevant staining patterns<sup>[55]</sup>. The use of the three tissues enables the simultaneous detection of virtually all the autoantibodies relevant to liver disease, namely SMA, ANA, anti-LKM1, AMA and anti-LC1<sup>[55]</sup>. The first serum dilution recommended for autoantibody detection (before titration) is for adults 1:40, and for children 1:20 for ANA and SMA and 1:10 for anti-LKM1 in children<sup>[55]</sup>.

#### **Autoantibodies detected by IFL and their reactants**

**ANA:** This autoantibody is readily detectable as nuclear staining in all the three tissues of the composite substrate. On the liver it is also possible to identify different patterns, the homogenous being typical of AIH-1<sup>[55]</sup>. A clearer definition of the different ANA patterns seen in PBC is best achieved by the use of the human larynx epithelioma cancer cell line (HEp-2) because these cells have large nuclei, and the mitotic phase of these cells permits the easy detection of anti-centromere antibodies (ACA) because they stain the chromosomes of cells in mitosis<sup>[56,57]</sup>. HEp-2 permit ready detection of the IFL patterns called multiple nuclear dot (MND) and rim-like membranous (RLM) typical of PBC<sup>[58,59]</sup>. Anti-MND stains 5-20 dots of variable size, distributed all over the nucleus but sparing the nucleoli<sup>[58]</sup>. The pattern can be confused with that of ACA but anti-MND do not stain the chromosomes of cells in mitosis whereas ACA do so<sup>[58]</sup>. Moreover, the dots of ACA are all of the same size while those of MND vary in size and number between individual cells<sup>[58]</sup>. In addition to homogenous ANA, speckled and nucleolar patterns are seen in AIH, and to a lesser extent in PBC, but are not disease-specific.

**SMA:** SMA of the VGT pattern is considered specific for AIH-1, though some 20%-40% of patients with AIH-1 do not have it<sup>[55]</sup>. SMA can also be detected, always by IFL, using fibroblasts or HEp-2 cells. The VGT pattern corresponds to the microfilament staining of isolated fibroblasts and represents a cable pattern across the cell<sup>[18]</sup>. Both patterns have been termed "anti-





**Figure 1** Immunofluorescence of anti-mitochondrial (A and B), and anti-liver kidney microsomal antibody (anti-LKM1) (C and D). AMA stain (A) stronger the smaller, distal tubules while anti-LKM1 the proximal tubules of the rat kidney (C). These specificities are frequently misdiagnosed, especially when only the kidney substrate is used and the sections do not contain both proximal and distal tubules. Thus, the use of rat stomach (B) and liver (D) is strongly recommended to prevent misinterpretation; AMA characteristically stain the gastric parietal cells while anti-LKM1 stain the rat liver but not the stomach.

actin” though there is no molecular proof as yet that actin is indeed the only or indeed the main target of VGT SMA.

**Anti-LKM1:** Anti-LKM1 brightly stains the third portion of the proximal renal tubules and the cytoplasm of the hepatocytes but it spares cells of the gastric mucosa<sup>[55]</sup>. Anti-LKM1 is a frequently undiagnosed autoantibody, being commonly misinterpreted as AMA<sup>[1,60]</sup>. AMA is extremely rare in pediatric patients and PBC is extremely rare in childhood<sup>[61,62]</sup>. So, when AMA is reported in a child with clinical and histological characteristics of AIH, the serological report is almost certainly incorrect.

**AMA:** The confusion between AMA and anti-LKM1 occurs because both autoantibodies stain the renal tubules, though with a pattern different to a trained eye and readily appreciated when the kidney tissue section contains both distal and proximal tubules (Figure 1). AMA stains strongly the mitochondria-rich distal tubules which are smaller than the proximal tubules stained by anti-LKM1 antibodies. AMA also stains the gastric parietal cells within the stomach, which are spared by anti LKM1, whereas AMA stains hepatocytes much less brightly than does anti-LKM1. The analysis therefore of the three-tissue substrate should allow a correct serological interpretation. Some serodiagnosticians claim

a utility of HEp-2 cells for recognition of AMA which gives a “string of pearls” pattern of cytoplasmic staining. Unfortunately interpretative problems are still frequent especially in those laboratories where only kidney is used as substrate, and particularly when the tissue is poorly oriented. Advice on how to orient and cut the kidney has been issued by the Autoimmune Serology Committee of IAIHG<sup>[55]</sup>.

**Anti-LC1:** This antibody stains the cytoplasm of hepatocytes with a zonal distribution within the liver, being particularly abundant on perivenous hepatocytes and the renal tubules. In most cases, however, anti-LC1 is obscured by the simultaneous presence of anti-LKM1<sup>[35,36]</sup>. Anti-LC1 can be also detected by gel diffusion techniques such as double dimension immunodiffusion and counter immunoelectrophoresis, techniques in which the cytosol of liver homogenate is used as antigen and the test serum is run with a positive control<sup>[63]</sup>.

**ANCA:** ANCA is detected by indirect IFL using neutrophils as substrate and can give a cytoplasmic (c-ANCA) or perinuclear (p-ANCA) pattern<sup>[64,65]</sup>. The pattern of p-ANCA is an artifact caused by the ethanol fixation of the neutrophils which leads to the migration of some positively charged cytoplasmic antigens to the negatively charged nuclear envelope, so giving the characteristic perinuclear fluorescence staining. An atypical p-ANCA staining, unaffected by ethanol fixation, gives a perinuclear staining subtly different from the classical p-ANCA. It recognizes components of the nuclear envelope and has been described, especially in patients with PSC<sup>[66]</sup>. In view of the location of the antigen, some groups are now describing these antibodies as perinuclear anti-neutrophil nuclear antibodies (p-ANNA)<sup>[67,68]</sup>.

## AUTOANTIGENS OF LIVER-RELATED AUTOANTIBODIES

### Nuclear antigens

No single AIH-1-specific nuclear antigen has been identified so far. A number of nuclear molecular targets has been detected, including centromere, histones, double-stranded DNA, chromatin, and ribonucleoprotein complexes with no single pattern or combination thereof being characteristic of AIH<sup>[3]</sup>, although most typical is a homogenous pattern attributable to anti-chromatin.

### Smooth muscle antigens

SMA giving the “anti-actin” IFL pattern has long been considered highly diagnostic for AIH type 1, its target deemed to be F-actin (noting that purified actin is a monomer G-actin, which is polymerized in the presence of ATP)<sup>[3,20,55,69]</sup>. The advent of commercial kits using highly purified F-actin as target has provided the opportunity both to test the molecular specificity of the SMA giving the IFL actin pattern and to assess the diagnostic performance of antibodies directed to molecularly pure F-actin (anti-FA)<sup>[70-75]</sup>. In Granito and



Villalta's studies, the IFL anti-actin pattern was strongly associated with AIH-1 and so was anti-FA, this latter being marginally more sensitive<sup>[70,74,75]</sup>. When disease specificity of the two reactivities was analysed the IFL pattern was found to be highly specific, being absent or extremely rare in diseases other than AIH-1. In sharp contrast, anti-FA was detectable in patients with viral hepatitis, PBC, primary sclerosing cholangitis, AIH-2 and celiac disease<sup>[70,74,75]</sup>. In a paper by Frenzel, positivity for anti-FA was found in some 75% of patients subsequently diagnosed as having AIH-1 but also in 24% non-AIH patients<sup>[71]</sup>. In an attempt to address the relatively high non-specificity of the molecular assay, Villalta *et al* performed a receiver operating curve (ROC) analysis, from which they deduced for this assay a cut off point giving a specificity similar to IFL: the cut off point had to be increased from the 30 arbitrary units (AU) suggested by the manufacturer to 53 AU<sup>[75]</sup>. At this cut-off point the specificity of the molecular assay was indeed comparable to that of IFL, but the sensitivity dropped by more than 10% below that of IFL.

The results obtained with the IFL and molecular assays overlap considerably, but by no means completely, with several instances of positivity with one test and not with the other<sup>[72,73,75]</sup>. With the availability of highly purified F-actin the question as to whether the antibody responsible for the anti-actin IFL pattern is directed against actin could be tested directly<sup>[70-75]</sup>. Three anti-SMA positive sera containing both reactivities were absorbed with solid phase F-actin: the reactivity against F-actin was abolished (absorbed out) but that giving the fluorescent pattern was unaltered in two of the 3 sera and reduced, but not abolished, in the third<sup>[72]</sup>. In summary, detection of the IFL anti-actin pattern continues to provide to date the best specificity/sensitivity compromise<sup>[55]</sup>. The antibody responsible for the IFL "actin" pattern targets, in addition to actin, molecules other than actin<sup>[3,72]</sup>. The question arises as to whether to maintain the tradition, and with it the term of "anti-actin" for the antibody recognised in IFL, or whether to call it anti-micro filament (MF) pattern as suggested by the Serology committee of the IAIHG<sup>[55]</sup>.

### **LKM1 antigen**

While the target antigens of ANA and SMA certainly need better molecular definition, that of anti-LKM1 in AIH-2 has been clearly identified as the microsomal enzyme cytochrome P450IID6 (CYP2D6)<sup>[5,26-28]</sup>. Its identification has enabled the establishment of assays based on the use of recombinant antigens which have proven useful in solving diagnostic uncertainties between AMA and anti-LKM1<sup>[1,60,76]</sup>. Such ELISAs, however, are not always able to detect anti-LKM1 antibodies in patients with chronic hepatitis C virus infection whereas IFL and radioligand assays can do so possibly because of their ability to identify conformational epitopes undetectable by ELISA<sup>[77-80]</sup>. Short CYP2D6 peptides used as antigenic preparations perform less well than those using full-length CYP2D6 and their diagnostic use is limited.

### **LC1 antigen**

ELISAs for detection of antibodies to FTCD, the target of anti-LC1, have been developed and used in diagnostic laboratories and their diagnostic and clinical relevance is under investigation<sup>[37,38]</sup>.

### **SLA/LP and ASGPR**

Progress has been made in the definition of other autoantibodies frequently present in AIH but undetectable by IFL including antibodies against SLA/LP<sup>[51,81-85]</sup> and ASGPR. Most of anti-SLA/LP positive patients are also positive for ANA, SMA or anti-LKM1, but occasionally anti-SLA is present in isolation and, in this case, its detection is of diagnostic importance<sup>[81,86]</sup>. The identification of the molecular target of anti-SLA/LP antibodies as the UGA serine tRNA-associated protein has led to the development of ELISA or dot-blot assays increasingly replacing the conventional inhibition ELISA originally used for anti-SLA antibody detection<sup>[51,83]</sup>. Recent studies investigating the exact role of this protein have shown that SLA/LP is a selenocysteine synthase but how the biosynthesis of selenocysteine may relate to the pathogenesis of AIH is not known<sup>[87]</sup>.

Anti-ASGPR antibody detection requires either purified or recombinant antigen. The lack of disease-specificity and the difficulty in developing a reliable molecular based assay for the detection of anti-ASGPR has limited its wider applicability in diagnostic practice.

### **Mitochondrial antigens**

The most recent advance in the immunodiagnosis of AMA is the availability of an ELISA using the triple MIT3 hybrid antigen preparation, developed in the Gershwin laboratory. This preparation contains all three immunodominant mitochondrial antigenic epitopes, namely PDC-E2, BCOADC-E2 and OGDC-E2<sup>[88]</sup>. Although assays based on MIT3 are reported to give positive results for PBC sera that test negative for AMA by conventional IFL techniques<sup>[89,90]</sup>, IFL testing for AMA should remain the screening procedure.

### **PBC-specific nuclear antigens**

As mentioned above, major target antigens of PBC-specific ANA have been identified. These include the nuclear body speckled 100 kDa (sp100), promyelocytic leukaemia (PML), and small ubiquitin-like modifier (SUMO) proteins corresponding to the MND pattern, and proteins within the nuclear pore complex (anti-NPC) including the 210 kDa glycoprotein (gp210) and the 62 kDa nucleoporin (NUP62), the major target antigens of anti-RLM antibodies and responsible for the RLM pattern<sup>[58,59,91]</sup>. New immunoassays testing autoantibodies to sp100, PML, gp210 and NUP62 have been developed using short peptides, polypeptides or full-length proteins as targets, but they have not been fully evaluated nor standardized<sup>[91-98]</sup>. They may be of diagnostic assistance, especially in those cases where it is difficult to interpret the IFL staining patterns due to concurrent autoantibody reactivities or in true AMA-negative PBC cases<sup>[1,92,99,100]</sup>. We note also the presence

of ACA reactivity in the combined PBC/CREST disease. Assays to detect multiple reactivities (multiplex) and to provide a full autoimmune serological profile of relevance to PBC are being developed<sup>[89]</sup>. At present, a lack of guidelines for the detection of PBC-specific autoantibodies by scientific bodies responsible for the standardization of autoimmune serological tests is a significant handicap and perpetuates uncertainties on which are the clinically relevant tests (see below).

### Atypical p-ANCA (pANNA) antigens

These are under current investigation<sup>[66-68]</sup>. The original description of a 50 kDa neutrophil-specific nuclear protein of the nuclear pore complex as the target antigen recognised by 90% of atypical p-ANCA from patients with PSC was followed by a study from the same group suggesting that the identity of the antigen is tubulin beta chain 5 (TBB5)<sup>[101]</sup>. However, when using the molecular target for their detection anti-TTB5 antibodies were found not only in PSC but also in other AiLDs.

## DIAGNOSTIC RELEVANCE OF LIVER-RELATED AUTOANTIBODIES

ANA, SMA, anti-LKM1, AMA and p-ANCA should be determined in all patients with biochemical, clinical and/or histological features suggestive of AiLD<sup>[3,5]</sup>. Autoantibody titres usually vary during the course of the disease. Hence seronegativity or low autoantibody titres on a single test cannot exclude the diagnosis of AiLD and repeat tests may allow autoantibody detection and correct disease classification. Conversely, the presence of autoantibodies even at high titres in the absence of any other clinical and laboratory features suggestive of AiLD is insufficient to make a diagnosis though a patient with high titre autoantibodies needs to be seen at regular intervals. Titres of ANA, SMA and LKM1 antibodies contribute in calculating the IAIHG diagnostic score for patients with a probable or definite diagnosis of AIH<sup>[5]</sup>. IFL titres of > 1:80 attract a +3 score; 1:80 a +2 score and 1:40 +1 score. A negative score of -4 is given to cases with hepatic features but detectable AMA at a titre of  $\geq$  1:40; such mixed serology points to “overlap syndrome”, discussed in another article in this issue. In children, titres of 1:20 for ANA or SMA and 1:10 for anti-LKM1 are sufficient to support the diagnosis of AIH if accompanied by other suggestive features<sup>[5,55]</sup>.

In AIH-1, ANA alone are present in 15% of patients, SMA alone in 35%, and ANA and SMA co-occur in 60%<sup>[3]</sup>. In the 5% or so of cases negative for these reactivities, anti-SLA/LP may be positive. In AIH-2 at presentation anti-LKM1 and/or anti-LC1 antibodies are positive in more than 90% of patients<sup>[25,35,36,63]</sup>. In PBC, AMA are detectable in more than 95% of patients and disease-specific ANA occur in 30%-70% of PBC patients according to different reports<sup>[9,58,59,100]</sup>. In PSC, atypical p-ANCA are present in up to 90% of patients but this reactivity also occurs in AIH (up to 70%) and PBC (5%),

as well as frequently in patients with inflammatory bowel disease<sup>[66-68,102,103]</sup>. In what is termed “*de novo*” AIH and in post-liver transplant patients, ANA, SMA, AMA and anti-LKM have been reported, at varying frequencies<sup>[85,94]</sup>. A diagnosis of AIH-2 is strongly supported by seropositivity for anti-LKM1 and/or anti-LC1, particularly in the absence of viral hepatitis C<sup>[5]</sup>. For PBC, the presence of AMA is one of the three widely accepted diagnostic criteria<sup>[9]</sup>.

Autoantibody positivity is part of the criteria used for the diagnosis of AiLD, though it is not diagnostic on its own. Elevated titres and certain patterns carry significant diagnostic connotations.

We are aware of various reports that, at first sight, might appear prejudicial to the diagnostic utility of liver-related autoantibodies<sup>[104]</sup>. Thus ANA and/or SMA are reported in PBC, PSC, *de novo* AIH, chronic viral hepatitis B, C and D, acute liver failure, drug-induced hepatitis, non-alcoholic steatohepatitis, alcohol-induced liver disease, hepatocellular carcinoma, and also in a variety of non-liver related diseases. Hence, the diagnostic significance of antibody positivity depends on the associated clinical features<sup>[3]</sup>, as well as the level of reactivity. Anti-LKM1 and anti-LC1 are reported in a proportion of adult (0%-6%) or pediatric (0%-11%) cases with chronic hepatitis C infection<sup>[36,105-107]</sup>. AMA are present (expectedly) in patients with AIH/PBC overlap syndrome, and also in chronic hepatitis C virus infected patients<sup>[1]</sup>, and most recently were described in patients with acute liver failure<sup>[108]</sup>; AMA occur also in various rheumatological disorders which may co-exist with PBC notably Sjögren's syndrome and systemic sclerosis<sup>[1,48,108-111]</sup> and are described in non-liver related conditions with asymptomatic recurrent bacteriuria in women, pulmonary tuberculosis and leprosy<sup>[112-114]</sup>. However we would submit that in the index disease (AIH or PBC) the frequency and titre of the relevant liver-related autoantibody is substantially higher than for the contrast disease.

Anti-ASGPR antibodies are found particularly in AIH-1 (approximately 90%) but are also present in patients with PBC (14%), chronic hepatitis B and C (7%) and alcoholic hepatitis (8%)<sup>[3,52,115]</sup>. Anti-SLA antibodies can be found in occasional seronegative AIH patients i.e. those who are negative for ANA, SMA or anti-LKM-1. Anti-SLA antibodies are also frequently present (up to 50%, depending on the sensitivity of the method used) in typical cases of AIH-1 and AIH-2, and also in ASC<sup>[86]</sup>. Their high specificity for AiLD has been questioned by reports of anti-SLA being present in some 10% of chronically infected HCV patients<sup>[115]</sup>. More recently, anti-SLA antibodies have been described in 22% of patients with acute liver failure (ALF)<sup>[111]</sup>. Since in most cases of ALF we do not know the cause, the presence of anti-SLA can either detract from their disease specificity or, alternatively, suggest an autoimmune pathogenesis (or an autoimmune component to the pathogenesis) of ALF. Monitoring of autoantibodies may be useful in the case of AIH as disappearance or sharp decrease of ANA, SMA and anti-LKM1 can be an indicator of response to

immunosuppressive treatment<sup>[3,6]</sup>. AMA titres do not relate to the stage of PBC and their fluctuation over time does not seem to have pathogenic significance<sup>[1,9,116]</sup>, although “activity” of the PBC process is not as readily measurable as that of AIH. Practically AMA are only tested at presentation to help establish the diagnosis and repeat tests are normally requested only in cases seronegative for AMA at presentation but with clinical or laboratory findings compatible with PBC<sup>[1,2,117]</sup>.

## PROGNOSTIC SIGNIFICANCE AND UTILITY OF LIVER-RELATED AUTOANTIBODIES

### AIH

Both SMA and ANA tend to lower in titre and even disappear during immunosuppressive therapy in most patients with AIH-1 although neither their titre at diagnosis nor their fluctuations during the disease are thought to predict disease course and outcome<sup>[3]</sup>. However, in 2002 Gregorio *et al* found a positive correlation between SMA titre and AST levels over time in pediatric AIH-1 cases, suggesting a potential use of these antibodies, together with IgG levels, to monitor disease activity<sup>[118]</sup>. There are no comparable adult sequential studies; this may be a reason why no correlation has been ascertained. Nevertheless, Czaja and colleagues have suggested that adult AIH-1 patients with antibodies to anti-actin have a disease onset earlier in life, respond less well to corticosteroids and progress to liver failure or require liver transplantation more frequently compared to those without anti-actin antibodies<sup>[69]</sup>. The presence of antibodies to double stranded DNA (dsDNA) has been associated with higher levels of immunoglobulin G and higher relapse rates during immunosuppressive treatment compared to seronegative cases<sup>[119]</sup>. Seropositivity for anti-ASGPR in patients with AIH correlates with histological activity with persistence indicating unresponsiveness to immunosuppressive treatment, and re-appearance being highly suggestive of relapse especially after corticosteroid withdrawal<sup>[3,52,115,120]</sup>. Anti-SLA antibodies denote patients with a more severe course of AIH and a propensity for relapse after corticosteroid withdrawal compared to their negative counterparts<sup>[49,81,121,122]</sup>. AIH-2 patients with anti-LC1 antibodies have histologically more severe disease compared to those without anti-LC1 antibodies<sup>[35,123,124]</sup>.

### PBC

AMA titres do not seem to be associated with disease severity but those of the IgG3 subclass may identify patients prone to develop more severe disease compared to those without AMA-IgG3<sup>[116,125]</sup>. PBC-specific ANA have been found more frequently in patients with advanced disease in a number of cross-sectional studies. Anti-NPC seropositivity is associated with accelerated progression to advanced disease and death<sup>[94,96,100,126-129]</sup> and also, ACA may identify patients with more severe PBC according to studies from USA and Japan<sup>[96,130]</sup>. These data

have obvious implications for the clinical management of PBC given that the only accepted index for estimating survival has been obtained and validated in patients with advanced PBC and hence is of limited use in early disease. Thus, anti-NPC and ACA testing may be important for identifying asymptomatic patients with a likely unfavourable disease course. Once PBC has progressed to advanced histological stages, and serum bilirubin levels have become abnormal, anti-NPC determinations do not appear to offer any additional advantage over other prognostic models such as the Mayo risk score.

## PATHOGENIC RELEVANCE OF LIVER-RELATED AUTOANTIBODIES

Despite their undoubted clinical relevance in diagnosis and classification of AiLD, the pathogenic role of autoantibodies and the mechanisms through which they may cause liver damage remains a topic for further research, mainly because of the difficulty in discriminating those actively involved in the immunopathogenic cascade, from those secondary to liver cell damage. The mechanism(s) responsible for the induction of liver-related autoantibodies is currently unknown; several possibilities including molecular mimicry and immunological cross-reactivity have been suggested<sup>[78,93,106,131-145]</sup>. Most liver-related autoantibodies have limited organ specificity and this notion militates against a direct pathogenic role in highly organ-specific autoimmune injury. For antibodies with a pathogenic potential, complement-dependent and/or antibody-dependent cell-mediated cytotoxicity (ADCC) are the likely effectors of damage<sup>[131,146]</sup>.

## EMERGING ISSUES: DIAGNOSTIC ACCURACY, QUALITY ASSURANCE AND STANDARDIZATION PROGRAMMES FOR LIVER AUTOIMMUNE SEROLOGY

There are a number of open issues on serum autoantibodies in AiLD. Their diagnostic significance is unquestioned, but problems concerning autoantibody detection and interpretation have not yet been resolved and are not being addressed with sufficient vigour. Several laboratories ignore, for example, the IFL cut-off points recommended by the Committee for Autoimmune Serology of the IAIHG and use their own, thus undermining comparability between different laboratories/centres. Worryingly, the cost per test seems a major reason for arbitrary elevation of cut-off points in routine practice: selecting 1:80 or even 1:160 as a screening dilution expands the number of “negatives” albeit reducing or eliminating the need for re-testing. In patients with AiLD and relatively low autoantibody titres, such as children with AIH, a report that is inaccurately indicative of negativity for autoantibodies can delay diagnosis and, harmfully, defer treatment<sup>[76,147]</sup>. Hence rigorously performed autoantibody testing may in fact

provide a more economical report than a “false negative” one if such leads the clinician to order additional costly diagnostic procedures.

Additional problems for autoantibody testing especially with IFL are intrinsic to the methodology itself. First, availability of tissue substrate comprised of freshly cut sections from cryostat blocks of unfixed liver, kidney stomach tissue is limited to relatively few specialised laboratories. Second, sections of commercial origin are of variable quality because, to lengthen shelf-life, they are treated with fixatives, which readily result in enhanced background staining<sup>[55]</sup>. Third, IFL requires highly-trained and experienced personnel, is time-consuming and cannot be automated, resulting in a low throughput and increased personnel costs leading to a significant shift from IFL towards ELISAs or blot assays based on liver-autoantibody profiles; these compared to IFL are less-time consuming, easy to perform and amenable to automation. However, the authors of this review reiterate the recommendations of the Committee for Autoimmune Serology of the IAIHG stating that the current ELISAs should complement but not replace IFL. Either technique has their *pros* and *cons*, and gives answers to different questions, such that results are not directly comparable<sup>[148-150]</sup>. Most liver-related autoantibodies can be detected by IFL when using a triple rodent tissue. HEP-2 cells can help to differentiate ANA patterns and ethanol-fixed neutrophils can be used for the detection of ANCA. In contrast, ELISAs give answers for (usually) pre-selected individual autoantibody specificities. While the analytical sensitivity of ELISAs is satisfactory, their specificity varies according to the manufacturer<sup>[150]</sup> whereas such problems are rather infrequent by IFL testing based on a triple rodent tissue substrate<sup>[151]</sup>.

Over the last decade there has been a steady increase in the use of the liver-related autoantibody tests to assist both diagnosis and clinical research into ALD<sup>[55]</sup>. This increase has been attributed mainly to the introduction of molecularly based assays for the testing of antibodies to F-actin<sup>[70-75]</sup>, CYP2D6<sup>[152]</sup> and SLA<sup>[83,122,153]</sup> in AIH, and for evaluating antibodies to sp100 and gp210 in PBC<sup>[92,94-96,154]</sup>. Of concern, results for these antibody specificities may be promulgated by laboratories without authentication from externally or independently monitored quality assurance programmes (QAP).

Quality assurance (QA) can occur at three levels. The first is at the level of commercial providers of assay kits, reagents etc who would establish QA “in house” before marketing but who often elect to participate also in QAPs for routine laboratories. The second are the formalised QAPs, run by semi-governmental agencies or other organizations, as described below. The third level, which scarcely exists, involves the end-user, the responsible clinician, who must order tests advisedly with good clinical data and interpret these in the light of the clinical information to make wise evidence-based decisions. Thus it behoves the clinician to become fully aware of the many contributions (and shortcomings) of contemporary diagnostic immunoserology.

**Table 1** Laboratories from various countries participating to the UK National External Quality Assessment Service (UK NEQAS)

Country	Number	Country	Number
Austria	3	Latvia	1
Belgium	7	Malaysia	1
Croatia	2	Malta	1
Cyprus	1	New Zealand	3
Denmark	2	Norway	9
Eire	15	Portugal	31
Estonia	1	Republic of Chile	1
Finland	5	Singapore	1
France	29	South Africa	3
Germany	9	Spain	68
Greece	16	Sweden	14
Hong kong	1	Switzerland	7
Hungary	5	The Netherlands	1
Israel	8	Turkey	2
Italy	65	UK	136
Kingdom of Saudi Arabia	1	United Arab Emirates	1
Kuwait	1	USA	3

## REPRESENTATIVE QUALITY ASSURANCE PROGRAMMES FOR DIAGNOSTIC SEROLOGY IN LIVER DISEASE

### USA

The College of American Pathologists (CAP, [www.cap.org](http://www.cap.org)) runs survey programmes which allow laboratories to evaluate regularly their autoantibody testing performance. Of relevance to liver, CAP circulates coded anti-M2 AMA, anti-LKM1 and SMA samples for testing. The participating laboratories analyse the sera and return their results for evaluation. In return, each laboratory receives an anonymised report of the performance of all participating laboratories.

### UK

A National External Quality Assessment Service (UK NEQAS) ([www.ukneqas.org.uk](http://www.ukneqas.org.uk)) is responsible for the objective assessment of the performance of autoantibody testing. The UK NEQAS for General Autoimmune Serology incorporates one sample in each of six distributions annually for AMA, anti-LKM1 and SMA. The performance reports of the participating laboratories also provide information on kit suppliers. Participation is not limited to UK but is open to non-UK Countries (Table 1).

### Germany

There are currently two regulatory and quality assurance agencies, namely INSTAND (Institut für Standardisierung, [www.instandev.de](http://www.instandev.de)) and DGKL (Deutsche Gesellschaft für Klinische Chemie und Laboratoriumsmedizin, [www.dgkl.de](http://www.dgkl.de)). INSTAND circulates twice per year two samples to be tested for AMA, SMA and anti-LKM1 antibody testing. Participants (150) report results quantitatively and semi-quantitatively (from 0-4 to evaluate antibody titre; 0 = negative; 1 = borderline; 2 = low; 3 = middle; and 4 =



high). There is no reference to specific manufacturers but only to test methods and overall percentage of consistent results. DGKL has a similar approach but evaluations are divided on the basis of the methods used and they provide also information in relation to the kits manufacturers. Target values are determined in two reference laboratories.

### France

Quality autoantibody assessment in France is organised by the French Health Products Safety Agency (AFSSAPS, Agence Française de Sécurité Sanitaire des Produits de Santé, <http://agmed.sante.gouv.fr/>). This Agency has the executive responsibility for proposing relevant QAPs to clinical laboratories, whether in the private or in the public sector. An autoantibody detection survey has been running on an annual basis since 1998.

### Italy

In Italy there are no formal regulatory and quality assurance programmes with several laboratories participating in the surveys by UK NEQAS or CAP. Recently, a study group has been formed (Forum Interdisciplinare per la Ricerca nelle Malattie Autoimmuni-FIRMA-[www.gruppofirma.com](http://www.gruppofirma.com)). FIRMA aims to provide guidelines for autotibody testing and to identify and collect sera of different autoantibody specificities that will be available for all of its member institutions.

### Finland

Labquality at Helsinki offers twice per year three samples for SMA, AMA and anti-LKM1 assessment. Qualitative target values are determined in a reference laboratory and results are listed according to manufacturer and method. Evaluation reports are confidential.

### Australia and New Zealand

QAPs have been established under the auspices of the Royal College of Pathologists of Australasia (RCPA) based on the selection by RCPA of expert organizing groups which distribute batches of sera to diagnostic laboratories that voluntarily elect to participate ([www.rcpaqap.com.au](http://www.rcpaqap.com.au)). Diagnostic laboratories from Australia, New Zealand, and several South East Asian countries together with manufacturers and purveyors of kits participate in this programme. The Tissue Antibodies module includes AMA, SMA and anti-LKM1 antibodies. Feedback to the laboratories is by a report to all participants in which any single laboratory can identify its own performance versus that of all other participants. The RCPA issues certification of participation in this QAP. The reports sent back to laboratories are inspected by the National Association of Testing Authorities (NATA) during laboratory assessment visits. In order to be accredited, laboratories must participate and perform satisfactorily in the relevant proficiency testing programmes. There is a 'regulatory' element here in that NATA certification is required for access to fees under the Medicare rebate scheme.

As expected, quality assurance programmes have highlighted difficulties encountered by peripheral laboratories. In mid-2007, UK NEQAS distributed a serum with a typical anti-LKM1 antibody staining; a substantial proportion (53 out of 356, 15%) of the laboratories reported negativity for anti-LKM1 antibody test and, among these 53 laboratories, 43 incorrectly reported positivity for AMA instead (Peter White, UK NEQAS, personal communication). Also, rather worryingly, several additional laboratories did not return reports on anti-LKM1 either because they themselves do not offer this test or because they ignore its significance (Peter White, UK NEQAS, personal communication).

It is clear that exchange of calibrated reference sera and rigorous standardization programmes on liver-related autoantibody serology are urgently needed. Such initiatives will need to involve initially researchers and laboratories with a special interest in the respective antibody specificities and subsequently clinical laboratories performing routine screening tests. To this end, efforts have been made recently by the IAIHG to arrange an exchange of sera at international level but whether such an initiative will take off depends on securing financial support. Administrative sponsorship should initially come from the International Association for the Study of Liver (IASL), the American Association for the Study of Liver Diseases (AASLD) or the European Association for the Study of Liver (EASL) or from Clinical Immunology Societies of developed countries.

In conclusion, practice guidelines on liver autoimmune serology based on consensus of experts in the field have been issued and need to be steadily updated<sup>[55]</sup>. The more the clinician is aware of these guidelines, the greater the chance of correct and clinically relevant autoantibody diagnosis. It is in the best interest of the patient to obtain eventually the highest possible commitment and coordination of all organizations, agencies, industrial partners and networks working in the field.

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## REFERENCES

- 1 **Bogdanos DP**, Baum H, Vergani D. Antimitochondrial and other autoantibodies. *Clin Liver Dis* 2003; **7**: 759-777, vi
- 2 **Invernizzi P**, Lleo A, Podda M. Interpreting serological tests in diagnosing autoimmune liver diseases. *Semin Liver Dis* 2007; **27**: 161-172
- 3 **Czaja AJ**, Homburger HA. Autoantibodies in liver disease. *Gastroenterology* 2001; **120**: 239-249
- 4 **Selmi C**, Mackay IR, Gershwin ME. The immunological milieu of the liver. *Semin Liver Dis* 2007; **27**: 129-139
- 5 **Alvarez F**, Berg PA, Bianchi FB, Bianchi L, Burroughs AK, Cancado EL, Chapman RW, Cooksley WG, Czaja AJ, Desmet VJ, Donaldson PT, Eddleston AL, Fainboim L,

- Heathcote J, Homberg JC, Hoofnagle JH, Kakumu S, Krawitt EL, Mackay IR, MacSween RN, Maddrey WC, Manns MP, McFarlane IG, Meyer zum Buschenfelde KH, Zeniya M. International Autoimmune Hepatitis Group Report: review of criteria for diagnosis of autoimmune hepatitis. *J Hepatol* 1999; **31**: 929-938
- 6 **Gregorio GV**, Portmann B, Reid F, Donaldson PT, Doherty DG, McCartney M, Mowat AP, Vergani D, Mieli-Vergani G. Autoimmune hepatitis in childhood: a 20-year experience. *Hepatology* 1997; **25**: 541-547
  - 7 **Gregorio GV**, Portmann B, Karani J, Harrison P, Donaldson PT, Vergani D, Mieli-Vergani G. Autoimmune hepatitis/sclerosing cholangitis overlap syndrome in childhood: a 16-year prospective study. *Hepatology* 2001; **33**: 544-553
  - 8 **Kerkar N**, Hadzic N, Davies ET, Portmann B, Donaldson PT, Rela M, Heaton ND, Vergani D, Mieli-Vergani G. De-novo autoimmune hepatitis after liver transplantation. *Lancet* 1998; **351**: 409-413
  - 9 **Kaplan MM**, Gershwin ME. Primary biliary cirrhosis. *N Engl J Med* 2005; **353**: 1261-1273
  - 10 **Miescher P**, Fauconnet M. [Absorption of L. E. factor by isolated cell nuclei.] *Experientia* 1954; **10**: 252-253
  - 11 **Hargraves M**, Richmond H, Morton R. Presentation of two bone marrow elements: The "tart" cells and the "L.E" cell. *Mayo Clin Proc* 1948; **27**: 25-28
  - 12 **Robbins WC**, Holman HR, Deicher H, Kunkel HG. Complement fixation with cell nuclei and DNA in lupus erythematosus. *Proc Soc Exp Biol Med* 1957; **96**: 575-579
  - 13 **Holman H**, Deicher HR. The reaction of the lupus erythematosus (L.E.) cell factor with deoxyribonucleoprotein of the cell nucleus. *J Clin Invest* 1959; **38**: 2059-2072
  - 14 **Cowling DC**, Mackay IR, Taft LI. Lupoid hepatitis. *Lancet* 1956; **271**: 1323-1326
  - 15 **Mackay IR**, Taft LI, Cowling DC. Lupoid hepatitis and the hepatic lesions of systemic lupus erythematosus. *Lancet* 1959; **1**: 65-69
  - 16 **Johnson GD**, Holborow EJ, Glynn LE. Antibody to smooth muscle in patients with liver disease. *Lancet* 1965; **2**: 878-879
  - 17 **Whittingham S**, Irwin J, Mackay IR, Smalley M. Smooth muscle autoantibody in "autoimmune" hepatitis. *Gastroenterology* 1966; **51**: 499-505
  - 18 **Bottazzo GF**, Florin-Christensen A, Fairfax A, Swana G, Doniach D, Groeschel-Stewart U. Classification of smooth muscle autoantibodies detected by immunofluorescence. *J Clin Pathol* 1976; **29**: 403-410
  - 19 **Gabbiani G**, Ryan GB, Lamelin JP, Vassalli P, Majno G, Bouvier CA, Cruchaud A, Luscher EF. Human smooth muscle autoantibody. Its identification as antiactin antibody and a study of its binding to "nonmuscular" cells. *Am J Pathol* 1973; **72**: 473-488
  - 20 **Lidman K**, Biberfeld G, Fagraeus A, Norberg R, Torstensson R, Utter G, Carlsson L, Luca J, Lindberg U. Anti-actin specificity of human smooth muscle antibodies in chronic active hepatitis. *Clin Exp Immunol* 1976; **24**: 266-272
  - 21 **Toh BH**. Smooth muscle autoantibodies and autoantigens. *Clin Exp Immunol* 1979; **38**: 621-628
  - 22 **Rizzetto M**, Swana G, Doniach D. Microsomal antibodies in active chronic hepatitis and other disorders. *Clin Exp Immunol* 1973; **15**: 331-344
  - 23 **Rizzetto M**, Bianchi FB, Doniach D. Characterization of the microsomal antigen related to a subclass of active chronic hepatitis. *Immunology* 1974; **26**: 589-601
  - 24 **Smith MG**, Williams R, Walker G, Rizzetto M, Doniach D. Hepatic disorders associated with liver-kidney microsomal antibodies. *Br Med J* 1974; **2**: 80-84
  - 25 **Manns MP**, Obermayer-Straub P. Cytochromes P450 and uridine triphosphate-glucuronosyltransferases: model autoantigens to study drug-induced, virus-induced, and autoimmune liver disease. *Hepatology* 1997; **26**: 1054-1066
  - 26 **Homberg JC**, Abuaf N, Bernard O, Islam S, Alvarez F, Khalil SH, Poupon R, Darnis F, Levy VG, Gripon P. Chronic active hepatitis associated with antiliver/kidney microsome antibody type 1: a second type of "autoimmune" hepatitis. *Hepatology* 1987; **7**: 1333-1339
  - 27 **Gueguen M**, Meunier-Rotival M, Bernard O, Alvarez F. Anti-liver kidney microsome antibody recognizes a cytochrome P450 from the IID subfamily. *J Exp Med* 1988; **168**: 801-806
  - 28 **Manns MP**, Johnson EF, Griffin KJ, Tan EM, Sullivan KF. Major antigen of liver kidney microsomal autoantibodies in idiopathic autoimmune hepatitis is cytochrome P450db1. *J Clin Invest* 1989; **83**: 1066-1072
  - 29 **Zanger UM**, Hauri HP, Loeper J, Homberg JC, Meyer UA. Antibodies against human cytochrome P-450db1 in autoimmune hepatitis type II. *Proc Natl Acad Sci USA* 1988; **85**: 8256-8260
  - 30 **Crivelli O**, Lavarini C, Chiaberge E, Amoroso A, Farci P, Negro F, Rizzetto M. Microsomal autoantibodies in chronic infection with the HBsAg associated delta (delta) agent. *Clin Exp Immunol* 1983; **54**: 232-238
  - 31 **Clemente MG**, Meloni A, Obermayer-Straub P, Frau F, Manns MP, De Virgiliis S. Two cytochromes P450 are major hepatocellular autoantigens in autoimmune polyglandular syndrome type 1. *Gastroenterology* 1998; **114**: 324-328
  - 32 **Boccaccio F**, Attali P, Nataf J, Ink O, Fabre M, Pelletier G. [Acute hepatitis caused by dihydralazine] *Gastroenterol Clin Biol* 1987; **11**: 614
  - 33 **Manns M**, Zanger U, Gerken G, Sullivan KF, Meyer zum Buschenfelde KH, Meyer UA, Eichelbaum M. Patients with type II autoimmune hepatitis express functionally intact cytochrome P-450 db1 that is inhibited by LKM-1 autoantibodies in vitro but not in vivo. *Hepatology* 1990; **12**: 127-132
  - 34 **Bourdi M**, Larrey D, Nataf J, Bernuau J, Pessayre D, Iwasaki M, Guengerich FP, Beaune PH. Anti-liver endoplasmic reticulum autoantibodies are directed against human cytochrome P-450IA2. A specific marker of dihydralazine-induced hepatitis. *J Clin Invest* 1990; **85**: 1967-1973
  - 35 **Martini E**, Abuaf N, Cavalli F, Durand V, Johanet C, Homberg JC. Antibody to liver cytosol (anti-LC1) in patients with autoimmune chronic active hepatitis type 2. *Hepatology* 1988; **8**: 1662-1666
  - 36 **Lenzi M**, Manotti P, Muratori L, Cataleta M, Ballardini G, Cassani F, Bianchi FB. Liver cytosolic 1 antigen-antibody system in type 2 autoimmune hepatitis and hepatitis C virus infection. *Gut* 1995; **36**: 749-754
  - 37 **Lapierre P**, Hajoui O, Homberg JC, Alvarez F. Formiminotransferase cyclodeaminase is an organ-specific autoantigen recognized by sera of patients with autoimmune hepatitis. *Gastroenterology* 1999; **116**: 643-649
  - 38 **Muratori L**, Sztul E, Muratori P, Gao Y, Ripalti A, Ponti C, Lenzi M, Landini MP, Bianchi FB. Distinct epitopes on formiminotransferase cyclodeaminase induce autoimmune liver cytosol antibody type 1. *Hepatology* 2001; **34**: 494-501
  - 39 **Mackay IR**. Primary biliary cirrhosis showing a high titer of autoantibody; report of a case. *N Engl J Med* 1958; **258**: 185-188
  - 40 **Asherson GL**, Dumonde DC. Characterization of auto-antibodies produced in the rabbit by the injection of rat liver. *Br J Exp Pathol* 1962; **43**: 12-20
  - 41 **Walker JG**, Doniach D, Roitt IM, Sherlock S. Serological Tests in Diagnosis of Primary Biliary Cirrhosis. *Lancet* 1965; **1**: 827-831
  - 42 **Berg PA**, Doniach D, Roitt IM. Mitochondrial antibodies in primary biliary cirrhosis. I. Localization of the antigen to mitochondrial membranes. *J Exp Med* 1967; **126**: 277-290
  - 43 **Berg PA**, Muscatello U, Horne RW, Roitt IM, Doniach D. Mitochondrial antibodies in primary biliary cirrhosis. II. The complement fixing antigen as a component of mitochondrial inner membranes. *Br J Exp Pathol* 1969; **50**: 200-208
  - 44 **Berg PA**, Roitt IM, Doniach D, Cooper HM. Mitochondrial antibodies in primary biliary cirrhosis. IV. Significance of membrane structure for the complement-fixing antigen. *Immunology* 1969; **17**: 281-293
  - 45 **Berg PA**, Roitt IM, Doniach D, Horne RW. Mitochondrial

- antibodies in primary biliary cirrhosis. 3. Characterization of the inner-membrane complement fixing antigen. *Clin Exp Immunol* 1969; **4**: 511-525
- 46 **Gershwin ME**, Mackay IR, Sturgess A, Coppel RL. Identification and specificity of a cDNA encoding the 70 kd mitochondrial antigen recognized in primary biliary cirrhosis. *J Immunol* 1987; **138**: 3525-3531
  - 47 **Yeaman SJ**, Fussey SP, Danner DJ, James OF, Mutimer DJ, Bassendine MF. Primary biliary cirrhosis: identification of two major M2 mitochondrial autoantigens. *Lancet* 1988; **1**: 1067-1070
  - 48 **Leung PS**, Coppel RL, Ansari A, Munoz S, Gershwin ME. Antimitochondrial antibodies in primary biliary cirrhosis. *Semin Liver Dis* 1997; **17**: 61-69
  - 49 **Manns M**, Gerken G, Kyriatsoulis A, Staritz M, Meyer zum Buschenfelde KH. Characterisation of a new subgroup of autoimmune chronic active hepatitis by autoantibodies against a soluble liver antigen. *Lancet* 1987; **1**: 292-294
  - 50 **Stechemesser E**, Klein R, Berg PA. Characterization and clinical relevance of liver-pancreas antibodies in autoimmune hepatitis. *Hepatology* 1993; **18**: 1-9
  - 51 **Wies I**, Brunner S, Henninger J, Herkel J, Kanzler S, Meyer zum Buschenfelde KH, Lohse AW. Identification of target antigen for SLA/LP autoantibodies in autoimmune hepatitis. *Lancet* 2000; **355**: 1510-1515
  - 52 **McFarlane IG**, Hegarty JE, McSorley CG, McFarlane BM, Williams R. Antibodies to liver-specific protein predict outcome of treatment withdrawal in autoimmune chronic active hepatitis. *Lancet* 1984; **2**: 954-956
  - 53 **McFarlane IG**, McFarlane BM, Major GN, Tolley P, Williams R. Identification of the hepatic asialo-glycoprotein receptor (hepatic lectin) as a component of liver specific membrane lipoprotein (LSP). *Clin Exp Immunol* 1984; **55**: 347-354
  - 54 **Weller TH**, Coons AH. Fluorescent antibody studies with agents of varicella and herpes zoster propagated in vitro. *Proc Soc Exp Biol Med* 1954; **86**: 789-794
  - 55 **Vergani D**, Alvarez F, Bianchi FB, Cancado EL, Mackay IR, Manns MP, Nishioka M, Penner E. Liver autoimmune serology: a consensus statement from the committee for autoimmune serology of the International Autoimmune Hepatitis Group. *J Hepatol* 2004; **41**: 677-683
  - 56 **Toolan HW**. Transplantable human neoplasms maintained in cortisone-treated laboratory animals: H.S. No. 1; H.Ep. No. 1; H.Ep. No. 2; H.Ep. No. 3; and H.Emb.Rh. No. 1. *Cancer Res* 1954; **14**: 660-666
  - 57 **Moore AE**, Sabachewsky L, Toolan HW. Culture characteristics of four permanent lines of human cancer cells. *Cancer Res* 1955; **15**: 598-602
  - 58 **Szosteki C**, Guldner HH, Will H. Autoantibodies against "nuclear dots" in primary biliary cirrhosis. *Semin Liver Dis* 1997; **17**: 71-78
  - 59 **Courvalin JC**, Worman HJ. Nuclear envelope protein autoantibodies in primary biliary cirrhosis. *Semin Liver Dis* 1997; **17**: 79-90
  - 60 **Czaja AJ**, Manns MP, Homburger HA. Frequency and significance of antibodies to liver/kidney microsome type 1 in adults with chronic active hepatitis. *Gastroenterology* 1992; **103**: 1290-1295
  - 61 **Dahlan Y**, Smith L, Simmonds D, Jewell LD, Wanless I, Heathcote EJ, Bain VG. Pediatric-onset primary biliary cirrhosis. *Gastroenterology* 2003; **125**: 1476-1479
  - 62 **Hannam S**, Bogdanos DP, Davies ET, Hussain MJ, Portmann BC, Mieli-Vergani G, Vergani D. Neonatal liver disease associated with placental transfer of anti-mitochondrial antibodies. *Autoimmunity* 2002; **35**: 545-550
  - 63 **Muratori L**, Cataleta M, Muratori P, Manotti P, Lenzi M, Cassani F, Bianchi FB. Detection of anti-liver cytosol antibody type 1 (anti-LC1) by immunodiffusion, counterimmunoelectrophoresis and immunoblotting: comparison of different techniques. *J Immunol Methods* 1995; **187**: 259-264
  - 64 **van der Woude FJ**, Rasmussen N, Lobatto S, Wiik A, Permin H, van Es LA, van der Giessen M, van der Hem GK, The TH. Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet* 1985; **1**: 425-429
  - 65 **Hagen EC**, Andrassy K, Chernok E, Daha MR, Gaskin G, Gross W, Lesavre P, Ludemann J, Pusey CD, Rasmussen N. The value of indirect immunofluorescence and solid phase techniques for ANCA detection. A report on the first phase of an international cooperative study on the standardization of ANCA assays. EEC/BCR Group for ANCA Assay Standardization. *J Immunol Methods* 1993; **159**: 1-16
  - 66 **Terjung B**, Herzog V, Worman HJ, Gestmann I, Bauer C, Sauerbruch T, Spengler U. Atypical antineutrophil cytoplasmic antibodies with perinuclear fluorescence in chronic inflammatory bowel diseases and hepatobiliary disorders colocalize with nuclear lamina proteins. *Hepatology* 1998; **28**: 332-340
  - 67 **Terjung B**, Spengler U, Sauerbruch T, Worman HJ. „Atypical p-ANCA“ in IBD and hepatobiliary disorders react with a 50-kilodalton nuclear envelope protein of neutrophils and myeloid cell lines. *Gastroenterology* 2000; **119**: 310-322
  - 68 **Terjung B**, Worman HJ, Herzog V, Sauerbruch T, Spengler U. Differentiation of antineutrophil nuclear antibodies in inflammatory bowel and autoimmune liver diseases from antineutrophil cytoplasmic antibodies (p-ANCA) using immunofluorescence microscopy. *Clin Exp Immunol* 2001; **126**: 37-46
  - 69 **Czaja AJ**, Cassani F, Cataleta M, Valentini P, Bianchi FB. Frequency and significance of antibodies to actin in type 1 autoimmune hepatitis. *Hepatology* 1996; **24**: 1068-1073
  - 70 **Granito A**, Muratori L, Muratori P, Pappas G, Guidi M, Cassani F, Volta U, Ferri A, Lenzi M, Bianchi FB. Antibodies to filamentous actin (F-actin) in type 1 autoimmune hepatitis. *J Clin Pathol* 2006; **59**: 280-284
  - 71 **Frenzel C**, Herkel J, Luth S, Galle PR, Schramm C, Lohse AW. Evaluation of F-actin ELISA for the diagnosis of autoimmune hepatitis. *Am J Gastroenterol* 2006; **101**: 2731-2736
  - 72 **Liaskos C**, Bogdanos DP, Davies ET, Dalekos GN. Diagnostic relevance of anti-filamentous actin antibodies in autoimmune hepatitis. *J Clin Pathol* 2007; **60**: 107-108
  - 73 **Zamanou A**, Tsirogianni A, Terzoglou C, Balafas A, Economidou I, Lymberi P. Anti-smooth muscle antibodies (ASMAs) and anti-cytoskeleton antibodies (ACTAs) in liver diseases: a comparison of classical indirect immunofluorescence with ELISA. *J Clin Lab Anal* 2002; **16**: 194-201
  - 74 **Granito A**, Muratori P, Muratori L, Georgios P, Lenzi M, Bianchi FB. Antifilamentous actin antibodies by ELISA for the diagnosis of type 1 autoimmune hepatitis. *Am J Gastroenterol* 2007; **102**: 1131-1132
  - 75 **Villalta D**, Bizzaro N, Da Re M, Tozzoli R, Komorowski L, Tonutti E. Diagnostic accuracy of four different immunological methods for the detection of anti-F-actin autoantibodies in type 1 autoimmune hepatitis and other liver-related disorders. *Autoimmunity* 2008; **41**: 105-110
  - 76 **Bogdanos DP**, Mieli-Vergani G, Vergani D. Liver-kidney microsomal antibody-positive autoimmune hepatitis in the United States. *Am J Gastroenterol* 2001; **96**: 3447-3448
  - 77 **Ma Y**, Gregorio G, Gaken J, Muratori L, Bianchi FB, Mieli-Vergani G, Vergani D. Establishment of a novel radioligand assay using eukaryotically expressed cytochrome P4502D6 for the measurement of liver kidney microsomal type 1 antibody in patients with autoimmune hepatitis and hepatitis C virus infection. *J Hepatol* 1997; **26**: 1396-1402
  - 78 **Bogdanos DP**, Lenzi M, Okamoto M, Rigopoulou EI, Muratori P, Ma Y, Muratori L, Tsantoulas D, Mieli-Vergani G, Bianchi FB, Vergani D. Multiple viral/self immunological cross-reactivity in liver kidney microsomal antibody positive hepatitis C virus infected patients is associated with the possession of HLA B51. *Int J Immunopathol Pharmacol* 2004; **17**: 83-92
  - 79 **Ma Y**, Peakman M, Lobo-Yeo A, Wen L, Lenzi M, Gaken J, Farzaneh F, Mieli-Vergani G, Bianchi FB, Vergani D. Dif-

- ferences in immune recognition of cytochrome P4502D6 by liver kidney microsomal (LKM) antibody in autoimmune hepatitis and chronic hepatitis C virus infection. *Clin Exp Immunol* 1994; **97**: 94-99
- 80 **Muratori L**, Lenzi M, Ma Y, Cataleta M, Mieli-Vergani G, Vergani D, Bianchi FB. Heterogeneity of liver/kidney microsomal antibody type 1 in autoimmune hepatitis and hepatitis C virus related liver disease. *Gut* 1995; **37**: 406-412
  - 81 **Ma Y**, Okamoto M, Thomas MG, Bogdanos DP, Lopes AR, Portmann B, Underhill J, Durr R, Mieli-Vergani G, Vergani D. Antibodies to conformational epitopes of soluble liver antigen define a severe form of autoimmune liver disease. *Hepatology* 2002; **35**: 658-664
  - 82 **Volkman M**, Martin L, Baurle A, Heid H, Strassburg CP, Trautwein C, Fiehn W, Manns MP. Soluble liver antigen: isolation of a 35-kd recombinant protein (SLA-p35) specifically recognizing sera from patients with autoimmune hepatitis. *Hepatology* 2001; **33**: 591-596
  - 83 **Baeres M**, Herkel J, Czaja AJ, Wies I, Kanzler S, Cancado EL, Porta G, Nishioka M, Simon T, Daehnrich C, Schlumberger W, Galle PR, Lohse AW. Establishment of standardised SLA/LP immunoassays: specificity for autoimmune hepatitis, worldwide occurrence, and clinical characteristics. *Gut* 2002; **51**: 259-264
  - 84 **Ballot E**, Bruneel A, Labas V, Johanet C. Identification of rat targets of anti-soluble liver antigen autoantibodies by serologic proteome analysis. *Clin Chem* 2003; **49**: 634-643
  - 85 **Bogdanos DP**, Bianchi I, Ma Y, Mitry RR, Mieli-Vergani G, Vergani D. Targets of antibodies to soluble liver antigen in patients with autoimmune hepatitis. *Clin Chem* 2004; **50**: 682-683; author reply 683-684
  - 86 **Vitozzi S**, Djilali-Saiah I, Lapiere P, Alvarez F. Anti-soluble liver antigen/liver-pancreas (SLA/LP) antibodies in pediatric patients with autoimmune hepatitis. *Autoimmunity* 2002; **35**: 485-492
  - 87 **Xu XM**, Carlson BA, Mix H, Zhang Y, Saira K, Glass RS, Berry MJ, Gladyshev VN, Hatfield DL. Biosynthesis of selenocysteine on its tRNA in eukaryotes. *PLoS Biol* 2007; **5**: e4
  - 88 **Moteki S**, Leung PS, Coppel RL, Dickson ER, Kaplan MM, Munoz S, Gershwin ME. Use of a designer triple expression hybrid clone for three different lipoyl domain for the detection of antimitochondrial autoantibodies. *Hepatology* 1996; **24**: 97-103
  - 89 **Oertelt S**, Rieger R, Selmi C, Invernizzi P, Ansari AA, Coppel RL, Podda M, Leung PS, Gershwin ME. A sensitive bead assay for antimitochondrial antibodies: Chipping away at AMA-negative primary biliary cirrhosis. *Hepatology* 2007; **45**: 659-665
  - 90 **Gabeta S**, Norman GL, Liaskos C, Papamichalis PA, Zografos T, Garagounis A, Rigopoulou EI, Dalekos GN. Diagnostic relevance and clinical significance of the new enhanced performance M2 (MIT3) ELISA for the detection of IgA and IgG antimitochondrial antibodies in primary biliary cirrhosis. *J Clin Immunol* 2007; **27**: 378-387
  - 91 **Janka C**, Selmi C, Gershwin ME, Will H, Sternsdorf T. Small ubiquitin-related modifiers: A novel and independent class of autoantigens in primary biliary cirrhosis. *Hepatology* 2005; **41**: 609-616
  - 92 **Muratori P**, Muratori L, Ferrari R, Cassani F, Bianchi G, Lenzi M, Rodrigo L, Linares A, Fuentes D, Bianchi FB. Characterization and clinical impact of antinuclear antibodies in primary biliary cirrhosis. *Am J Gastroenterol* 2003; **98**: 431-437
  - 93 **Bogdanos DP**, Baum H, Butler P, Rigopoulou EI, Davies ET, Ma Y, Burroughs AK, Vergani D. Association between the primary biliary cirrhosis specific anti-sp100 antibodies and recurrent urinary tract infection. *Dig Liver Dis* 2003; **35**: 801-805
  - 94 **Bogdanos DP**, Liaskos C, Pares A, Norman G, Rigopoulou EI, Caballeria L, Dalekos GN, Rodes J, Vergani D. Anti-gp210 antibody mirrors disease severity in primary biliary cirrhosis. *Hepatology* 2007; **45**: 1583; author reply 1583-1583; author reply 1584
  - 95 **Bogdanos DP**, Pares A, Rodes J, Vergani D. Primary biliary cirrhosis specific antinuclear antibodies in patients from Spain. *Am J Gastroenterol* 2004; **99**: 763-764; author reply 765
  - 96 **Nakamura M**, Kondo H, Mori T, Komori A, Matsuyama M, Ito M, Takii Y, Koyabu M, Yokoyama T, Migita K, Daikoku M, Abiru S, Yatsushashi H, Takezaki E, Masaki N, Sugi K, Honda K, Adachi H, Nishi H, Watanabe Y, Nakamura Y, Shimada M, Komatsu T, Saito A, Saoshiro T, Harada H, Sodeyama T, Hayashi S, Masumoto A, Sando T, Yamamoto T, Sakai H, Kobayashi M, Muro T, Koga M, Shums Z, Norman GL, Ishibashi H. Anti-gp210 and anti-centromere antibodies are different risk factors for the progression of primary biliary cirrhosis. *Hepatology* 2007; **45**: 118-127
  - 97 **Bauer A**, Habior A. Measurement of gp210 autoantibodies in sera of patients with primary biliary cirrhosis. *J Clin Lab Anal* 2007; **21**: 227-231
  - 98 **Wesierska-Gadek J**, Klima A, Komina O, Ranftler C, Invernizzi P, Penner E. Characterization of autoantibodies against components of the nuclear pore complexes: high frequency of anti-p62 nucleoporin antibodies. *Ann N Y Acad Sci* 2007; **1109**: 519-530
  - 99 **Vergani D**, Bogdanos DP. Positive markers in AMA-negative PBC. *Am J Gastroenterol* 2003; **98**: 241-243
  - 100 **Invernizzi P**, Selmi C, Ranftler C, Podda M, Wesierska-Gadek J. Antinuclear antibodies in primary biliary cirrhosis. *Semin Liver Dis* 2005; **25**: 298-310
  - 101 **Terjung B**, Gottwein J, Muennich M, Muennich M, Gottwein J, Sauerbruch T, Spengler U. Identifizierung des Zielantigens atypischer p-ANCA bei autoimmunem Lebererkrankungen als Protein der Tubulin-beta Genfamilie. *Z Gastroenterol* 2005; **43**: Abstract
  - 102 **Targan SR**, Landers C, Vidrich A, Czaja AJ. High-titer antineutrophil cytoplasmic antibodies in type-1 autoimmune hepatitis. *Gastroenterology* 1995; **108**: 1159-1166
  - 103 **Mulder AH**, Horst G, Haagsma EB, Limburg PC, Kleibeuker JH, Kallenberg CG. Prevalence and characterization of neutrophil cytoplasmic antibodies in autoimmune liver diseases. *Hepatology* 1993; **17**: 411-417
  - 104 **Pratt DS**, Kaplan MM. Evaluation of abnormal liver-enzyme results in asymptomatic patients. *N Engl J Med* 2000; **342**: 1266-1271
  - 105 **Bogdanos DP**, Mieli-Vergani G, Vergani D. Non-organ-specific autoantibodies in children with chronic hepatitis C virus infection. *Clin Infect Dis* 2004; **38**: 1505; author reply 1505-1506
  - 106 **Bogdanos DP**, Mieli-Vergani G, Vergani D. Non-organ-specific autoantibodies in hepatitis C virus infection: do they matter? *Clin Infect Dis* 2005; **40**: 508-510
  - 107 **Bogdanos DP**, Mieli-Vergani G, Vergani D. Virus, liver and autoimmunity. *Dig Liver Dis* 2000; **32**: 440-446
  - 108 **Leung PS**, Rossaro L, Davis PA, Park O, Tanaka A, Kikuchi K, Miyakawa H, Norman GL, Lee W, Gershwin ME. Antimitochondrial antibodies in acute liver failure: implications for primary biliary cirrhosis. *Hepatology* 2007; **46**: 1436-1442
  - 109 **Bogdanos DP**, Liaskos C, Rigopoulou EI, Dalekos GN. Antimitochondrial antibodies in patients with systemic lupus erythematosus: revealing the unforeseen. *Clin Chim Acta* 2006; **373**: 183-184; author reply 185
  - 110 **Liaskos C**, Bogdanos DP, Rigopoulou EI, Dalekos GN. Development of antimitochondrial antibodies in patients with autoimmune hepatitis: art of facts or an artifact? *J Gastroenterol Hepatol* 2007; **22**: 454-455
  - 111 **Bernal W**, Meda F, Ma Y, Bogdanos DP, Vergani D. Disease-specific autoantibodies in patients with acute liver failure: the King's College London Experience. *Hepatology* 2008; **47**: 1096-1097; author reply 1097
  - 112 **Butler P**, Hamilton-Miller J, Baum H, Burroughs AK. Detection of M2 antibodies in patients with recurrent urinary tract infection using an ELISA and purified PBC specific antigens. Evidence for a molecular mimicry mechanism in the pathogenesis of primary biliary cirrhosis? *Biochem Mol Biol*



*Int* 1995; **35**: 473-485

- 113 **Klein R**, Wiebel M, Engelhart S, Berg PA. Sera from patients with tuberculosis recognize the M2a-epitope (E2-subunit of pyruvate dehydrogenase) specific for primary biliary cirrhosis. *Clin Exp Immunol* 1993; **92**: 308-316
- 114 **Gilburd B**, Ziporen L, Zharhary D, Blank M, Zurgil N, Scheinberg MA, Guedes LH, Gershwin ME, Shoenfeld Y. Antimitochondrial (pyruvate dehydrogenase) antibodies in leprosy. *J Clin Immunol* 1994; **14**: 14-19
- 115 **Czaja AJ**, Pfeifer KD, Decker RH, Vallari AS. Frequency and significance of antibodies to asialoglycoprotein receptor in type 1 autoimmune hepatitis. *Dig Dis Sci* 1996; **41**: 1733-1740
- 116 **Van Norstrand MD**, Malinchoc M, Lindor KD, Therneau TM, Gershwin ME, Leung PS, Dickson ER, Homburger HA. Quantitative measurement of autoantibodies to recombinant mitochondrial antigens in patients with primary biliary cirrhosis: relationship of levels of autoantibodies to disease progression. *Hepatology* 1997; **25**: 6-11
- 117 **Invernizzi P**, Crosignani A, Battezzati PM, Covini G, De Valle G, Larghi A, Zuin M, Podda M. Comparison of the clinical features and clinical course of antimitochondrial antibody-positive and -negative primary biliary cirrhosis. *Hepatology* 1997; **25**: 1090-1095
- 118 **Gregorio GV**, McFarlane B, Bracken P, Vergani D, Mieli-Vergani G. Organ and non-organ specific autoantibody titres and IgG levels as markers of disease activity: a longitudinal study in childhood autoimmune liver disease. *Autoimmunity* 2002; **35**: 515-519
- 119 **Czaja AJ**, Morshed SA, Parveen S, Nishioka M. Antibodies to single-stranded and double-stranded DNA in antinuclear antibody-positive type 1-autoimmune hepatitis. *Hepatology* 1997; **26**: 567-572
- 120 **Treichel U**, Gerken G, Rossol S, Rotthauwe HW, Meyer zum Buschenfelde KH, Poralla T. Autoantibodies against the human asialoglycoprotein receptor: effects of therapy in autoimmune and virus-induced chronic active hepatitis. *J Hepatol* 1993; **19**: 55-63
- 121 **Kanzler S**, Weidemann C, Gerken G, Lohr HF, Galle PR, Meyer zum Buschenfelde KH, Lohse AW. Clinical significance of autoantibodies to soluble liver antigen in autoimmune hepatitis. *J Hepatol* 1999; **31**: 635-640
- 122 **Czaja AJ**, Shums Z, Norman GL. Frequency and significance of antibodies to soluble liver antigen/liver pancreas in variant autoimmune hepatitis. *Autoimmunity* 2002; **35**: 475-483
- 123 **Abuaf N**, Johanet C, Chretien P, Martini E, Soulier E, Laperche S, Homberg JC. Characterization of the liver cytosol antigen type 1 reacting with autoantibodies in chronic active hepatitis. *Hepatology* 1992; **16**: 892-898
- 124 **Muratori L**, Cataleta M, Muratori P, Lenzi M, Bianchi FB. Liver/kidney microsomal antibody type 1 and liver cytosol antibody type 1 concentrations in type 2 autoimmune hepatitis. *Gut* 1998; **42**: 721-726
- 125 **Rigopoulou EI**, Davies ET, Bogdanos DP, Liaskos C, Mytilinaiou M, Koukoulis GK, Dalekos GN, Vergani D. Antimitochondrial antibodies of immunoglobulin G3 subclass are associated with a more severe disease course in primary biliary cirrhosis. *Liver Int* 2007; **27**: 1226-1231
- 126 **Invernizzi P**, Podda M, Battezzati PM, Crosignani A, Zuin M, Hitchman E, Maggioni M, Meroni PL, Penner E, Wiesierska-Gadek J. Autoantibodies against nuclear pore complexes are associated with more active and severe liver disease in primary biliary cirrhosis. *J Hepatol* 2001; **34**: 366-372
- 127 **Miyachi K**, Hankins RW, Matsushima H, Kikuchi F, Inomata T, Horigome T, Shibata M, Onozuka Y, Ueno Y, Hashimoto E, Hayashi N, Shibuya A, Amaki S, Miyakawa H. Profile and clinical significance of anti-nuclear envelope antibodies found in patients with primary biliary cirrhosis: a multicenter study. *J Autoimmun* 2003; **20**: 247-254
- 128 **Kerkar N**, Choudhuri K, Ma Y, Mahmoud A, Bogdanos DP, Muratori L, Bianchi F, Williams R, Mieli-Vergani G, Vergani D. Cytochrome P450D6(193-212): a new immunodominant epitope and target of virus/self cross-reactivity in liver kidney microsomal autoantibody type 1-positive liver disease. *J Immunol* 2003; **170**: 1481-1489
- 129 **Wesierska-Gadek J**, Penner E, Battezzati PM, Selmi C, Zuin M, Hitchman E, Worman HJ, Gershwin ME, Podda M, Invernizzi P. Correlation of initial autoantibody profile and clinical outcome in primary biliary cirrhosis. *Hepatology* 2006; **43**: 1135-1144
- 130 **Yang WH**, Yu JH, Nakajima A, Neuberger D, Lindor K, Bloch DB. Do antinuclear antibodies in primary biliary cirrhosis patients identify increased risk for liver failure? *Clin Gastroenterol Hepatol* 2004; **2**: 1116-1122
- 131 **Vergani D**, Mieli-Vergani G. The impact of autoimmunity on hepatocytes. *Semin Liver Dis* 2007; **27**: 140-151
- 132 **Bogdanos DP**, Baum H, Sharma UC, Grasso A, Ma Y, Burroughs AK, Vergani D. Antibodies against homologous microbial caseinolytic proteases P characterise primary biliary cirrhosis. *J Hepatol* 2002; **36**: 14-21
- 133 **Longhi MS**, Ma Y, Bogdanos DP, Cheeseman P, Mieli-Vergani G, Vergani D. Impairment of CD4(+)CD25(+) regulatory T-cells in autoimmune liver disease. *J Hepatol* 2004; **41**: 31-37
- 134 **Bogdanos DP**, Baum H, Grasso A, Okamoto M, Butler P, Ma Y, Rigopoulou E, Montalto P, Davies ET, Burroughs AK, Vergani D. Microbial mimics are major targets of cross-reactivity with human pyruvate dehydrogenase in primary biliary cirrhosis. *J Hepatol* 2004; **40**: 31-39
- 135 **Bogdanos DP**, Baum H, Gunsar F, Arioli D, Polymeros D, Ma Y, Burroughs AK, Vergani D. Extensive homology between the major immunodominant mitochondrial antigen in primary biliary cirrhosis and *Helicobacter pylori* does not lead to immunological cross-reactivity. *Scand J Gastroenterol* 2004; **39**: 981-987
- 136 **Bogdanos DP**, Baum H, Okamoto M, Montalto P, Sharma UC, Rigopoulou EI, Vlachogiannakos J, Ma Y, Burroughs AK, Vergani D. Primary biliary cirrhosis is characterized by IgG3 antibodies cross-reactive with the major mitochondrial autoepitope and its *Lactobacillus* mimic. *Hepatology* 2005; **42**: 458-465
- 137 **Bogdanos DP**, Choudhuri K, Vergani D. Molecular mimicry and autoimmune liver disease: virtuous intentions, malign consequences. *Liver* 2001; **21**: 225-232
- 138 **Bogdanos DP**, Koutsoumpas A, Baum H, Vergani D. Borrelia burgdorferi: a new self-mimicking trigger in primary biliary cirrhosis. *Dig Liver Dis* 2006; **38**: 781-782; author reply 782-783
- 139 **Bogdanos DP**, McFarlane IG. Cytochrome P450 2A6 meets P450 2D6: an enigma of viral infections and autoimmunity. *J Hepatol* 2003; **39**: 860-863
- 140 **Bogdanos DP**, Pares A, Baum H, Caballeria L, Rigopoulou EI, Ma Y, Burroughs AK, Rodes J, Vergani D. Disease-specific cross-reactivity between mimicking peptides of heat shock protein of *Mycobacterium gordonae* and dominant epitope of E2 subunit of pyruvate dehydrogenase is common in Spanish but not British patients with primary biliary cirrhosis. *J Autoimmun* 2004; **22**: 353-362
- 141 **Bogdanos DP**, Rigopoulou EI. Viral/self-mimicry and immunological cross-reactivity as a trigger of hepatic C virus associated autoimmune diabetes. *Diabetes Res Clin Pract* 2007; **77**: 155-156
- 142 **Bogdanos DP**, Vergani D. Origin of cross-reactive autoimmunity in primary biliary cirrhosis. *Liver Int* 2006; **26**: 633-635
- 143 **Xu L**, Shen Z, Guo L, Fodera B, Keogh A, Joplin R, O'Donnell B, Aitken J, Carman W, Neuberger J, Mason A. Does a betaretrovirus infection trigger primary biliary cirrhosis? *Proc Natl Acad Sci USA* 2003; **100**: 8454-8459
- 144 **Mackay IR**. The etiopathogenesis of autoimmunity. *Semin Liver Dis* 2005; **25**: 239-250
- 145 **Selmi C**, Balkwill DL, Invernizzi P, Ansari AA, Coppel RL, Podda M, Leung PS, Kenny TP, Van De Water J, Nantz MH, Kurth MJ, Gershwin ME. Patients with primary biliary cirrhosis react against a ubiquitous xenobiotic-metabolizing bacterium. *Hepatology* 2003; **38**: 1250-1257

- 146 **Vergani D**, Mieli-Vergani G, Mondelli M, Portmann B, Eddleston AL. Immunoglobulin on the surface of isolated hepatocytes is associated with antibody-dependent cell-mediated cytotoxicity and liver damage. *Liver* 1987; **7**: 307-315
- 147 **Duchini A**, McHutchison JG, Pockros PJ. LKM-positive autoimmune hepatitis in the western United States: a case series. *Am J Gastroenterol* 2000; **95**: 3238-3241
- 148 **Tan EM**, Smolen JS, McDougal JS, Fritzler MJ, Gordon T, Hardin JA, Kalden JR, Lahita RG, Maini RN, Reeves WH, Rothfield NF, Takasaki Y, Wiik A, Wilson M, Koziol JA. A critical evaluation of enzyme immunoassay kits for detection of antinuclear autoantibodies of defined specificities. II. Potential for quantitation of antibody content. *J Rheumatol* 2002; **29**: 68-74
- 149 **Fritzler MJ**, Wiik A, Tan EM, Smolen JS, McDougal JS, Chan EK, Gordon TP, Hardin JA, Kalden JR, Lahita RG, Maini RN, Reeves WH, Rothfield NF, Takasaki Y, Wilson M, Byrd MG, Slivka L, Koziol JA. A critical evaluation of enzyme immunoassay kits for detection of antinuclear autoantibodies of defined specificities. III. Comparative performance characteristics of academic and manufacturers' laboratories. *J Rheumatol* 2003; **30**: 2374-2381
- 150 **Fritzler MJ**, Wiik A, Fritzler ML, Barr SG. The use and abuse of commercial kits used to detect autoantibodies. *Arthritis Res Ther* 2003; **5**: 192-201
- 151 **Rondeel JM**, van Gelder W, van der Leeden H, Dinkelaar RB. Different strategies in the laboratory diagnosis of autoimmune disease: immunofluorescence, enzyme-linked immunosorbent assay or both? *Ann Clin Biochem* 1999; **36** (Pt 2): 189-195
- 152 **Kerkar N**, Ma Y, Davies ET, Cheeseman P, Mieli-Vergani G, Vergani D. Detection of liver kidney microsomal type 1 antibody using molecularly based immunoassays. *J Clin Pathol* 2002; **55**: 906-909
- 153 **Bogdanos DP**, Gilbert D, Bianchi I, Leoni S, Mitry RR, Ma Y, Mieli-Vergani G, Vergani D. Antibodies to soluble liver antigen and alpha-enolase in patients with autoimmune hepatitis. *J Autoimmune Dis* 2004; **1**: 4
- 154 **Bogdanos DP**, Vergani D, Muratori P, Muratori L, Bianchi FB. Specificity of anti-sp100 antibody for primary biliary cirrhosis. *Scand J Gastroenterol* 2004; **39**: 405-406; author reply 407

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## TOPIC HIGHLIGHT

Pietro Invernizzi, MD; Ian R Mackay, MD, Series Editors

# Transplantation in autoimmune liver diseases

Marcus Mottershead, James Neuberger

Marcus Mottershead, James Neuberger, Liver Unit, Queen Elizabeth Hospital, Birmingham B15 2TH, United Kingdom  
Correspondence to: James Neuberger, MD, Liver Unit, Queen Elizabeth Hospital, Birmingham B15 2TH, United Kingdom. [j.m.neuberger@bham.ac.uk](mailto:j.m.neuberger@bham.ac.uk)  
Telephone: +44-6-272414 Fax: +44-6-272449  
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## Abstract

Liver transplantation remains an effective treatment for those with end-stage disease and with intractable liver-related symptoms. The shortage of organs for transplantation has resulted in the need for rationing. A variety of approaches to selection and allocation have been developed and vary from country to country. The shortage of donors has meant that new approaches have to be adopted to make maximal use of the available organs; these include splitting grafts, use of extended criteria livers, livers from non-heart-beating donors and from living donors. Post transplantation, most patients will need life-long immunosuppression, although a small proportion can have immunosuppression successfully withdrawn. Newer immunosuppressive drugs and different strategies may allow a more targeted approach with a reduction in side-effects and so improve the patient and graft survival. For autoimmune diseases, transplantation is associated with significant improvement in the quality and length of life. Disease may recur after transplantation and may affect patient and graft survival.

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**Key words:** Liver transplantation; Autoimmune disease; Recurrence; Immunosuppression

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## INTRODUCTION

The three major autoimmune liver diseases that may

require liver transplantation are primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC) and autoimmune hepatitis (AIH). In this review, we will discuss the role, timing and outcome of transplantation for these indications.

Criteria for liver transplantation for patients with autoimmune diseases are relatively well defined<sup>[1]</sup>. As with other indications, liver transplantation is indicated either to relieve intractable symptoms of liver disease (such as pruritus or encephalopathy which do not respond to conventional therapy) or to prolong life. Life after transplantation is normally excellent but is never normal. Furthermore, survival is reduced when compared to an age and sex-matched population<sup>[2]</sup>. Reasons for the reduction in survival include the mortality of the procedure itself, the risks of recurrent disease and the consequences of immunosuppression which may be class related (such as an increased risk of sepsis and some malignancies) or more-specifically related to the individual drugs used (such as renal failure and cerebro- and cardio-vascular death). Thus, for most patients with chronic liver disease, timing of transplantation has to be done with consideration of the risks and balance of remaining with the native liver and of the procedure.

Some guidance is given by prognostic models, of which the most commonly used is the model for end-stage liver disease (MELD) formula<sup>[3]</sup>. MELD, initially used to predict short-term survival after stent insertion has been shown to be accurate in prediction of most patients with chronic liver disease. The score, which is derived from serum bilirubin, creatinine and prothrombin time, is useful; for the average patient, there is a survival benefit when transplanted with a score of 16 or more. Addition of other analytes to the formula, such as serum sodium, may increase the accuracy<sup>[4]</sup>. In some situations, the model does not predict outcome. For example, in those with a liver cell cancer where the prognosis without transplant is dependent on the cancer rather than parenchymal function. Other exceptions occur with hepatopulmonary syndromes, for example. There are several recent reviews of the general indications and contra-indications (see for example<sup>[5,6]</sup>).

There is an increasing gap between the number of patients who would benefit from a transplant and the availability of suitable organs for transplantation. In order to fulfil demand, differing strategies have been utilized: this includes use of split livers, non-heart beating donors, marginal donors and to a lesser extent,

living donor programmes. These strategies have, to some extent, masked the shortage of organs. Extended criteria or marginal livers are being utilized in greater numbers, (these are grafts where there is concerns that their use might impact on the outcome of the patient). These include those grafts where there is a greater risk of non-function (characterized by steatosis in the graft, older donors and prolonged cold ischemia times), technical problems (such as the use of split, partial or reduced grafts) or those grafts that carry a risk of transmission of viral infection or malignancy.

Living donor transplantation accounts for around 2%-5% of transplants in Europe and North America but for almost all transplants carried out in Asia, where donation rates from deceased donors are very low. Limitations on living donation focus on the risk to the donor: too much liver volume removed from donor may induce liver failure in the donor, too little may cause recipient graft failure. The mortality for the donor in left lobe is 0.05% rising to 0.4%-0.5% in right lobe transplants and donor morbidity is 20% with long-term outcomes unknown<sup>[7]</sup>.

The shortage of grafts means that rationing must occur: the competing interests of equity, justice and utility have to be recognised. Thus, criteria for selection (that is admission to the list) and allocation (identification of the recipient for a graft) need to be agreed. Different health care systems have adopted different principles. Conflict may exist where transplantation is considered for some indications, such as liver disease from alcohol where the medical views are not in accordance with those of the public<sup>[8]</sup>. The immunological processes that operate in an allografted liver are complex, since the immune system of the host can react against alloantigens, human leucocyte antigen (HLA) molecules and "minor" transplantation antigens of the donor. Concurrently, passenger leucocytes of the donor may react against HLA or other antigens of the host, resulting in "two-way" immune responsiveness. The liver above all other organs has a propensity to generate a state of intra-hepatic immune tolerance that limits harmful immune reactivities, sometimes to the degree that immunosuppressant drugs become dispensable after a liver transplant. The immunological issues involved, which are beyond the scope of this article, are discussed informatively in several reviews<sup>[9,10]</sup>.

Post transplant, most patients will need life-long immunosuppression. However, in the last decade there have been developments in the management of immunosuppression. In the early days of liver transplantation, the principles of immunosuppression after liver transplant were extrapolated from renal transplant programmes. However, there are some major differences: early acute rejection after liver transplantation is not associated with an adverse outcome; the requirement of immunosuppression is less and sometimes, as mentioned, it is even possible to withdraw immunosuppression completely. It is not possible reliably to identify those patients in whom immunosuppression can be safely withdrawn: however, those with good graft

function at 5 years and with minimal inflammation on histology and were not transplanted for autoimmune diseases are most likely to benefit from a planned withdrawal of immunosuppression<sup>[11,12]</sup>.

Tailoring immunosuppression to the individual is a much discussed but little practiced approach. For instance, those grafted for hepatitis C virus infection need to be protected against rejection since major changes in corticosteroids will increase the consequences of viral re-infection. The advent of newer biological agents including humanized monoclonal antibodies such as Campath-1H (alemtuzumab), antibodies to interleukin-2 receptor (IL-2R), and CTLA-4Ig, may permit more selective approaches to immunosuppression. Campath-1H is a humanized monoclonal antibody against CD52, a molecule expressed on the surface of human B and T lymphocytes. Antibodies to the IL2Ra chain target the CD25 molecule on activated T lymphocytes. Cytolytic T-lymphocyte-associated antigen 4 (CTLA-4) Ig is an immunoglobulin fusion protein with CTLA-4, a natural down-regulatory molecule expressed by T lymphocytes. There are other biologicals under investigation. Induction of full tolerance has long been the goal of solid organ transplantation but, despite advances in the laboratory, this goal has so far remained elusive in the human. The adoption of approaches allowing for early immunological engagement (the Window of Opportunity for Immunological Engagement) as suggested by Calne<sup>[13]</sup> or use of Campath-1 or other biologicals may offer a new and effective approach<sup>[14]</sup>.

## AIH

AIH is a relatively uncommon indication for liver transplantation, currently accounting for no more than 5% of cases<sup>[15]</sup>. As with cirrhosis from other causes, liver transplantation is indicated in those with end-stage disease characterized by a MELD score > 16, signs of decompensation on treatment such as hepatic encephalopathy, ascites or variceal haemorrhage or, rarely, with hepatocellular carcinoma development. In those who present with acute or fulminant liver failure, liver transplantation should be considered early in the course. Outcomes are good with 1 year and 5 years patient survival rates of about 87% and 80%-90%. Graft survival rates at 1 year and 5 years are 84% and 74%-76%<sup>[16-18]</sup>.

### **Recurrence after transplantation**

Diagnostic criteria for recurrent AIH (rAIH) have been developed and are summarised in Table 1. The reported recurrence rate of AIH following transplantation is variable 17%-42% at 5 years<sup>[19,20]</sup>. Table 2 shows the reports of recurrent AIH. Gautam's systematic review of 13 papers concluded disease recurrence occurred in 22% of recipients at a median interval of 26.4 mo<sup>[21]</sup>. Czaja suggested that a loss of self-tolerance and molecular mimicry would explain the repopulation of the allograft with recipient antigen-presenting cells and that the already primed promiscuous recipient cytotoxic



Table 1 Criteria for the diagnosis of recurrent AIH

Criteria
Liver transplant for autoimmune hepatitis
Auto-antibodies in significant titre (> 1:40)
Sustained rise in serum aminotransferase activity (> 2 times normal)
Elevated serum immunoglobulins
Compatible liver histology (infiltration of portal tracts by plasma cells, piecemeal necrosis and bridging necrosis <sup>[21]</sup> )
Corticosteroid dependency
Exclusion of other causes of graft dysfunction (such as rejection and HCV infection)

T cells are likely factors for recurrent disease<sup>[22]</sup>.

Many studies have been published in the literature, but most include relatively small numbers, use different criteria for the diagnosis and are retrospective. Reich retrospectively reviewed 24 AIH transplant recipients; 6 patients developed biopsy proven recurrence at 15 mo, 3 proceeded to regrafting and 2 of these patients developed recurrent AIH in the second graft. No patient transplanted for fulminant hepatic failure developed recurrence compared to 1/3 of those with chronic disease<sup>[15]</sup>. Duclos-Vallee performed protocol biopsies and demonstrated histological recurrence preceded biochemical abnormality by 1-5 years in 23.5%<sup>[25]</sup>. There was no difference in survival or recurrence between the three sub-types of AIH. Rates of rejection were high both in the control and AIH groups but greater in those grafted for AIH. (50% and 88%)<sup>[27]</sup>. No patient required re-transplant because of recurrent disease and there was no difference in patient survival or graft survival<sup>[18]</sup>.

There are no consistent risk factors for recurrence identified. Pre-transplant disease duration, donor/recipient gender distribution, HLA studies, and rejection episodes did not correlate with AIH recurrence but the degree of necro-inflammation in the native liver was significantly greater in those with recurrence in one study<sup>[24]</sup>. The choice of immunosuppression is controversial but a recent systematic review by Gautam found no difference in recurrence rates between recipients on tacrolimus (31%) or cyclosporin<sup>[21]</sup>.

Khalaf reported a histological recurrence in 18.7% (median follow up of 530 d) which was successfully treated by optimizing immunosuppression. Steroid withdrawal failed in all recipients and was always accompanied by almost immediate elevation of liver enzymes<sup>[28]</sup>. A case of AIH recurrence 6 years after a living donor related liver transplant, in the absence of autoantibodies was reported. The patient had steroids discontinued 1 year post orthotopic liver transplant (OLT) whilst maintained on tacrolimus but became antinuclear antibody (ANA) positive again 3 years later, 2 years prior to the histological diagnosis but in the absence of abnormal LFTs<sup>[29]</sup>.

### De novo AIH

De novo AIH has features of a steroid responsive AIH in patients transplanted for other non-immune indications and is characterized by a biochemical

Table 2 Reports of recurrent autoimmune hepatitis

Author	Follow up (mo)	n	Recurrence	Period recurrence occurred	Re-OLT/Cirrhosis
Milkiewicz 1999 <sup>[23]</sup>	29	47	13/47	29 mo	3/47
Ayata 2000 <sup>[24]</sup>	67	12	5/12	35-280 d	2/12
Reich 2000 <sup>[15]</sup>	27	24	6/12	At 15 mo	3/24
Molmenti 2002 <sup>[18]</sup>	29	55	11/55	At end	
Duclos-Vallee 2003 <sup>[25]</sup>	120	17	7/17	2.5 yr <sup>1</sup>	2/17
Núñez-Martínez 2003 <sup>[26]</sup>	38	15	1/15	At end	
Vogel 2004 <sup>[27]</sup>	24	28	9/28	5 yr	4/28
Gautam 2006 <sup>[21]</sup>			23%	2.4 mo <sup>2</sup>	

<sup>1</sup>Mean; <sup>2</sup>Median.

hepatitis, circulating auto-antibodies, elevated immunoglobulins and an inflammatory infiltration with interface hepatitis. The first report of de novo AIH was in 7 children at a median of 2 years post-transplant<sup>[30]</sup>. Children are more at risk than adults but the condition is still relatively uncommon with an incidence of around 3%. There is usually a good response to additional immunosuppression with corticosteroids, but in some cases there is progression to cirrhosis and subsequent graft failure<sup>[31]</sup>. Whether this is truly a de novo autoimmune phenomenon or merely a form of rejection is not certain: early studies suggesting an immune response to graft antigens are controversial<sup>[32]</sup> and studies suggesting an immune response to graft isoforms of glutathione-S transferase remain unconfirmed.

### Conclusion

The outcome for OLT in AIH is good and is merited in those with chronic disease and a much smaller cohort will have an acute or fulminating course the prognosis of which is relatively unaffected by corticosteroids. Recurrence of disease is relatively common in the allograft and may be detected on protocol biopsy at an asymptomatic stage before biochemical or clinical clues. Generally recurrent AIH responds well to increases in immunosuppression or addition of corticosteroids. This should be taken into account when considering long term immunosuppression and especially on reduction should be in conjunction with immunoglobulins, autoantibody profile and histology. Most data are retrospective with relatively small numbers and studies are lacking in long term reduction and withdrawal of immunosuppression and further controlled studies are required.

### PBC

#### Indications

Indications for transplantation are listed in Table 3. Unlike pruritus, which is rapidly reversed after transplantation, lethargy is not an indication since often

**Table 3** Indications for transplantation in PBC

Indications
Symptom based
Intractable pruritus refractory to medical therapy
Hepatic encephalopathy
End-stage liver disease
Recurrent variceal haemorrhage
Episode of spontaneous bacterial peritonitis
Pulmonary hypertension
Hepato-pulmonary syndrome
Diuretic resistant ascites
Progressive osteopaenia
Muscle-wasting
Hepatoma (Milan criteria)
Biochemistry
Serum bilirubin > 150 µmol/L
Serum albumin < 25 g/L

it does not improve with transplantation<sup>[33]</sup>. The need for transplantation for PBC is falling (United Network for Organ Sharing (UNOS) data shows, of 2391 cadaveric liver transplants in 1991, 18% were for cholestatic liver disease compared with 10% of 4579 in 2000 and was the second most common indication for transplantation) and the impact of ursodeoxycholic acid (UDCA) is a tempting but controversial explanation.

### Timing of transplantation

A variety of disease-specific prognostic models have been developed but for short term survival a MELD score is effective and a score > 16 indicates a survival benefit from transplantation. Serum bilirubin > 100 µmol/L<sup>[34]</sup> as well as significant poor liver function with length of life attributed to disease limited to 1 year are indicators for transplantation assessment<sup>[35]</sup>. A Mayo risk score > 7.8 has also been validated to indicate survival in the absence of transplantation<sup>[36,37]</sup>.

### Survival after OLT

The 1, 3 and 5 year actuarial patient and graft survival was 94%, 91%, and 82%, and 89%, 83%, and 75%, respectively in a series of 301 PBC transplant recipients in the UK<sup>[38]</sup> which is comparable to European transplant registry data. The commonest indication for re-transplantation in the first year is chronic rejection<sup>[39]</sup>. Survival rates remain consistently better than other indications, even after adjusting for case-mix and other risk factors. Immunosuppression is usually a standard triple regimen of calcineurin inhibitor (tacrolimus or cyclosporin), corticosteroids (withdrawn over 3 mo) and azathioprine or mycophenolate mofetil.

### Recurrent disease

Recurrent disease (Table 4) is diagnosed by characteristic histology and absence of other causes of graft damage. The histology of recurrence is comparable to pre-transplant PBC<sup>[40]</sup>. Patients with anti-mitochondrial antibodies and normal liver function tests in the presence of normal histology may develop recurrence with hallmark granulomatous cholangitis<sup>[41]</sup>. Elevated serum

**Table 4** Criteria for the diagnosis of recurrent PBC

Criteria
Transplantation for PBC
Characteristic histological features of PBC
Mononuclear inflammatory infiltrates
Lymphoid aggregates
Epithelioid granulomas
Bile duct damage
Persistence of anti-mitochondrial antibodies
Elevated immunoglobulins
Exclusion of other causes of graft damage

Definite recurrent PBC is made when all 4 of these criteria are present, and in the presence of at least 3 of the 4 histological features. Probable recurrence when only 2 histological features are present<sup>[40]</sup>.

immunoglobulins and persisting anti-mitochondrial antibodies do not in themselves indicate recurrent disease. Recurrent PBC is seen in 17% of patients at a mean of 36 mo<sup>[42]</sup> rising to 30% at 10 years. Recurrence rates on biopsy as high as 35% at 1 year have been reported<sup>[43]</sup>. The reported median time to recurrence is between 3.7 and 5 years<sup>[44,45]</sup>. Recurrence may not be diagnosed unless a protocol biopsy is taken as the liver tests may be normal<sup>[42]</sup>; indeed only half will have biochemical abnormality<sup>[44]</sup>. Liver tests may remain normal for 5 years after histological diagnosis<sup>[45]</sup>.

The role of UDCA in the treatment or prevention of recurrent PBC remains uncertain. A retrospective review of 154 PBC liver transplant recipients followed at the Mayo Clinic for least 1 year reported that recurrent PBC was not associated with death or liver re-transplantation. 38 patients with recurrent PBC received UDCA at an average dose of 12 mg/kg per day for a mean duration of 55 mo. Over a 36-mo period, an estimated 52% of UDCA-treated patients experienced normalization of serum alkaline phosphatase and alanine aminotransferase compared to 22% of untreated patients but no significant difference in the rate of histological progression was noted between subgroups. UDCA did not influence patient and graft survival<sup>[46]</sup>. It should be noted that this experience does not concord with our own unpublished data where graft loss from recurrent PBC is 4%.

Should all those transplanted for PBC be offered UDCA? The agent is safe and improves all serological parameters and may retard progression so, even in the absence of clear evidence, we would advocate its routine use.

### Risk factors for recurrence

Many studies have evaluated risk factors for recurrence. The literature is mixed concerning donor and recipient age as well as cold and warm ischaemia time<sup>[42,43,47,48]</sup>. The type of immunosuppression used is also controversial<sup>[45,49]</sup>. In a study of 485 recipients followed up over 79 mo, the recurrence rate with tacrolimus was 23% with OR 2.73 and time to recurrence 62 mo compared to 123 mo on cyclosporin ( $P < 0.001$ )<sup>[47]</sup>. Guy found similar results with OR 2.5 for tacrolimus<sup>[43]</sup>. No differences between cyclosporin and tacrolimus were

seen in other trials, though protocol liver biopsies were not performed or were only done in the context of graft dysfunction<sup>[50,51]</sup>. Sanchez reported a 156 patient cohort using protocol biopsies at 1, 2, 5, 10 and 15 year intervals with recurrence in 8.4% of recipients taking cyclosporin, azathioprine and steroids, compared with 12.2% of those receiving cyclosporin and steroids alone and 16.7% of patients taking tacrolimus and steroids ( $P = 0.11$ )<sup>[52]</sup>. Thus the evidence does suggest that cyclosporin is, compared with tacrolimus, associated with a slower rate of progression of recurrent disease. Whether this indicates that those grafted for PBC should be offered cyclosporin-based immunosuppression rather than that based on tacrolimus, and whether those with recurrent PBC should be switched from tacrolimus to cyclosporin is uncertain.

### Implications of recurrence

The consequences of recurrent disease appear to be relatively small<sup>[53]</sup>. In our series of 486 PBC transplant recipients, 3 were re-grafted as a consequence of recurrent disease, all of whom have recurrence in the re-graft<sup>[54]</sup>.

### Quality of life issues

Pruritus may resolve within days of transplantation. Fatigue persists and does not appear to improve post liver transplant<sup>[33]</sup> although there is a great improvement in quality of life<sup>[55]</sup>. Gross studied 157 adult patients with PBC or PSC before and 1 year after liver transplantation. The quality of life following transplantation was significantly better than before transplantation in all aspects but at 1-year follow-up, was not predictable by the pre-transplant subjective health status or clinical factors<sup>[56]</sup>.

## PSC

### Survival after transplantation

Indications for transplantation are as for other end-stage liver disease complications. European data show patient survival at 1, 3, 5 and 10 years was 86%, 79%, 76% and 66% respectively from Jan 1988-June 2006 (www.eltr.org).

### PSC recurrence

Recurrent PSC (Table 5) must be distinguished from secondary sclerosing cholangitis; Characteristic histological features are not always present so the diagnosis may be made on imaging the biliary tree. PSC recurrence is relatively common with figures of 37% at 36 mo and 60 % at 5 years<sup>[57,58]</sup>. Gautam's systematic review of 14 reports revealed a recurrence rate of 17% but was unable to comment upon possible risk factors<sup>[21]</sup>. Sheng studied the prevalence of stricturing disease in 100 patients who underwent transplantation for PSC and 543 controls without PSC. 27% PSC liver recipients compared to 13% of controls showed intra-hepatic strictures by cholangiography. Intra-hepatic and non-anastomotic extra-hepatic strictures were significantly more frequent in the PSC group<sup>[59]</sup>. In a small cohort who underwent living donor liver transplantation with a median follow up of 3.5 years, half developed recurrent

**Table 5** Criteria for the diagnosis of recurrent primary sclerosing cholangitis<sup>[72]</sup>

#### Criteria

Transplant for PSC

Multiple non-anastomotic strictures, headings and irregularity more than 90 d post OLT

Characteristic liver histology (fibrous cholangitis and/or fibro-obliterative lesions) with or without ductopenia, biliary fibrosis or biliary cirrhosis may be seen (but absence of characteristic features does not exclude the diagnosis).

Exclusion of other causes of secondary sclerosing cholangitis & stricturing (due to surgery, trauma, ischaemia, hepatic artery stenosis/thrombosis, established ductopaenic rejection, blood type ABO incompatibility and infections)

Cholestatic liver tests

PSC with the mean time to recurrence 3.3 years (1.1-5.4 years). There was no direct comparison to their cadaveric cohort<sup>[60]</sup>. Khettry retrospectively analysed 51 PSC patients with a follow-up of 2 to 14 years. Of the remaining 42 patients, 6 had recurrent PSC with typical histological and cholangiographic findings, 12 had autoimmune liver disease that was not otherwise specified with histology of AIH/overlap syndrome, 3 had chronic rejection, 4 had ischemic cholangiopathy, and 17 had no recurrence. Post-transplant malignancies were significantly more common in the non-recurrent cases compared with all others combined ( $P = 0.031$ ) and caused death in four. The majority of deaths (11/13) in other groups were due to sepsis. In conclusion, allograft autoimmune liver disease was seen in 18 (43%) of 42 long-term post-LT PSC patients, with progression in 5 of 18 patients. Features of PSC were seen in 6 (33%) of 18<sup>[61]</sup>.

Many factors have been associated with recurrence including steroid-resistant rejection, OKT3 use, preservation injury, ABO incompatibility, cytomegalovirus infection, male sex, donor-recipient gender mismatch and steroid resistant rejection but not specific calcineurin inhibitor use or frequency of rejection<sup>[61-67]</sup>.

Although there is some controversy as to the effect of pre-transplant colectomy on the recurrence rate, our own data consistently show that colectomy either before or during transplant is not associated with recurrent disease whereas the incidence of recurrence in those who had a colectomy post transplant is no different to those with an intact colon. Overall, recurrence of PSC leads to patient and graft loss.

### Colitis and colonic neoplasia after transplantation

Evidence linking immunosuppression with inflammatory bowel disease-free survival is mixed<sup>[68,69]</sup>. Colitis is variable and may present *de novo* after transplantation, with an incidence of 6% at 1 year and 20% at 5 years<sup>[70]</sup>. In a study of 20 PSC transplant recipients with coexisting ulcerative colitis followed over a median period of 11.9 years before OLT and 4.4 years after OLT, there was a significantly higher relapse rate after OLT than pre-transplant. 35% of recipients went onto colectomy after OLT (3 for disease severity and 4 for neoplasia/

dysplasia)<sup>[71]</sup>. These results were mirrored in a study from Birmingham which looked at 152 patients with PSC (100 with coexisting IBD). The incidence of colorectal cancer after transplant was 5.3% compared with 0.6% in non-PSC cases. All cancers in the PSC group were in patients with IBD and an intact colon. The cumulative risk of developing cancer in the 83 patients with an intact colon and IBD was 14% and 17% after 5 and 10 years, respectively. The multivariate analysis identified colonic dysplasia after transplant ( $P < 0.0003$ ), duration of colitis more than 10 years ( $P < 0.002$ ), and pancolitis ( $P < 0.004$ ) as risk factors<sup>[69]</sup>. Colonoscopy is thus recommended annually following transplant.

## FUTURE PROSPECTS

Over the last three decades, liver transplantation has evolved from an experimental, high risk procedure to a routine operation with a high success rate. Indications have widened and contra-indications decreased. Currently, the major limitation remains the shortage of organs so that not everyone who might benefit from the procedure can receive a graft and surgeons have to use extended criteria organs. The use of stem cell therapy and liver cell transplants, remain in their infancy. There still remain considerable challenges ahead: major causes of graft loss include recurrent disease, especially Hepatitis C but also autoimmune diseases, and the side-effects and complications of immunosuppression. The goal of achieving tolerance remains elusive but the development of new agents, especially biologicals, may allow for more effective strategies. The stimulus and challenges of liver transplantation have advanced our understanding of the mechanisms of alloantigen immune recognition and target cell damage and helped introduce new immune-modifying agents and strategies. They have also helped our understanding of the anatomy, physiology and pathophysiology of the liver. However, the long-term goal of clinical research must be the treatment of disease in the native liver so that transplantation becomes redundant.

## REFERENCES

- 1 Devlin J, O'Grady J. Indications for referral and assessment in adult liver transplantation: a clinical guideline. British Society of Gastroenterology. *Gut* 1999; **45** Suppl 6: VI1-VI22
- 2 Adam R, McMaster P, O'Grady JG, Castaing D, Klempnauer JL, Jamieson N, Neuhaus P, Lerut J, Salizzoni M, Pollard S, Muhlbacher F, Rogiers X, Garcia Valdecasas JC, Berenguer J, Jaeck D, Moreno Gonzalez E. Evolution of liver transplantation in Europe: report of the European Liver Transplant Registry. *Liver Transpl* 2003; **9**: 1231-1243
- 3 Kamath PS, Wiesner RH, Malinchoc M, Kremers W, Therneau TM, Kosberg CL, D'Amico G, Dickson ER, Kim WR. A model to predict survival in patients with end-stage liver disease. *Hepatology* 2001; **33**: 464-470
- 4 Londoño MC, Cárdenas A, Guevara M, Quintó L, de Las Heras D, Navasa M, Rimola A, Garcia-Valdecasas JC, Arroyo V, Ginès P. MELD score and serum sodium in the prediction of survival of patients with cirrhosis awaiting liver transplantation. *Gut* 2007; **56**: 1283-1290
- 5 Ahmed A, Keefe EB. Current indications and contraindications for liver transplantation. *Clin Liver Dis* 2007; **11**: 227-247
- 6 Consensus conference: Indications for Liver Transplantation, January 19 and 20, 2005, Lyon-Palais Des Congres: text of recommendations (long version). *Liver Transpl* 2006; **12**: 998-1011
- 7 Florman S, Miller CM. Live donor liver transplantation. *Liver Transpl* 2006; **12**: 499-510
- 8 O'Grady JG. Liver transplantation alcohol related liver disease: (deliberately) stirring a hornet's nest! *Gut* 2006; **55**: 1529-1531
- 9 Martinez OM, Rosen HR. Basic concepts in transplant immunology. *Liver Transpl* 2005; **11**: 370-381
- 10 Benseler V, McCaughan GW, Schlitt HJ, Bishop GA, Bowen DG, Bertolino P. The liver: a special case in transplantation tolerance. *Semin Liver Dis* 2007; **27**: 194-213
- 11 McCaughan GW. Withdrawal of immunosuppression in liver transplant recipients: is this as good as it gets? *Liver Transpl* 2002; **8**: 408-410
- 12 Hirose R, Vincenti F. Immunosuppression: today, tomorrow, and withdrawal. *Semin Liver Dis* 2006; **26**: 201-210
- 13 Calne R. WOFIE hypothesis: some thoughts on an approach toward allograft tolerance. *Transplant Proc* 1996; **28**: 1152
- 14 Calne RY. Prope tolerance with alemtuzumab. *Liver Transpl* 2005; **11**: 361-363
- 15 Reich DJ, Fiel I, Guarrera JV, Emre S, Guy SR, Schwartz ME, Miller CM, Sheiner PA. Liver transplantation for autoimmune hepatitis. *Hepatology* 2000; **32**: 693-700
- 16 Krawitt EL. Autoimmune hepatitis. *N Engl J Med* 2006; **354**: 54-66
- 17 Futagawa Y, Terasaki PI. An analysis of the OPTN/UNOS Liver Transplant Registry. *Clin Transpl* 2004; 315-329
- 18 Molmenti EP, Netto GJ, Murray NG, Smith DM, Molmenti H, Crippin JS, Hoover TC, Jung G, Marubashi S, Sanchez EQ, Gogel B, Levy MF, Goldstein RM, Fasola CG, Gonwa TA, Klintmalm GB. Incidence and recurrence of autoimmune/alloimmune hepatitis in liver transplant recipients. *Liver Transpl* 2002; **8**: 519-526
- 19 González-Koch A, Czaja AJ, Carpenter HA, Roberts SK, Charlton MR, Porayko MK, Rosen CB, Wiesner RH. Recurrent autoimmune hepatitis after orthotopic liver transplantation. *Liver Transpl* 2001; **7**: 302-310
- 20 Vogel A, Heinrich E, Bahr MJ, Rifai K, Flemming P, Melter M, Klempnauer J, Nashan B, Manns MP, Strassburg CP. Long-term outcome of liver transplantation for autoimmune hepatitis. *Clin Transplant* 2004; **18**: 62-69
- 21 Gautam M, Cheruvattath R, Balan V. Recurrence of autoimmune liver disease after liver transplantation: a systematic review. *Liver Transpl* 2006; **12**: 1813-1824
- 22 Czaja AJ. Autoimmune hepatitis after liver transplantation and other lessons of self-intolerance. *Liver Transpl* 2002; **8**: 505-513
- 23 Milkiewicz P, Hubscher SG, Skiba G, Hathaway M, Elias E. Recurrence of autoimmune hepatitis after liver transplantation. *Transplantation* 1999; **68**: 253-256
- 24 Ayata G, Gordon FD, Lewis WD, Pomfret E, Pomposelli JJ, Jenkins RL, Khettry U. Liver transplantation for autoimmune hepatitis: a long-term pathologic study. *Hepatology* 2000; **32**: 185-192
- 25 Duclos-Vallée JC, Sebagh M, Rifai K, Johanet C, Ballot E, Guettier C, Karam V, Hurtova M, Feray C, Reynes M, Bismuth H, Samuel D. A 10 year follow up study of patients transplanted for autoimmune hepatitis: histological recurrence precedes clinical and biochemical recurrence. *Gut* 2003; **52**: 893-897
- 26 Núñez-Martínez O, De la Cruz G, Salcedo M, Molina J, De Diego A, Ripoll C, Calleja J, Alvarez E, Clemente G. Liver transplantation for autoimmune hepatitis: fulminant versus chronic hepatitis presentation. *Transplant Proc* 2003; **35**: 1857-1858
- 27 Vogel A, Heinrich E, Bahr MJ, Rifai K, Flemming P, Melter M, Klempnauer J, Nashan B, Manns MP, Strassburg CP.



- Long-term outcome of liver transplantation for autoimmune hepatitis. *Clin Transplant* 2004; **18**: 62-69
- 28 **Khalaf H**, Mourad W, El-Sheikh Y, Abdo A, Helmy A, Medhat Y, Al-Sofayan M, Al-Sagheir M, Al-Sebayel M. Liver transplantation for autoimmune hepatitis: a single-center experience. *Transplant Proc* 2007; **39**: 1166-1170
  - 29 **Yao H**, Michitaka K, Tokumoto Y, Murata Y, Mashiba T, Abe M, Hiasa Y, Horiike N, Onji M. Recurrence of autoimmune hepatitis after liver transplantation without elevation of alanine aminotransferase. *World J Gastroenterol* 2007; **13**: 1618-1621
  - 30 **Kerkar N**, Hadzić N, Davies ET, Portmann B, Donaldson PT, Rela M, Heaton ND, Vergani D, Mieli-Vergani G. De-novo autoimmune hepatitis after liver transplantation. *Lancet* 1998; **351**: 409-413
  - 31 **Heneghan MA**, Portmann BC, Norris SM, Williams R, Muiesan P, Rela M, Heaton ND, O'Grady JG. Graft dysfunction mimicking autoimmune hepatitis following liver transplantation in adults. *Hepatology* 2001; **34**: 464-470
  - 32 **Aguilera I**, Wichmann I, Sousa JM, Bernardos A, Franco E, García-Lozano JR, Núñez-Roldán A. Antibodies against glutathione S-transferase T1 (GSTT1) in patients with de novo immune hepatitis following liver transplantation. *Clin Exp Immunol* 2001; **126**: 535-539
  - 33 **Goldblatt J**, Taylor PJ, Lipman T, Prince MI, Baragiotta A, Bassendine MF, James OF, Jones DE. The true impact of fatigue in primary biliary cirrhosis: a population study. *Gastroenterology* 2002; **122**: 1235-1241
  - 34 **Shapiro JM**, Smith H, Schaffner F. Serum bilirubin: a prognostic factor in primary biliary cirrhosis. *Gut* 1979; **20**: 137-140
  - 35 **Devlin J**, O'Grady J. Indications for referral and assessment in adult liver transplantation: a clinical guideline. British Society of Gastroenterology. *Gut* 1999; **45** Suppl 6: VII-VI22
  - 36 **Dickson ER**, Grambsch PM, Fleming TR, Fisher LD, Langworthy A. Prognosis in primary biliary cirrhosis: model for decision making. *Hepatology* 1989; **10**: 1-7
  - 37 **Kim WR**, Wiesner RH, Thorneau TM, Poterucha JJ, Porayko MK, Evans RW, Klintmalm GB, Crippin JS, Krom RA, Dickson ER. Optimal timing of liver transplantation for primary biliary cirrhosis. *Hepatology* 1998; **28**: 33-38
  - 38 **Garcia CE**, Garcia RF, Gunson B, Christensen E, Neuberger J, McMaster P, Mirza DF. Analysis of marginal donor parameters in liver transplantation for primary biliary cirrhosis. *Exp Clin Transplant* 2004; **2**: 183-188
  - 39 **MacQuillan GC**, Neuberger J. Liver transplantation for primary biliary cirrhosis. *Clin Liver Dis* 2003; **7**: 941-956, ix
  - 40 **Hubscher SG**, Elias E, Buckels JA, Mayer AD, McMaster P, Neuberger JM. Primary biliary cirrhosis. Histological evidence of disease recurrence after liver transplantation. *J Hepatol* 1993; **18**: 173-184
  - 41 **Mitchison HC**, Bassendine MF, Hendrick A, Bennett MK, Bird G, Watson AJ, James OF. Positive antimitochondrial antibody but normal alkaline phosphatase: is this primary biliary cirrhosis? *Hepatology* 1986; **6**: 1279-1284
  - 42 **Mackie J**, Groves K, Hoyle A, Garcia C, Garcia R, Gunson B, Neuberger J. Orthotopic liver transplantation for alcoholic liver disease: a retrospective analysis of survival, recidivism, and risk factors predisposing to recidivism. *Liver Transpl* 2001; **7**: 418-427
  - 43 **Guy JE**, Qian P, Lowell JA, Peters MG. Recurrent primary biliary cirrhosis: peritransplant factors and ursodeoxycholic acid treatment post-liver transplant. *Liver Transpl* 2005; **11**: 1252-1257
  - 44 **Sylvestre PB**, Batts KP, Burgart LJ, Poterucha JJ, Wiesner RH. Recurrence of primary biliary cirrhosis after liver transplantation: Histologic estimate of incidence and natural history. *Liver Transpl* 2003; **9**: 1086-1093
  - 45 **Jacob DA**, Neumann UP, Bahra M, Klupp J, Puhl G, Neuhaus R, Langrehr JM. Long-term follow-up after recurrence of primary biliary cirrhosis after liver transplantation in 100 patients. *Clin Transplant* 2006; **20**: 211-220
  - 46 **Charatcharoenwittaya P**, Pimentel S, Talwalkar JA, Enders FT, Lindor KD, Krom RA, Wiesner RH. Long-term survival and impact of ursodeoxycholic acid treatment for recurrent primary biliary cirrhosis after liver transplantation. *Liver Transpl* 2007; **13**: 1236-1245
  - 47 **Neuberger J**, Gunson B, Hubscher S, Nightingale P. Immunosuppression affects the rate of recurrent primary biliary cirrhosis after liver transplantation. *Liver Transpl* 2004; **10**: 488-491
  - 48 **Balan V**, Abu-Elmagd K, Demetris AJ. Autoimmune liver diseases. Recurrence after liver transplantation. *Surg Clin North Am* 1999; **79**: 147-152
  - 49 **Dmitrewski J**, Hubscher SG, Mayer AD, Neuberger JM. Recurrence of primary biliary cirrhosis in the liver allograft: the effect of immunosuppression. *J Hepatol* 1996; **24**: 253-257
  - 50 **Khettry U**, Anand N, Faul PN, Lewis WD, Pomfret EA, Pomposelli J, Jenkins RL, Gordon FD. Liver transplantation for primary biliary cirrhosis: a long-term pathologic study. *Liver Transpl* 2003; **9**: 87-96
  - 51 **Levitsky J**, Hart J, Cohen SM, Te HS. The effect of immunosuppressive regimens on the recurrence of primary biliary cirrhosis after liver transplantation. *Liver Transpl* 2003; **9**: 733-736
  - 52 **Sanchez EQ**, Levy MF, Goldstein RM, Fasola CG, Tillery GW, Netto GJ, Watkins DL, Weinstein JS, Murray NG, Byers D, Christensen LL, Klintmalm GB. The changing clinical presentation of recurrent primary biliary cirrhosis after liver transplantation. *Transplantation* 2003; **76**: 1583-1588
  - 53 **Jacob DA**, Neumann UP, Bahra M, Langrehr JM, Neuhaus P. Liver transplantation for primary biliary cirrhosis: influence of primary immunosuppression on survival. *Transplant Proc* 2005; **37**: 1691-1692
  - 54 **Neuberger J**. Liver transplantation for primary biliary cirrhosis: indications and risk of recurrence. *J Hepatol* 2003; **39**: 142-148
  - 55 **Kim WR**, Lindor KD, Malinchoc M, Petz JL, Jorgensen R, Dickson ER. Reliability and validity of the NIDDK-QA instrument in the assessment of quality of life in ambulatory patients with cholestatic liver disease. *Hepatology* 2000; **32**: 924-929
  - 56 **Gross CR**, Malinchoc M, Kim WR, Evans RW, Wiesner RH, Petz JL, Crippin JS, Klintmalm GB, Levy MF, Ricci P, Thorneau TM, Dickson ER. Quality of life before and after liver transplantation for cholestatic liver disease. *Hepatology* 1999; **29**: 356-364
  - 57 **Vera A**, Moledina S, Gunson B, Hubscher S, Mirza D, Olliff S, Neuberger J. Risk factors for recurrence of primary sclerosing cholangitis of liver allograft. *Lancet* 2002; **360**: 1943-1944
  - 58 **Neuberger J**. Liver Transplantation for Cholestatic Liver Disease. *Curr Treat Options Gastroenterol* 2003; **6**: 113-121
  - 59 **Sheng R**, Zajko AB, Campbell WL, Abu-Elmagd K. Biliary strictures in hepatic transplants: prevalence and types in patients with primary sclerosing cholangitis vs those with other liver diseases. *AJR Am J Roentgenol* 1993; **161**: 297-300
  - 60 **Tamura S**, Sugawara Y, Kaneko J, Matsui Y, Togashi J, Makuuchi M. Recurrence of primary sclerosing cholangitis after living donor liver transplantation. *Liver Int* 2007; **27**: 86-94
  - 61 **Khettry U**, Huang WY, Simpson MA, Pomfret EA, Pomposelli JJ, Lewis WD, Jenkins RL, Gordon FD. Patterns of recurrent hepatitis C after liver transplantation in a recent cohort of patients. *Hum Pathol* 2007; **38**: 443-452
  - 62 **Goss JA**, Shackleton CR, Farmer DG, Arnaout WS, Seu P, Markowitz JS, Martin P, Stribling RJ, Goldstein LI, Busuttil RW. Orthotopic liver transplantation for primary sclerosing cholangitis. A 12-year single center experience. *Ann Surg* 1997; **225**: 472-481; discussion 481-483
  - 63 **Graziadei IW**. Recurrence of primary sclerosing cholangitis after liver transplantation. *Liver Transpl* 2002; **8**: 575-581
  - 64 **Gopal DV**, Corless CL, Rabkin JM, Olyaei AJ, Rosen HR. Graft failure from severe recurrent primary sclerosing

- cholangitis following orthotopic liver transplantation. *J Clin Gastroenterol* 2003; **37**: 344-347
- 65 **Kugelmas M**, Spiegelman P, Osgood MJ, Young DA, Trotter JF, Steinberg T, Wachs ME, Bak T, Kam I, Everson GT. Different immunosuppressive regimens and recurrence of primary sclerosing cholangitis after liver transplantation. *Liver Transpl* 2003; **9**: 727-732
- 66 **Vera A**, Gunson BK, Ussatoff V, Nightingale P, Candinas D, Radley S, Mayer AD, Buckels JA, McMaster P, Neuberger J, Mirza DF. Colorectal cancer in patients with inflammatory bowel disease after liver transplantation for primary sclerosing cholangitis. *Transplantation* 2003; **75**: 1983-1988
- 67 **Brandsaeter B**, Schrumpf E, Bentdal O, Brabrand K, Smith HJ, Abildgaard A, Clausen OP, Bjoro K. Recurrent primary sclerosing cholangitis after liver transplantation: a magnetic resonance cholangiography study with analyses of predictive factors. *Liver Transpl* 2005; **11**: 1361-1369
- 68 **Haagsma EB**, Van Den Berg AP, Kleibeuker JH, Slooff MJ, Dijkstra G. Inflammatory bowel disease after liver transplantation: the effect of different immunosuppressive regimens. *Aliment Pharmacol Ther* 2003; **18**: 33-44
- 69 **Vera A**, Gunson BK, Ussatoff V, Nightingale P, Candinas D, Radley S, Mayer AD, Buckels JA, McMaster P, Neuberger J, Mirza DF. Colorectal cancer in patients with inflammatory bowel disease after liver transplantation for primary sclerosing cholangitis. *Transplantation* 2003; **75**: 1983-1988
- 70 **Papatheodoridis GV**, Hamilton M, Mistry PK, Davidson B, Rolles K, Burroughs AK. Ulcerative colitis has an aggressive course after orthotopic liver transplantation for primary sclerosing cholangitis. *Gut* 1998; **43**: 639-644
- 71 **Ho GT**, Seddon AJ, Therapondos G, Satsangi J, Hayes PC. The clinical course of ulcerative colitis after orthotopic liver transplantation for primary sclerosing cholangitis: further appraisal of immunosuppression post transplantation. *Eur J Gastroenterol Hepatol* 2005; **17**: 1379-1385
- 72 **Graziadei IW**, Wiesner RH, Marotta PJ, Porayko MK, Hay JE, Charlton MR, Poterucha JJ, Rosen CB, Gores GJ, LaRusso NF, Krom RA. Long-term results of patients undergoing liver transplantation for primary sclerosing cholangitis. *Hepatology* 1999; **30**: 1121-1127

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REVIEW

# Why, who and how should perform liver biopsy in chronic liver diseases

Ioan Sporea, Alina Popescu, Roxana Sirli

Ioan Sporea, Alina Popescu, Roxana Sirli, Department of Gastroenterology, University of Medicine and Pharmacy, Timisoara 700736, Romania

Author contributions: Sporea I wrote the paper and revised data from the literature, Popescu A and Sirli R researched for data from the literature and revised the manuscript.

Correspondence to: Ioan Sporea, Department of Gastroenterology, University of Medicine and Pharmacy, Timisoara 700736, Romania. [isporea@excite.com](mailto:isporea@excite.com)

Telephone: +40-256-309455 Fax: +40-256-488003

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**Peer reviewer:** Marek Hartleb, Professor, Department of Gastroenterology, Silesian Medical School, ul. Medyków 14, Katowice 40-752, Poland

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## Abstract

Chronic viral hepatitis is a common disease in the general population. During chronic hepatitis, the prognosis and clinical management are highly dependent on the extent of liver fibrosis. The fibrosis evaluation can be performed by FibroTest (using serological markers), by Elastography or FibroScan (a noninvasive percutaneous technique using the elastic properties of the hepatic tissue) and by liver biopsy (LB), considered to be the "gold standard". Currently, there are three techniques for performing LB: percutaneous, transjugular and laparoscopic. The percutaneous LB can be performed blind, ultrasound (US) guided or US assisted. There are two main categories of specialists who perform LB: gastroenterologists (hepatologists) and radiologists, and the specialty of the individual who performs the LB determines if the LB is performed under ultrasound guidance or not. There are two types of biopsy needles used for LB: cutting needles (Tru-Cut, Vim-Silverman) and suction needles (Menghini, Klatzkin, Jamshidi). The rate of major complications after percutaneous LB ranges from 0.09% to 2.3%, but the echo-guided percutaneous liver biopsy is a safe method for the diagnosis of chronic diffuse hepatitis (cost-effective as compared to blind biopsy) and the rate of complications seems to be related to the experience of the physician and the type of the needle used (Menghini type needle seems to be safer). Maybe, in a few years we will use non-invasive markers of fibrosis, but at this time, most authorities in the field consider that the LB is useful and necessary for the evaluation of chronic hepatopathies, despite the fact that it is not a perfect test.

## INTRODUCTION

Chronic viral hepatitis is a common disease in the general population. Chronic hepatitis B virus (HBV) infection affects 350 million individuals globally, and approximately 15%-40% may develop serious complications, including end-stage liver disease and hepatocellular carcinoma<sup>[1]</sup>. Chronic hepatitis C virus (HCV) infection is also an important cause of chronic hepatitis, data from World Health Organization (WHO) suggesting that 170 million people are infected world-wide with HCV, 10 million of them in Western Europe<sup>[2]</sup>. It is estimated that at least 3.9 million persons (1.8% of the population) in the United States are anti-HCV seropositive, and that 2.7 million are chronically viremic<sup>[3]</sup>.

At the same time, alcoholic steatohepatitis (ASH) and non-alcoholic steatohepatitis (NASH) are frequent in the developed countries. Regarding NASH, long-term follow up studies on obese patients showed increased cirrhosis-related morbidity and mortality<sup>[4]</sup>. Also, studies on cohorts of diabetic patients showed increased incidence of non-alcoholic chronic liver disease and hepatocellular carcinoma<sup>[5]</sup>.

The complete evaluation of a patient with diffuse liver diseases requires: clinical evaluation, biological evaluation and morphopathological exam-liver biopsy (LB), for the grading and staging of the liver disease. The clinical evaluation is often irrelevant, only the presence of spider naevi on the anterior thorax or an enlarged and firmer liver could suggest that the patient already has liver cirrhosis (suspicion that has to be confirmed or infirmed by further tests). The biological evaluation by means of

the usual tests is also often irrelevant, especially in chronic hepatitis C, known to induce sometimes severe hepatic lesions, while the aminotransferases are normal or only slightly elevated. Thus, it is considered that the liver biopsy has a key role for the diagnosis and follow-up of chronic diffuse hepatopathies, especially for the staging of chronic hepatitis C<sup>[6-9]</sup>.

So, what is the utility of LB in chronic liver disease? Fontollet<sup>[6]</sup> stated that LB has the following roles: to confirm the diagnosis of chronic hepatitis; to assess the necro-inflammatory activity (grading) and the severity of fibrosis (staging); to exclude another hepatopathy or an associated disease; to certify the diagnosis of cirrhosis (when present).

A review of the short history of LB shows us that: Paul Ehrlich is credited with performing the first percutaneous LB in 1883 in Germany; Sheila Sherlock described the percutaneous LB technique in 1945 and after Menghini reported a technique for the “one-second needle biopsy of the liver” in 1958, the procedure became more widely used<sup>[10]</sup>.

But did the LB become a method of diagnosis unanimously accepted by the patients and the doctors? To answer this question we will present the results of a French study, performed on 1177 general practitioners that showed that 59% of the patients infected with HCV refused the LB, opinion shared by 22% of the general practitioners<sup>[11]</sup>.

All these being said, we would like to discuss in this paper several aspects concerning the LB, trying to answer the following questions: (1) Why? (2) Who? (3) How to perform the LB?

## WHY TO PERFORM A LB IN CHRONIC LIVER DISEASE?

During chronic hepatitis, the prognosis and clinical management are highly dependent on the extent of liver fibrosis<sup>[12]</sup>. The fibrosis evaluation can be performed by means of: FibroTest-using serological markers; Elastography or FibroScan-a noninvasive percutaneous technique using the elastic property of the hepatic tissue; liver biopsy-that seems to be the “gold standard”.

### FibroTest

The non-invasive tests for the assessment of the severity of chronic liver diseases are an interesting alternative, more and more evaluated in the last years, aimed to replace, maybe, the LB. After 2000, the non-invasive tests predictive of liver damage were studied more and more, especially in chronic hepatitis C<sup>[13]</sup> and, more recently, also in chronic hepatitis B<sup>[14]</sup> and NASH<sup>[15-17]</sup>.

FibroTest-ActiTest (FT-AT) was developed using biochemical markers and repeatedly demonstrated a high predictive value for fibrosis and necroinflammatory histological activity, in patients with chronic hepatitis C<sup>[18-21]</sup>. In two separate studies the FT-AT has been proven valuable also in patients with chronic hepatitis B<sup>[14, 22]</sup>.

FT-AT is a noninvasive blood test that combines the

quantitative results of six serum biochemical markers (alfa2-macroglobulin, haptoglobin, gamma glutamyl transpeptidase, total bilirubin, apolipoprotein A1 and ALT) with patients' age and gender in a patented artificial intelligence algorithm (USPTO 6631330) in order to generate a measure of fibrosis and necroinflammatory activity in the liver<sup>[14]</sup>. Previously validated FT-AT are used (Biopredictive, Paris, France; Fibro-SURE LabCorp, Burlington, NC), that provide an accurate measurement of bridging fibrosis and/or moderate necroinflammatory activity with AUROC (Area Under Receiver-Operating Characteristic Curve) predictive value between 0.70 and 0.80, when compared to the liver biopsy<sup>[23]</sup>.

It is recommended that FibroTest-ActiTest should not be performed during Ribavirin therapy, because it can induce hemolysis and low haptoglobin levels, nor in patients with Gilbert's syndrome, with acute hepatitis or extra hepatic cholestasis<sup>[23]</sup>, cases in which falsely elevated fibrosis and activity scores can be obtained.

In a recently published editorial in the American Journal of Gastroenterology, Paul Thuluvath<sup>[24]</sup> discusses the FibroTest, stating that it was extensively studied only by Poynard *et al* and that there are only few independent studies. It is also considered that there are significant inter-laboratory variations, thus some studies demonstrated that significant fibrosis can be over-looked or over-rated in approximately 15%-20% of the cases. As a conclusion of this editorial, Paul Thuluvath considers that “we may be approaching a time when serum biomarkers may become an integral part of the assessment of patients with chronic liver disease, but published evidence suggests that these markers are not yet ready for prime time”.

### Elastography or FibroScan

Another non-invasive method of assessment of liver fibrosis is transient elastography. This technique enables the assessment the liver's stiffness and it is performed by a device called FibroScan (Echosens). The main component of the FibroScan is an ultrasound probe mounted on a vibrating device (piston). The patient to be examined lies down on his back and the ultrasound probe is applied to the skin surface between the ribs, thus examining the right liver lobe. The piston induces elastic vibrations, with low frequency and small amplitude that propagate through the liver. The reflected waves are captured by the transducer, their velocity being directly related to the elasticity (stiffness) of the liver. After several elastographic measurements are performed, the mean value must be calculated, thus enabling a correct assessment of the fibrosis.

This method of evaluation is totally painless and lasts only a few minutes. The stiffness of the liver is measured up to 2 cm in depth and on a surface with the diameter of approximately 1 cm (thus enabling the evaluation of a portion of the liver 500 times bigger than by LB)<sup>[25]</sup>. In the study performed by Foucher *et al*<sup>[26]</sup> the stiffness of the liver was measured up to a depth of 4 cm and on a surface with the diameter of 1 cm, so that 1/500 of the liver was evaluated. However the FibroScan device is ex-



ceedingly expensive rising to more than 60 000 Euros.

The value of the FibroScan method for the assessment of the severity of fibrosis in chronic hepatitis is under evaluation, but in the last 2 years several papers were published that demonstrate that this non-invasive method is precise enough to be compared to LB<sup>[12,25-28]</sup>.

In the study performed by Castera *et al*, the elasticity of the liver, measured with the FibroScan device, varied between 2.4 and 75.4 kilopascals (kPa), with a median of 7.4 kPa<sup>[27]</sup>. When comparing the FibroScan to the LB in patients with chronic hepatitis C, the cut-off values were 7.1 kPa for  $F \leq 2$ ; 9.5 kPa for  $F \leq 3$  and 12.5 kPa for  $F = 4$ <sup>[27]</sup>. The area under the receiver operating characteristic curve (AUROC) of FibroScan was 0.83 for  $F \leq 2$ ; 0.90 for  $F \leq 3$  and 0.95 for  $F = 4$ <sup>[27]</sup>. The same authors demonstrated that, when combined with FibroTest, FibroScan was more precise than the LB, the AUROC values reaching 0.88, 0.95 and 0.95 respectively, for fibrosis  $\leq 2$ ,  $\leq 3$  or 4.

In a study performed by Ziolkowski *et al*<sup>[12]</sup> the AUROC value of FibroScan as compared to the LB was 0.79 for  $F \leq 2$ ; 0.91 for  $F \leq 3$  and 0.97 for  $F = 4$ . Thus, the authors concluded that transient elastography appears reliable to detect significant fibrosis or cirrhosis in patients with chronic hepatitis C.

The majority of studies that compared the FibroScan to the FibroTest and to the LB showed a slight superiority of FibroScan *vs* FibroTest<sup>[27,28]</sup>.

Starting from the encouraging results of FibroScan in patients with chronic hepatitis C, this method was also used to evaluate the severity of fibrosis in patients with chronic hepatitis B and in patients with primary biliary cirrhosis (PBC)<sup>[29,30]</sup>.

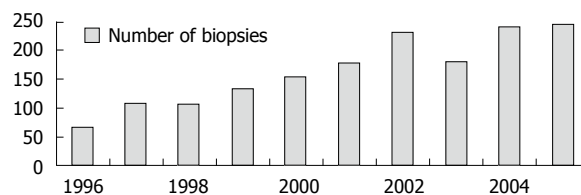
It is probable that in a not too far future, the combination of FibroScan with FibroTest could avoid biopsy in most patients with chronic hepatopathies<sup>[27]</sup>.

FibroTest and Elastography have a good value for the cases with no fibrosis or with important fibrosis (cirrhosis), but for the intermediate stages the value is low. This is the reason why LB is still the most used method of assessment of the severity of liver lesions in chronic hepatitis, several guidelines recommending that the decision to treat should be made after liver biopsy<sup>[31-32]</sup>.

### Liver biopsy

At this moment LB is still the "gold standard" for the evaluation of chronic hepatitis<sup>[33]</sup>, but the method is not perfect. There are some problems regarding the diagnosis of cirrhosis by LB<sup>[33]</sup> and regarding the differences of the severity of fibrosis when twin LB are performed in both liver lobes<sup>[34]</sup>. This is why we consider appropriate a review of the advantages of LB, of its limits, of the best techniques to perform LB and also of the possible complications.

How representative can be a needle biopsy? The size of a biopsy specimen, which varies between 1 cm and 4 cm in length and between 1.2 mm and 1.8 mm in diameter, represents 1/50 000 of the total mass of the



**Figure 1** Liver biopsies performed in our department in the last years (1996-2005).

liver<sup>[31]</sup>. The British guideline about LB<sup>[31]</sup> considers that most hepatologists are satisfied with a biopsy specimen containing at least six to eight portal triads. A critical review of the literature reveals that biopsy samples 2 cm or more in length, containing at least 11 complete portal tracts, should be reliable for grading and staging chronic viral hepatitis<sup>[35]</sup>. Another study, concerning the dimensions of the biopsy specimen needed in order to perform an accurate pathological diagnosis, demonstrated that a fragment of at least 10 mm is enough for a correct staging and grading<sup>[36]</sup>.

But which is the main reason to perform liver biopsy? The answer is: chronic hepatitis C. In our Department of Gastroenterology, the last 1500 LB were performed to evaluate: chronic C viral infection in 56.0% of the cases; chronic B viral infection in 34.2% of the cases; chronic viral coinfection in 3.2% of the cases; NASH and ASH in 4.5% of the cases and other liver diseases in 1.7% of the cases<sup>[37]</sup>.

In France, in a nationwide survey, Cadranet *et al*<sup>[38]</sup> showed that 54% of the LB were performed for chronic C viral infection. The total number of LB performed each year in France is approximately 16 000<sup>[39]</sup>.

The number of LB performed in every department increased in the last 15 years, mainly due to the increasing number of cases with chronic hepatitis C discovered in the last period, but we do not know the future trend, in connection to the introduction of non-invasive tests of fibrosis. The evolution of the number of ultrasound guided LB in our department in the last 10 years is shown in Figure 1.

### WHO SHOULD PERFORM THE LIVER BIOPSY?

There are two main categories of specialists who perform LB: gastroenterologists/hepatologists and the radiologists. The specialty of the individual who performs the LB determines if the LB is performed under ultrasound (US) guidance or not.

In many countries the ultrasound examination is performed both by radiologists and by clinicians (Germany, Italy, Austria, Switzerland and Romania). In other countries, the ultrasound examination is performed only by radiologists (USA, UK, The Netherlands and Denmark).

Currently it is estimated that in the USA, 50% of the LB are performed by radiologists<sup>[40]</sup>. In the same country, a questionnaire regarding the LB practice, answered by 112 gastroenterologists/hepatologists,

showed that 30% of them do not perform LB (due to concern about risks, low reimbursement and logistical issues)<sup>[41]</sup>. Another important fact is that in countries in which gastroenterologists do not perform US examinations, the LB performed by the clinician are “blind”, or done by the radiologist. It was suggested in USA that, by installing a US machine in the endoscopy unit, the cost of LB would decrease because a previous US examination in the Radiology Department would not be necessary before the LB, but that would require the gastroenterologist and hepatologist to become proficient in US technique and interpretation<sup>[40]</sup>.

The European Diploma of Gastroenterology stipulates that, in order to become specialists, all the gastroenterologists should perform at least 300 US examinations<sup>[42]</sup>. In the opinion of Vautier's team, issued years ago, “the ideal liver biopsy may be one that is performed in the ward by a gastroenterologist using ultrasonographic guidance”<sup>[43]</sup>.

## HOW TO PERFORM LB?

Currently, there are 3 techniques for performing a liver biopsy<sup>[31]</sup>: percutaneous, transjugular and laparoscopic. The percutaneous liver biopsy (PLB) can be performed: blind, US guided or US assisted.

The most important question regarding the percutaneous liver biopsy that should be addressed is: *blind or echo-guided techniques?* The answer depends on the skills of the gastroenterologist (hepatologist) and on the technical possibilities (accessibility to the ultrasound machine).

However, it is still debatable whether ultrasound-guided LB has an advantage over the blind one or not<sup>[43,44]</sup>. In a prospective study in France, Cadranet *et al.*<sup>[38]</sup> showed that from 2084 liver biopsies, only 56% were echo-guided. Also, many studies showed that the complications of LB seem to be related to the type of the technique, blind or echo-guided, respectively: (1) Younossi *et al.*<sup>[45]</sup> showed that the complications appeared in 4% of the cases with “blind” biopsies and in 2% of the cases with “ultrasound-guided” biopsies (the study revealed the cost-effectiveness of echo-guided biopsy); (2) Farrell *et al.*<sup>[46]</sup> showed complications in 1.8% of the cases with “ultrasound-guided” biopsies and in 7.7% of the cases with “blind” biopsies ( $P < 0.05$ ); (3) Pasha *et al.*<sup>[44]</sup> showed that severe complications occurred in 0.5% of the cases with “ultrasound-guided” biopsies and in 2.2% of the cases with “blind” biopsies ( $P < 0.05$ ). The same author revealed that the pain appeared more often (50% of the cases) in the “blind” biopsy group as compared to the “ultrasound-guided” biopsy group (37% of the cases,  $P = 0.003$ ).

But how often does the ultrasound guidance change the liver biopsy site? In a prospective study, Riley<sup>[47]</sup> showed that by ultrasound examination the site of biopsy was changed in 15.1% of the cases (21/165 patients). The reasons for changing the place of biopsy were the interposition of: lung, gall bladder, large central

vessel, ascites, colonic loop, and slim liver edge.

Considering all these facts, it is reasonable and cost-efficient to perform the LB under US guidance<sup>[40,45]</sup>, recent data suggesting a decrease in severe postbiopsy complications by up to 30% and less postbiopsy pain<sup>[40]</sup>.

## Type of needle

There are two types of biopsy needles used to perform a LB: “cutting needles” (Tru-Cut, Vim-Silverman) and “suction needles” (Menghini needle, Klatzkin needle, Jamshidi needle). Regarding how we use the needle, we can perform the LB manually or automatic, using spring-loaded devices (the so called “gun system”).

Data from literature showed that there is a correlation between the rate of complications and the type of the needle used for biopsy: 3.5‰ for the Tru-Cut needle and 1‰ for the Menghini type needle<sup>[48]</sup>.

Usually the choice of the biopsy instrument/needle is based on operator preference, instrument availability and clinical scenario<sup>[40]</sup>. The choice between the automatic biopsy gun *vs* manual activated needle depends on the experience of the center (operator). In a Dutch study<sup>[49]</sup> that compared standard Tru-Cut needle with a new automatic biopsy gun (Acecut), the performance of the automatic needle was superior and more consistent with respect to tissue yield, but post-biopsy pain and post-biopsy use of analgesics was superior after automatic biopsy gun. Thus, the authors<sup>[49]</sup> conclude that the automatic Tru-Cut needle offers an advantage, particularly for physicians with no or limited experience in liver biopsy.

Another group<sup>[50]</sup> did not find either type of needle to offer more safety when comparing the Tru-Cut needle with an automatic biopsy needle.

In a personal prospective study<sup>[37]</sup>, we compared the number of portal spaces obtained after PLB performed with a modified Menghini needle (manual activated needle) to the number of portal spaces obtained by PLB performed with an automatic needle (Auto Vac). The mean number of portal spaces obtained by Menghini needle biopsy was  $14.03 \pm 7.48$ , and by automatic needle biopsy was  $8.81 \pm 4.35$  ( $P < 0.0001$ ).

## The size of the needle

Usually the size of the biopsy needle used for LB in chronic hepatopathies varies between 1.2 and 1.8 mm. Gazelle's group<sup>[51]</sup> showed that larger needles produce more bleeding after LB in anaesthetized pigs (by comparing 2.1 mm with 1.6 mm needles and also by comparing 1.6 mm with 1.2 mm needles). Another study performed by Plecha *et al.*<sup>[52]</sup>, using cutting needles of 14, 18 and 22 gauge on porcine models, showed that the larger is the caliber of the needle, the greater is the absolute blood loss, but the conclusion of the study was that the use of larger-caliber needles is more efficient, despite the greater amount of blood loss, because more tissue can be recovered and because fewer passes are necessary, thus reducing the chance of complications.

However, other studies concerning the size of the

needle in connection with the rate of hemorrhagic complications, performed in humans, did not show any difference<sup>[46]</sup>.

### The number of passes of the needle into the liver

It has been demonstrated that taking more than one biopsy can increase the diagnostic value, but may have an effect on morbidity<sup>[38,39,47,53,54]</sup>. In a study performed by Riley<sup>[47]</sup> on 165 patients, only in 1.8% of cases multiple passes were necessary (noting that a low multiple pass rate was observed when applying ultrasound guidance), but in another study<sup>[55]</sup>, two needle passes were required in 20% of the patients and 3 needle passes in 0.2% of the cases.

From our point of view, after a long experience in performing PLB, we consider that the visual inspection of the hepatic fragment obtained by LB represents the guarantee that enough histological material was obtained. If we are unhappy with the size of the specimen, we perform another hepatic pass in the same session, rather than make a new biopsy later.

### The experience of the operator

There are controversial results regarding this issue. In one study, Gilmore *et al*<sup>[55]</sup> showed that the rate of complications in PLB was 3.2% if the operator had performed less than 20 biopsies and only 1.1% if the operator had performed more than 100 biopsies. In the study of Chevalier's group<sup>[56]</sup> the operator's experience did not influence either the final histological diagnosis or the degree of pain suffered by the patients.

### The safety of PLB

In a very large multicentric retrospective study concerning 98 445 liver biopsies, Poynard *et al*<sup>[57]</sup> showed that the LB was followed by severe adverse events in 3.1% of the cases and by mortality in 0.3% of the cases. In another large study the mortality rate from fatal hemorrhage after PLB was 0.11%<sup>[58]</sup>. In the well-known retrospective study performed by Piccinino *et al*<sup>[48]</sup> on 68 276 PLB, death was infrequent (0.09/1000 biopsies). The rate of major complications after PLB ranges from 0.09% to 2.3%<sup>[40]</sup>, while in a French study, severe complications appeared in 0.57% of cases<sup>[38]</sup>.

Another important question is: when did the post biopsy complications appear? From the retrospective multicentric study of Piccinino<sup>[48]</sup> we found that 61% of the complications appeared in the first 2 hours after the biopsy, 82% in the first 10 hours and 96% in the first 24 hours after biopsy. Some studies showed that the rate of complications is similar in out or inpatients<sup>[58,59]</sup>.

## CONCLUSION

Percutaneous echo-guided liver biopsy is a safe method for the diagnosis of chronic diffuse liver diseases (cost-effective in comparison with blind biopsy) and the rate of complications seems to be related to the experience of the physician and the type of the needle used (the Menghini type needle seems to be safer).

Perhaps the use of non-invasive markers will be

used in the future. For now, liver biopsy is useful and necessary for the evaluation of chronic hepatopathies, despite the fact that it is not a perfect test.

## REFERENCES

- 1 **Lai CL**, Ratziu V, Yuen MF, Poynard T. Viral hepatitis B. *Lancet* 2003; **362**: 2089-2094
- 2 **Global surveillance and control of hepatitis C**. Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. *J Viral Hepat* 1999; **6**: 35-47
- 3 **Alter MJ**, Kruszon-Moran D, Nainan OV, McQuillan GM, Gao F, Moyer LA, Kaslow RA, Margolis HS. The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. *N Engl J Med* 1999; **341**: 556-562
- 4 **Ioannou GN**, Weiss NS, Kowdley KV, Dominitz JA. Is obesity a risk factor for cirrhosis-related death or hospitalization? A population-based cohort study. *Gastroenterology* 2003; **125**: 1053-1059
- 5 **El-Serag HB**, Tran T, Everhart JE. Diabetes increases the risk of chronic liver disease and hepatocellular carcinoma. *Gastroenterology* 2004; **126**: 460-468
- 6 **Fontolliet CH**. Rôle de la biopsie dans le diagnostic et le traitement de l'hépatite chronique C. *Med Hyg* 2001; **59**: 2179-2182. Available from: URL: <http://www.revmed.ch/article.php3?sid=21734>
- 7 **Everhart JE**, Stolar M, Hoofnagle JH. Management of hepatitis C: a national survey of gastroenterologists and hepatologists. *Hepatology* 1997; **26**: 78S-82S
- 8 **Perrillo RP**. The role of liver biopsy in hepatitis C. *Hepatology* 1997; **26**: 57S-61S
- 9 **Saadeh S**, Cammell G, Carey WD, Younossi Z, Barnes D, Easley K. The role of liver biopsy in chronic hepatitis C. *Hepatology* 2001; **33**: 196-200
- 10 **Bravo AA**, Sheth SG, Chopra S. Liver biopsy. *N Engl J Med* 2001; **344**: 495-500
- 11 **Rayssiguier R**, Bonny C, Abergel A, Ughetto S, Aublet-Cuvelier B, Bommelaer G, Baranger J, Blanchet G, Delteil J, Hautefeuille P, Lapalus F, Ordonio B. Pratiques et attentes des medecins generalistes en matiere d'hepatite c dans la region auvergne. Proceedings of Les 25-emes Journees Francophones de Patologie Digestive, Paris, France, Abstract 76. Available from: URL: <http://www.snfge.asso.fr/01-bibliotheque/0a-resumes-jfpd/2001/lundi/posters/76.htm>
- 12 **Ziol M**, Handra-Luca A, Kettaneh A, Christidis C, Mal F, Kazemi F, de Ledinghen V, Marcellin P, Dhumeaux D, Trinchet JC, Beaugrand M. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with chronic hepatitis C. *Hepatology* 2005; **41**: 48-54
- 13 **Gebo KA**, Herlong HF, Torbenson MS, Jenckes MW, Chander G, Ghanem KG, El-Kamary SS, Sulkowski M, Bass EB. Role of liver biopsy in management of chronic hepatitis C: a systematic review. *Hepatology* 2002; **36**: S161-S172
- 14 **Poynard T**, Zoulim F, Ratziu V, Degos F, Imbert-Bismut F, Deny P, Landais P, El Hasnaoui A, Slama A, Blin P, Thibault V, Parvaz P, Munteanu M, Trepo C. Longitudinal assessment of histology surrogate markers (FibroTest-ActiTest) during lamivudine therapy in patients with chronic hepatitis B infection. *Am J Gastroenterol* 2005; **100**: 1970-1980
- 15 **Laine F**, Bendavid C, Moirand R, Tessier S, Perrin M, Guillygomarc'h A, Guyader D, Calon E, Renault A, Brissot P, Turlin B, Deugnier Y. Prediction of liver fibrosis in patients with features of the metabolic syndrome regardless of alcohol consumption. *Hepatology* 2004; **39**: 1639-1646
- 16 **Ratzin W**, Le Calvez S, Imbert-Bismut F, Messous D, Charlotte F, Bonyhay L, Munteanu M, Poynard T. Diagnostic value of biochemical markers (FibroTest) for the prediction of liver fibrosis in patients with non-alcoholic fatty liver disease. Proceedings of the 54th Annual Meeting of AASLD,

- 2003, nov. 25-29, Boston, USA. Abstract 729. *Hepatology* 2003; **38** Suppl 1: 510-511. Available from: URL: <http://www3.interscience.wiley.com/cgi-bin/fulltext/1122120/PDFSTART>
- 17 **Rosenberg WM**, Voelker M, Thiel R, Becka M, Burt A, Schuppan D, Hubscher S, Roskams T, Pinzani M, Arthur MJ. Serum markers detect the presence of liver fibrosis: a cohort study. *Gastroenterology* 2004; **127**: 1704-1713
  - 18 **Imbert-Bismut F**, Ratziu V, Pieroni L, Charlotte F, Benhamou Y, Poynard T. Biochemical markers of liver fibrosis in patients with hepatitis C virus infection: a prospective study. *Lancet* 2001; **357**: 1069-1075
  - 19 **Poynard T**, Imbert-Bismut F, Ratziu V, Chevret S, Jardel C, Moussalli J, Messous D, Degos F. Biochemical markers of liver fibrosis in patients infected by hepatitis C virus: longitudinal validation in a randomized trial. *J Viral Hepat* 2002; **9**: 128-133
  - 20 **Myers RP**, Ratziu V, Imbert-Bismut F, Charlotte F, Poynard T. Biochemical markers of liver fibrosis: a comparison with historical features in patients with chronic hepatitis C. *Am J Gastroenterol* 2002; **97**: 2419-2425
  - 21 **Rossi E**, Adams L, Prins A, Bulsara M, de Boer B, Garas G, MacQuillan G, Speers D, Jeffrey G. Validation of the FibroTest biochemical markers score in assessing liver fibrosis in hepatitis C patients. *Clin Chem* 2003; **49**: 450-454
  - 22 **Myers RP**, Tainturier MH, Ratziu V, Piton A, Thibault V, Imbert-Bismut F, Messous D, Charlotte F, Di Martino V, Benhamou Y, Poynard T. Prediction of liver histological lesions with biochemical markers in patients with chronic hepatitis B. *J Hepatol* 2003; **39**: 222-230
  - 23 **Poynard T**, McHutchison J, Manns M, Myers RP, Albrecht J. Biochemical surrogate markers of liver fibrosis and activity in a randomized trial of peginterferon alfa-2b and ribavirin. *Hepatology* 2003; **38**: 481-492
  - 24 **Thuluvath PJ**, Krok KL. Noninvasive markers of fibrosis for longitudinal assessment of fibrosis in chronic liver disease: are they ready for prime time? *Am J Gastroenterol* 2006; **101**: 1497-1499
  - 25 **Sandrin L**, Fourquet B, Hasquenoph JM, Yon S, Fournier C, Mal F, Christidis C, Ziol M, Poulet B, Kazemi F, Beaugrand M, Palau R. Transient elastography: a new noninvasive method for assessment of hepatic fibrosis. *Ultrasound Med Biol* 2003; **29**: 1705-1713
  - 26 **Foucher J**, Vergniol J, Castira L, Le Bail B, Chanteloup E, Adhoute X, Darriet M, Bertet J, Couzigou P, de Ledinghen V. Fibrosis evaluation in chronic liver diseases: comparison of FibroScan with liver biopsy, FibroTest, Forns score, APRI, hyaluronan, prothrombin time and AST/ALT ratio. 40-th Annual Meeting of the EASL, April 13-17, 2004, Paris, France
  - 27 **Castera L**, Vergniol J, Foucher J, Le Bail B, Chanteloup E, Haaser M, Darriet M, Couzigou P, De Ledinghen V. Prospective comparison of transient elastography, Fibrotest, APRI, and liver biopsy for the assessment of fibrosis in chronic hepatitis C. *Gastroenterology* 2005; **128**: 343-350
  - 28 **Colletta C**, Smirne C, Fabris C, Toniutto P, Rapetti R, Minisini R, Pirisi M. Value of two noninvasive methods to detect progression of fibrosis among HCV carriers with normal aminotransferases. *Hepatology* 2005; **42**: 838-845
  - 29 **Marcellin P**, De Ledinghen V, Dhumeaux D, Poupon R, Ziol M, Bedossa P, Beaugrand M. Non-invasive assessment of liver fibrosis in chronic hepatitis B using FibroScan. Poster to 56th Annual Meeting of AASLD, 2005, nov. 11-15, San Francisco, USA
  - 30 **Pares A**, Caballeria L, Lazaro E, Garcia-Criado MA, Navasa M, Gilabert R. Transient elastography: a new and useful non-invasive method for assessing liver damage progression in primary biliary cirrhosis. Proceedings of the 56th Annual Meeting of AASLD, 2005, nov. 11-15, San Francisco, USA. Abstract 679. *Hepatology* 2005, **42** Suppl 1: 464A. Available from: URL: <http://www3.interscience.wiley.com/cgi-bin/fulltext/112099834/PDFSTART>
  - 31 **Grant A**, Neuberger J. Guidelines on the use of liver biopsy in clinical practice. British Society of Gastroenterology. *Gut* 1999; **45** Suppl 4: IV1-IV11
  - 32 **European Association for the Study of the Liver**. Consensus statement. EASL: International consensus Conference in hepatitis C. *J Hepatol* 1999; **26** Suppl: 2S-10S
  - 33 **Abdi W**, Millan JC, Mezey E. Sampling variability on percutaneous liver biopsy. *Arch Intern Med* 1979; **139**: 667-669
  - 34 **Ratziu V**, Charlotte F, Heurtier A, Gombert S, Giral P, Bruckert E, Grimaldi A, Capron F, Poynard T. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. *Gastroenterology* 2005; **128**: 1898-1906
  - 35 **Guido M**, Ruge M. Liver biopsy sampling in chronic viral hepatitis. *Semin Liver Dis* 2004; **24**: 89-97
  - 36 **Schiano TD**, Azeem S, Bodian CA, Bodenheimer HC Jr, Merati S, Thung SN, Hytioglou P. Importance of specimen size in accurate needle liver biopsy evaluation of patients with chronic hepatitis C. *Clin Gastroenterol Hepatol* 2005; **3**: 930-935
  - 37 **Sporea I**, Popescu A, Stirli R, Danila M, Strain M. Ultrasound assisted liver biopsy for the staging of diffuse chronic hepatopathies. *Rom J Gastroenterol* 2004; **13**: 287-290
  - 38 **Cadranel JF**, Rufat P, Degos F. Practices of liver biopsy in France: results of a prospective nationwide survey. For the Group of Epidemiology of the French Association for the Study of the Liver (AFEF). *Hepatology* 2000; **32**: 477-481
  - 39 **Cadranel JF**, Rufat P, Degos F. [Practices of transcutaneous liver biopsies in France. Results of a retrospective nationwide study] *Gastroenterol Clin Biol* 2001; **25**: 77-80
  - 40 **Adams T**, Lewis JH. Percutaneous liver biopsy. *Clin Perspectives Gastroenterol* 2002; **2**: 117-121
  - 41 **Muir AJ**, Trotter JF. A survey of current liver biopsy practice patterns. *J Clin Gastroenterol* 2002; **35**: 86-88
  - 42 **Anonymous**-The blue book of the European Board of Gastroenterology. Available from: URL: <http://www.gastrohep.com/eums/UEMS1.pdf>
  - 43 **Vautier G**, Scott B, Jenkins D. Liver biopsy: blind or guided? *BMJ* 1994; **309**: 1455-1456
  - 44 **Pasha T**, Gabriel S, Therneau T, Dickson ER, Lindor KD. Cost-effectiveness of ultrasound-guided liver biopsy. *Hepatology* 1998; **27**: 1220-1226
  - 45 **Younossi ZM**, Teran JC, Ganiats TG, Carey WD. Ultrasound-guided liver biopsy for parenchymal liver disease: an economic analysis. *Dig Dis Sci* 1998; **43**: 46-50
  - 46 **Farrell RJ**, Smiddy PF, Pilkington RM, Tobin AA, Mooney EE, Temperley IJ, McDonald GS, Bowmer HA, Wilson GF, Kelleher D. Guided versus blind liver biopsy for chronic hepatitis C: clinical benefits and costs. *J Hepatol* 1999; **30**: 580-587
  - 47 **Riley TR 3rd**. How often does ultrasound marking change the liver biopsy site? *Am J Gastroenterol* 1999; **94**: 3320-3322
  - 48 **Piccinino F**, Sagnelli E, Pasquale G, Giusti G. Complications following percutaneous liver biopsy. A multicentre retrospective study on 68,276 biopsies. *J Hepatol* 1986; **2**: 165-173
  - 49 **de Man RA**, van Buuren HR, Hop WC. A randomised study on the efficacy and safety of an automated Tru-Cut needle for percutaneous liver biopsy. *Neth J Med* 2004; **62**: 441-445
  - 50 **Lindor KD**, Bru C, Jorgensen RA, Rakela J, Bordas JM, Gross JB, Rodes J, McGill DB, Reading CC, James EM, Charboneau JW, Ludwig J, Batts KP, Zinsmeister AR. The role of ultrasonography and automatic-needle biopsy in outpatient percutaneous liver biopsy. *Hepatology* 1996; **23**: 1079-1083
  - 51 **Gazelle GS**, Haaga JR, Rowland DY. Effect of needle gauge, level of anticoagulation, and target organ on bleeding associated with aspiration biopsy. Work in progress. *Radiology* 1992; **183**: 509-513
  - 52 **Plecha DM**, Goodwin DW, Rowland DY, Varnes ME, Haaga JR. Liver biopsy: effects of biopsy needle caliber on bleeding and tissue recovery. *Radiology* 1997; **204**: 101-104
  - 53 **Maharaj B**, Bhoora IG. Complications associated with percutaneous needle biopsy of the liver when one, two or



- three specimens are taken. *Postgrad Med J* 1992; **68**: 964-967
- 54 **Firpi RJ**, Soldevila-Pico C, Abdelmalek MF, Morelli G, Judah J, Nelson DR. Short recovery time after percutaneous liver biopsy: should we change our current practices? *Clin Gastroenterol Hepatol* 2005; **3**: 926-929
- 55 **Gilmore IT**, Burroughs A, Murray-Lyon IM, Williams R, Jenkins D, Hopkins A. Indications, methods, and outcomes of percutaneous liver biopsy in England and Wales: an audit by the British Society of Gastroenterology and the Royal College of Physicians of London. *Gut* 1995; **36**: 437-441
- 56 **Chevallier P**, Ruitort F, Denys A, Staccini P, Saint-Paul MC, Ouzan D, Motamedi JP, Tran A, Schnyder P, Bruneton JN. Influence of operator experience on performance of ultrasound-guided percutaneous liver biopsy. *Eur Radiol* 2004; **14**: 2086-2091
- 57 **Poynard T**, Ratziu V, Bedossa P. Appropriateness of liver biopsy. *Can J Gastroenterol* 2000; **14**: 543-548
- 58 **McGill DB**, Rakela J, Zinsmeister AR, Ott BJ. A 21-year experience with major hemorrhage after percutaneous liver biopsy. *Gastroenterology* 1990; **99**: 1396-1400
- 59 **Douds AC**, Joseph AE, Finlayson C, Maxwell JD. Is day case liver biopsy underutilised? *Gut* 1995; **37**: 574-575
- 60 **Spiezia S**, Salvio A, Di Somma C, Scelzi C, Assanti AP, Giannattasio F, Varriale M, Visconti M. The efficacy of liver biopsy under ultrasonographic guidance on an outpatient basis. *Eur J Ultrasound* 2002; **15**: 127-131

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# Updating magnetic resonance imaging of small bowel: Imaging protocols and clinical indications

Jiong Zhu, Jian-Rong Xu, Hong-Xia Gong, Yan Zhou

Jiong Zhu, Jian-Rong Xu, Hong-Xia Gong, Yan Zhou, Department of Radiology, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200127, China  
Author contributions: Zhu J and Xu JR contributed equally to this work.

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Correspondence to: Jian-Rong Xu, MD, Professor of Radiology, Department of Radiology, Ren Ji Hospital, Shanghai Jiao Tong University School of Medicine, 1630 Dong Fang Road, Shanghai 200127, China. [xujianr@hotmail.com](mailto:xujianr@hotmail.com)  
Telephone: +86-21-58752345

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## INTRODUCTION

Magnetic resonance imaging (MRI) of the small bowel has been an unexplored field of application for years. Since 1998 the number of the publications has started to increase<sup>[1-6]</sup>. The reason was not lack of interest, but the technical inadequacy of the MR scanners to perform motion-free examinations. With the development of hardware (gradients, multi-channel coils) and software (fast and ultrafast sequences), which enabled breath-held studies, freezing voluntary (respiratory) and involuntary (peristaltic) motion artifacts, it opened the access to modern abdominal MRI.

High soft tissue contrast resolution, acquisition of multi-planar images and the possibility to obtain functional information make MR an interesting imaging technique to evaluate the small bowel disease. The absence of ionizing radiation is an important feature of MRI examinations because inflammatory diseases such as Crohn's disease (CD) are studied most frequently, which are prevalent among children and young adults<sup>[7-9]</sup>.

The major advantage of MRI, compared with conventional barium radiographic studies, is direct visualization of small bowel wall. This feature dramatically changes the image interpretation process. Radiologists must shift their attention from analysis of mucosal profile and lumen caliber to direct evaluation of bowel wall thickness and parietal inflammatory changes.

## IMAGING PROTOCOLS

### Small bowel distension

Bowel distension is a most important requisite for any method of the small bowel. A collapsed bowel loop can hide lesions or simulate pathologic wall thickening. The presence of the lesion that generates small bowel obstruction creates a natural distention of lumen and the possibility of examining the patient without any preparation<sup>[6,10-11]</sup>. In contrast, the relative collapse of bowel loops under standard conditions has led researchers to study a variety of methods of luminal distension.

## Abstract

High soft tissue contrast resolution, acquisition of multi-planar images and the possibility to obtain functional information make magnetic resonance an interesting imaging technique to evaluate the small bowel disease. The absence of ionizing radiation is an important feature of magnetic resonance imaging (MRI) examinations because inflammatory diseases such as Crohn's disease (CD) are studied most frequently, which are prevalent among children and young adults. MRI, using modern equipment and a rigorous technical approach, can offer detailed morphologic information and functional data on the small bowel. This article discusses the MRI protocols for small bowel and the MR imaging findings of small bowel diseases, such as CD and small bowel neoplasms.

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**Key words:** Magnetic resonance imaging; Small bowel; Crohn's; Neoplasm

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There are two main approaches for MRI of the small bowel: (1) study following oral administration of contrast material; and (2) study with distension of lumen obtained with contrast material that is introduced through a naso-jejunal tube (MR enteroclysis).

### **Oral contrast agents for small bowel MRI**

Oral contrast agents can be classified into positive, negative and biphasic categories according to their action on the signal intensity of bowel lumen.

A positive agent is a paramagnetic substance that produces a high signal intensity on T1-weighted sequences. It reduces T1 relaxation time without, or only minimally, influencing T2 relaxation time. Because of the water content of the contrast solution, it also results in high signal intensity on T2-weighted images. Positive contrast agents include paramagnetic substances, such as gadolinium chelates, ferrous and manganic ions and manganese ions<sup>[12-16]</sup>. The use of positive oral contrast agents has been abandoned almost completely because a hyperintense lumen does not enable a clear differentiation with inflammatory parietal enhancement.

A negative agent is a substance that produces a low signal intensity on T1- and T2-weighted sequences. These substances induce local inhomogeneity in the magnetic field that affects T1 and T2 relaxation time. T2 effects predominate and are caused by spin dephasing with a consequent loss of signal intensity. Negative contrast agents include perfluorooctyl bromide<sup>[17]</sup>, iron oxides<sup>[15,18]</sup>, and oral magnetic particles<sup>[14,15]</sup>. Barium sulfate, if used at high concentrations, can be considered a negative contrast agent<sup>[19]</sup>. Negative contrast agents are more favorable if hyperintense signal of the bowel wall and the surrounding fat tissue signs of acute inflammation have to be detected on T2-weighted sequences<sup>[15]</sup>. However, magnetic susceptibility on gradient echo sequences may alter image quality on breath-held T1-weighted images.

The term “biphasic” recently was introduced to define those substances that show different signal intensities depending on different sequences<sup>[20]</sup>. The first group (hyperintense signal on T1-weighted images and hypointense signal on T2-weighted images) included manganese and substances that contain manganese, and gadolinium chelates, which can act as biphasic contrast agents if administered at high concentrations<sup>[15]</sup>. The second group (hypointense signal on T1-weighted images and hyperintense signal on T2-weighted images) included water, hyperosmolar and isosmolar watery solutions, and barium sulfate<sup>[15]</sup>. Although water is the safest and cheapest agent, it has the limitation of intestinal absorption, which compromises an adequate distension of distal ileum in many patients<sup>[21]</sup>. To obviate this problem, hyperosmolar solutions, such as mannitol-based solutions, have been used. Mannitol reduces water absorption and distends the distal ileal loops well. Major drawbacks are undesirable side effects, such as diarrhea, meteorism and abdominal cramps<sup>[21-25]</sup>.

In an attempt to reduce undesirable side effects and to obtain better distension of the distal ileum, some new oral mixtures, such as Polyethylene glycol, a water

solution combined with low concentration sorbitol and locus bean gum (LBG), were used as the oral contrast agents<sup>[19,26,27]</sup>. They are all hyperosmolar. Some of them can reduce the side effect and ensure optimal intestinal distension with appropriate concentration and reasonable transit time.

### **Magnetic resonance enteroclysis**

MR enteroclysis (MRE) is an emerging technique for the evaluation of small intestinal diseases. Administration of an iso-osmotic water solution through a nasojejunal catheter can guarantee adequate luminal distention, and in combination with ultrafast sequences, such as single shot TSE, true FISP, HASTE and 3D FLASH, resulting in excellent anatomic demonstration of the small intestine. MR fluoroscopy can be performed during MRE examination to monitor the filling process and might be useful in studying low-grade stenosis or motility related disorders. MRE is a very promising technique for the detection and characterization of involved small bowel segments in patients with Crohn's disease while its diagnostic performance in disclosing lumen narrowing and extramural manifestations and complications of the disease is outstanding. Initial experience shows that MRE is very efficient in the diagnosis of small bowel tumors and can be used in the evaluation of small bowel obstruction<sup>[20,28,29]</sup>.

### **Sequences**

Fast sequences that are able to acquire T1- and T2-weighted images within a single breath-hold are essential requisites for MRI evaluation of small bowel. In T2-weighted images, several studies<sup>[11,5,6,28,29]</sup> support the validity of single-shot sequences, including half-Fourier single-shot turbo spin-echo (HASTE) and single-shot fast spin-echo. Because these sequences, based on the half-Fourier reconstruction technique, have extremely fast acquisition time (approximately 1 second per image), they are able to freeze motion artifacts. Single-shot sequences differ from each other depending on echo time (TE). Using long TE (e.g. about 600 m) can obtain selective images of fluids with cancellation of surrounding organs (similar to magnetic resonance cholangiopancreatography). Using shorter TE (60-90 m) can obtain simultaneous evaluation of fluids, bowel wall and surrounding structures. The use of fat saturation pulses is a useful complement to the acquisition of T2-weighted sequences<sup>[2,13,14,29-31]</sup>. Fat saturation causes an increase in contrast between bowel wall and the surrounding fat tissue. This can help assess the bowel wall inflammation and identify the inflammatory changes in peritoneal fat tissue.

The “balanced” or “hybrid” gradient-echo sequence has been introduced in clinical practice. This sequence, known as true fast imaging with steady-state precession (true-FISP), presents with an intermediate contrast between T1- and T2-weighted images<sup>[29,31-33]</sup>. Shorter repetition times (TR) are used (< 3 m) and the acquisition time is short. The true FISP sequence provides motion-free, high-resolution images similar to T2-weighted images of the intestine, mesentery and



**Figure 1** A 36-year-old man with Crohn's disease, the small bowel thickness exceeds 4-5 mm on T2W image, and stratified appearance (so-called "target" or "double halo" appearance) can be seen.

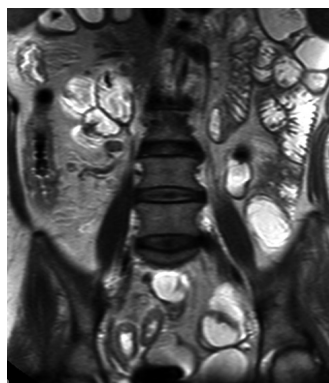


**Figure 2** A 25-year-old man with Crohn's disease and inflammation of ileocecal junction. T2W image shows "double halo" appearance (arrows) of thickened (8 mm) bowel wall.

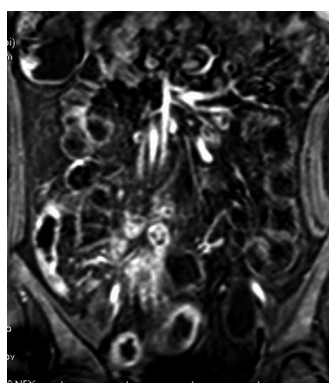
vasculature in 1.5 s. However, this sequence is prone to susceptibility artifacts from intraluminal air and from "black boundary" artifact due to the chemical shift phenomenon, which may obscure the subtle bowel wall thickening. The black boundary artifact can be eliminated with use of fat suppression.

T1-weighted images are obtained with fast spoiled-gradient-echo sequences, using 2-D or 3-D acquisition. The acquisition time ranges from 15 to 20 seconds. T1-weighted sequences generally are used following intravenous injection of contrast material to evaluate enhancement, a useful parameter to assess disease activity, especially the inflammatory activity. T1-weighted images also benefit from the use of fat saturation. The gadolinium-enhanced fat-suppressed spoiled gradient-echo sequence provides T1-weighted images with excellent visualization of the enhanced bowel wall, which contrasts well with the low-signal-intensity mesenteric fat and negative intraluminal contrast material<sup>[34,35]</sup>.

The latest technical development to speed up the acquisition process is parallel imaging, based on simultaneous acquisition of spatial harmonics, or sensitivity encoding techniques. Marked improvement in spatial resolution can be achieved in shorter acquisition times<sup>[36]</sup>. Parallel imaging can reduce the number of phase encodings to be acquired per TR. Consequently, the spatial resolution can be increased while maintaining an acquisition time that is compatible with a single breath-hold, or the number of scans to be acquired, which allows a larger volume coverage. Alternatively,



**Figure 3** A 42-year-old man with Crohn's disease, T2W image shows skip lesions of ileum with thickened (7 mm) bowel wall.



**Figure 4** A 36-year-old woman with Crohn's disease, the bowel wall of the involved segment has a homogeneous enhancement at CE-T1W image. And the "comb sign" also can be seen.

the acquisition time can be reduced drastically. The drawback in the use of parallel imaging is the reduction of signal-to-noise ratio (SNR) and the need to perform a calibration of equipment immediately before image acquisition<sup>[37]</sup>.

## CLINICAL INDICATIONS

### **Inflammatory bowel disease (CD) (Figures 1-4)**

Chronic inflammatory disease, and in particular, CD, represents the most common application of MRI of small bowel<sup>[1-5,8,9,15,28-35,38]</sup>.

### **Imaging findings**

With MRI, both inflammatory changes of the bowel wall and extramural complications of Crohn's disease can be assessed. The non-invasiveness of this technique, as well as its lack of ionizing radiation, has prompted many radiologists to perform systematic studies of MRI for evaluation of Crohn's disease.

In patients having proved suspected CD, cross-sectional images, including CT and MRI, should be analyzed specifically for the presence and character of a pathologically altered bowel segment (wall thickness, pattern of attenuation, degree of enhancement, length of involvement), stenosis and prestenotic dilatation, skip lesions, fistulas, abscess, fibrofatty proliferation, increased vascularity of the vasa recta (comb sign), mesenteric adenopathy, and other extraintestinal disease involvement.

The normal small bowel wall thickness is between 1 mm and 3 mm when the lumen is well distended. Any portion of the bowel wall that exceeds 4-5 mm is



considered abnormal<sup>[19,12,17,19,26,29,39,40]</sup>. An adequate intestinal distension is mandatory because collapsed loops or spastic intestinal segments may mimic wall thickening. Most optimal distension is obtained with MR enteroclysis with instillation of contrast medium after nasojejunal intubation under fluoroscopic guidance. Although many authors reporting on MR enteroclysis administer antiperistaltic drugs to reduce motion artifacts, reflex atony is induced by high flow rates, theoretically allowing images (almost) free of motion artifacts<sup>[41,42]</sup>. Drawbacks are that this technique is uncomfortable for patients and exposes them to a considerable dose of ionizing radiation of up to 8 mSv during intubation<sup>[43]</sup>. To avoid such disadvantages, MRI has been performed by many researchers using oral contrast media. Many contrast media have been proposed, but no oral contrast medium has yet been accepted universally as optimal for use<sup>[2,3,7,14-19,22-26]</sup>.

Small bowel wall thickening is a sensitive, but not pathognomonic, sign of CD. It is observed in several other intestinal diseases, such as ischemic disorders and infections.

Although superficial mucosal lesions are missed easily as a result of inadequate spatial resolution, MR imaging can detect early inflammatory changes of the bowel wall, based on enhancement after intravenous injection of contrast medium. The bowel wall of the involved segment may have a homogeneous or stratified appearance at MR imaging after enhancement. The homogeneous enhancement is diffuse and transmural with no recognition of different bowel layers. The stratified appearance (so-called "target" or "double halo" appearance) is related to alternating layers of higher or lower attenuation or signal intensity. The stratified appearance also can be seen on T2-weighted imaging. "Target" or "double halo" appearance is often seen in active lesions, particularly after the intravenous administration of contrast medium, and related to submucosal edema. The intensity of enhancement correlates with the degree of inflammatory lesion activity. Inactive disease is characterized by no abnormalities or bowel wall thickening with relative low signal intensity representing fibrosis with limited, homogeneous contrast enhancement. Absence of stratification on T2-weighted images with stratified enhancement on T1-weighted images is often due to fibrosis, which is a typical long-standing CD<sup>[29,33,35,44]</sup>. This sign (stratified enhancement) also can be seen on MSCT (multi-slice CT) images<sup>[45,46]</sup>.

Increased vascularity of the vasa recta (comb sign) is a sign of active inflammation. It arises from the combination of vascular engorgement of vasa recta and fibro-fatty proliferation and is demonstrated as multiple tubular, tortuous opacities on mesenteric side of ileum, aligned as the teeth of a comb<sup>[29]</sup>. "Comb sign" is frequently seen on enhanced MSCT images<sup>[46,47]</sup>. Abscess and phlegmon can occur in the small bowel mesentery, abdominal wall, or psoas muscle or around the anus. Abscesses and phlegmon are well demonstrated at fat-saturated T2-weighted MR imaging and can be distinguished reliably, which aids in management planning. Fistulas and sinus tracts are also depicted, however, the

reported sensitivity of MR imaging for depicting sinus tracts is 50%-75% when a conventional enteroclysis study is used as a reference<sup>[32,39,45]</sup>. Mesenteric lymphadenopathy ranging from 3 to 8 mm in size is depicted at MR imaging with a true fast imaging with steady state precession (FISP) or T2-weighted turbo spin-echo sequence<sup>[32,41]</sup>. If these sequences are not available, axial T1- or T2-weighted spin-echo imaging should be added. When lymph nodes are larger than 10 mm, lymphoma and carcinoma must be excluded.

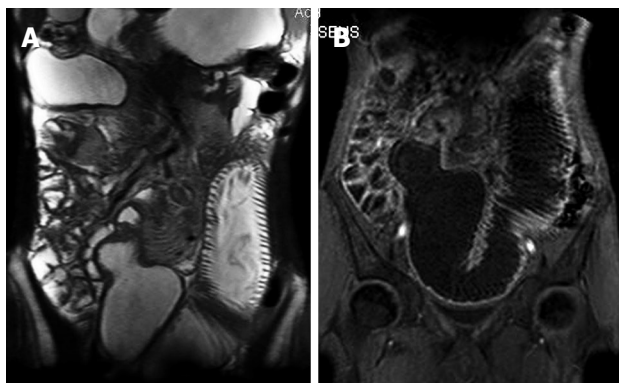
### Assessment of disease activity

Imaging techniques form a very important part of the evaluation of CD. However, several clinical scoring systems have been developed as well to assess disease activity and response to therapy, especially in trials. The Crohn's disease activity index (CDAI) is currently the gold standard for clinical evaluation of disease activity<sup>[47,48]</sup>. This index is relatively subjective since an important part of the total score is derived from items that reflect the patient's perception of disease (general well being and "intensity of abdominal pain"). However, in many studies, this index has been used as gold standard for disease activity since it is a validated and extensively used clinical index. Scores ranging from 0 to approximately 600 with values below 150 are considered as remission and values over 150 as active disease. MR imaging was used to evaluate disease activity<sup>[9,30-35,38,41,44,48,49]</sup>. Based on different experiences, contrast-enhanced (CE) fat-suppressed T1-weighted images offer the best correlation between MR findings and CDAI, although a correlation that used fat-suppressed T2-weighted images is also demonstrated. MRI can clearly distinct pathologic from normal bowel wall in CD, as it detects significant variations in bowel wall thickness with clinical improvement and is able to reflect pathologic inflammatory changes at the bowel wall based on variations in the CE. In most patients with active disease, abnormal bowel identified on MR imaging was isointense or slightly hypointense to the psoas muscles on T1-weighted imaging. On T2-weighted imaging, the abnormal bowel segments were usually isointense or slightly hyperintense compared with the psoas muscle. MR imaging can correctly identify active disease, the enhancement pattern of abnormal bowel is diffuse and layered. The layered pattern is seen only in patients with active disease. Consequently, this technique is reliably applicable to the follow-up of patients with CD. MRI is able to detect significant variations in bowel wall thickness and contrast enhancement (CE), reflecting favorable clinical response to medical treatment of CD's relapse<sup>[30,34,49]</sup>.

## NEOPLASMS (Figure 5)

### Benign masses

Benign and malignant small intestinal tumors are uncommon. Adenomas, leiomyomas and lipomas constitute the three most common primary benign small intestinal tumors<sup>[50]</sup>. In general, benign tumors occur less commonly in the duodenum and increase in frequency



**Figure 5** A: A 68-year-old man with adenocarcinomas, T2W image shows the tumor with similar signal intensity, the proximate jejunum dilating conspicuously; B: The same patient, tumor shows heterogeneous enhancement greater than adjacent bowel on gadolinium-enhanced image.

in the ileum. The term “polyp” is a clinical term for any tumorous mass that projects above the surrounding normal mucosa. Hamartomatous, hyperplastic and inflammatory polyps are benign, non-neoplastic lesions and adenomatous polyps are true neoplastic tumors containing dysplastic epithelium and are precursors of carcinoma. Polyps are infrequently symptomatic and are usually incidental findings at autopsy. Current convention is that leiomyomas should be classified as gastrointestinal stromal tumors (GIST), and benignancy can never be determined with absolute certainty. Small bowel GIST accounts for 25% of these tumors. As in the stomach, these may be large and ulcerating.

### Malignant masses

Adenocarcinomas account for 50% of all small bowel malignancies, but only account for less than 1% of all gastrointestinal malignancies<sup>[51]</sup>. The most common site for small bowel adenocarcinoma is the duodenum. This tumor frequently occurs in close proximity to the ampulla and as a result may cause obstructive jaundice<sup>[52]</sup>. Adenocarcinoma and metastases can be seen rarely in the jejunum.

Most primary gastrointestinal non-Hodgkin lymphomas are of B-cell type, and appear to arise from B cells of mucosa-associated lymphoid tissue (MALT). In the small intestine, the terminal ileum is the most common site affected, which may reflect the relatively greater amount of lymphoid tissue present in this segment compared with the duodenum and jejunum.

Carcinoids are the most common primary neoplasm of the small bowel. They are well-differentiated neuroendocrine neoplasms that occur primarily in the distal ileum. Men and women are affected with equal frequency. Most patients present with tumor-related symptoms of bleeding and bowel obstruction or intussusception. Ileal carcinoids are regional mesenteric metastases and vascular sclerosis. The primary tumor may be quite small with the accompanying lymphadenopathy and desmoplastic reaction in the root of the mesentery presenting as the only visible manifestation of disease. Liver metastases are responsible for the “carcinoid syndrome”,

which is characterized by vasomotor instability, intestinal hypomotility and bronchoconstriction<sup>[53]</sup>.

### Imaging findings

Tumors had similar signal intensity to normal small bowel on precontrast images. Tumors showed heterogeneous enhancement greater than adjacent bowel on gadolinium-enhanced images. Tumor local extent was best shown on precontrast-spoiled gradient-echo images and postgadolinium T1-weighted fat-suppressed images. Image quality was most consistent on breath-hold images. Precontrast breath-hold T1-weighted spoiled gradient-echo images and gadolinium-enhanced fat suppressed images demonstrate tumor extent most reliably. The accuracy of the technique in cases of non-occlusive tumors of the lumen is not known, given the lack of large case series<sup>[28,54]</sup>.

## CONCLUSION

MR imaging, using modern equipment and a rigorous technical approach, can offer detailed morphologic information and functional data on the small bowel. The optimal study technique is debatable, although the oral administration of contrast material as a first-line approach is less expensive, faster, easier to perform and better tolerated by patients. MR enteroclysis might be reserved for selected cases as a second-line study.

The major clinical indication is the evaluation of patients who have suspected or known CD. The absence of ionizing radiation, in view of the young age of most of the patients and the frequency of the examinations, is an important advantage over other techniques (radiography and CT enteroclysis).

## REFERENCES

- 1 Ernst O, Asselah T, Cablan X, Sergent G. Breath-hold fast spin-echo MR imaging of Crohn's disease. *AJR Am J Roentgenol* 1998; **170**: 127-128
- 2 Rieber A, Wruk D, Nussle K, Aschoff AJ, Reinshagen M, Adler G, Brambs HJ, Tomczak R. [MRI of the abdomen combined with enteroclysis in Crohn disease using oral and intravenous Gd-DTPA] *Radiologie* 1998; **38**: 23-28
- 3 Holzknecht N, Helmberger T, von Ritter C, Gauger J, Faber S, Reiser M. [MRI of the small intestine with rapid MRI sequences in Crohn disease after enteroclysis with oral iron particles] *Radiologie* 1998; **38**: 29-36
- 4 Ha HK, Lee EH, Lim CH, Shin YM, Jeong YK, Yoon KH, Lee MG, Min YI, Auh YH. Application of MRI for small intestinal diseases. *J Magn Reson Imaging* 1998; **8**: 375-383
- 5 Lee JK, Marcos HB, Semelka RC. MR imaging of the small bowel using the HASTE sequence. *AJR Am J Roentgenol* 1998; **170**: 1457-1463
- 6 Regan F, Beall DP, Bohlman ME, Khazan R, Sufi A, Schaefer DC. Fast MR imaging and the detection of small-bowel obstruction. *AJR Am J Roentgenol* 1998; **170**: 1465-1469
- 7 Adamek HE, Breer H, Karschkes T, Albert J, Riemann JF. Magnetic resonance imaging in gastroenterology: time to say good-bye to all that endoscopy? *Endoscopy* 2000; **32**: 406-410
- 8 Wong SH, Wong VW, Sung JJ. Virtual colonoscopy-induced perforation in a patient with Crohn's disease. *World J Gastroenterol* 2007; **13**: 978-979

- 9 **Saibeni S**, Rondonotti E, Iozzelli A, Spina L, Tontini GE, Cavallaro F, Ciscato C, de Franchis R, Sardanelli F, Vecchi M. Imaging of the small bowel in Crohn's disease: a review of old and new techniques. *World J Gastroenterol* 2007; **13**: 3279-3287
- 10 **Chou CK**, Liu GC, Chen LT, Jaw TS. The use of MRI in bowel obstruction. *Abdom Imaging* 1993; **18**: 131-135
- 11 **Beall DP**, Fortman BJ, Lawler BC, Regan F. Imaging bowel obstruction: a comparison between fast magnetic resonance imaging and helical computed tomography. *Clin Radiol* 2002; **57**: 719-724
- 12 **Hirohashi S**, Uchida H, Yoshikawa K, Fujita N, Ohtomo K, Yuasa Y, Kawamura Y, Matsui O. Large scale clinical evaluation of bowel contrast agent containing ferric ammonium citrate in MRI. *Magn Reson Imaging* 1994; **12**: 837-846
- 13 **Rieber A**, Nussle K, Reinshagen M, Brambs HJ, Gabelmann A. MRI of the abdomen with positive oral contrast agents for the diagnosis of inflammatory small bowel disease. *Abdom Imaging* 2002; **27**: 394-399
- 14 **Vlahos L**, Gouliamos A, Athanasopoulou A, Kotoulas G, Claus W, Hatzioannou A, Kalovidouris A, Papavasiliou C. A comparative study between Gd-DTPA and oral magnetic particles (OMP) as gastrointestinal (GI) contrast agents for MRI of the abdomen. *Magn Reson Imaging* 1994; **12**: 719-726
- 15 **Rieber A**, Aschoff A, Nussle K, Wruk D, Tomczak R, Reinshagen M, Adler G, Brambs HJ. MRI in the diagnosis of small bowel disease: use of positive and negative oral contrast media in combination with enteroclysis. *Eur Radiol* 2000; **10**: 1377-1382
- 16 **Hiraishi K**, Narabayashi I, Fujita O, Yamamoto K, Sagami A, Hisada Y, Saika Y, Adachi I, Hasegawa H. Blueberry juice: preliminary evaluation as an oral contrast agent in gastrointestinal MR imaging. *Radiology* 1995; **194**: 119-123
- 17 **Brown JJ**, Duncan JR, Heiken JP, Balfe DM, Corr AP, Mirowitz SA, Eilenberg SS, Lee JK. Perfluorooctylbromide as a gastrointestinal contrast agent for MR imaging: use with and without glucagon. *Radiology* 1991; **181**: 455-460
- 18 **Johnson WK**, Stoupis C, Torres GM, Rosenberg EB, Ros PR. Superparamagnetic iron oxide (SPIO) as an oral contrast agent in gastrointestinal (GI) magnetic resonance imaging (MRI): comparison with state-of-the-art computed tomography (CT). *Magn Reson Imaging* 1996; **14**: 43-49
- 19 **Ajaj W**, Goyen M, Schneemann H, Kuehle C, Nuefer M, Ruehm SG, Goehde SC, Lauenstein TC. Oral contrast agents for small bowel distension in MRI: influence of the osmolarity for small bowel distention. *Eur Radiol* 2005; **15**: 1400-1406
- 20 **Maglinte DD**, Siegelman ES, Kelvin FM. MR enteroclysis: the future of small-bowel imaging? *Radiology* 2000; **215**: 639-641
- 21 **Lomas DJ**, Graves MJ. Small bowel MRI using water as a contrast medium. *Br J Radiol* 1999; **72**: 994-997
- 22 **Grubnic S**, Padhani AR, Revell PB, Husband JE. Comparative efficacy of and sequence choice for two oral contrast agents used during MR imaging. *AJR Am J Roentgenol* 1999; **173**: 173-178
- 23 **Ajaj W**, Goehde SC, Schneemann H, Ruehm SG, Debatin JF, Lauenstein TC. Dose optimization of mannitol solution for small bowel distension in MRI. *J Magn Reson Imaging* 2004; **20**: 648-653
- 24 **Kuehle CA**, Ajaj W, Ladd SC, Massing S, Barkhausen J, Lauenstein TC. Hydro-MRI of the small bowel: effect of contrast volume, timing of contrast administration, and data acquisition on bowel distention. *AJR Am J Roentgenol* 2006; **187**: W375-W385
- 25 **Lauenstein TC**, Schneemann H, Vogt FM, Herborn CU, Ruhm SG, Debatin JF. Optimization of oral contrast agents for MR imaging of the small bowel. *Radiology* 2003; **228**: 279-283
- 26 **Ajaj W**, Goehde SC, Schneemann H, Ruehm SG, Debatin JF, Lauenstein TC. Oral contrast agents for small bowel MRI: comparison of different additives to optimize bowel distension. *Eur Radiol* 2004; **14**: 458-464
- 27 **McKenna DA**, Roche CJ, Murphy JM, McCarthy PA. Polyethylene glycol solution as an oral contrast agent for MRI of the small bowel in a patient population. *Clin Radiol* 2006; **61**: 966-970
- 28 **Umschaden HW**, Szolar D, Gasser J, Umschaden M, Haselbach H. Small-bowel disease: comparison of MR enteroclysis images with conventional enteroclysis and surgical findings. *Radiology* 2000; **215**: 717-725
- 29 **Wiarda BM**, Kuipers EJ, Heitbrink MA, van Oijen A, Stoker J. MR Enteroclysis of inflammatory small-bowel diseases. *AJR Am J Roentgenol* 2006; **187**: 522-531
- 30 **Low RN**, Sebrechts CP, Politoske DA, Bennett MT, Flores S, Snyder RJ, Pressman JH. Crohn disease with endoscopic correlation: single-shot fast spin-echo and gadolinium-enhanced fat-suppressed spoiled gradient-echo MR imaging. *Radiology* 2002; **222**: 652-660
- 31 **Albert JG**, Martiny F, Krummenerl A, Stock K, Lesske J, Gobel CM, Lotterer E, Nietsch HH, Behrmann C, Fleig WE. Diagnosis of small bowel Crohn's disease: a prospective comparison of capsule endoscopy with magnetic resonance imaging and fluoroscopic enteroclysis. *Gut* 2005; **54**: 1721-1727
- 32 **Prassopoulos P**, Papanikolaou N, Grammatikakis J, Rousomoustakaki M, Maris T, Gourtsoyiannis N. MR enteroclysis imaging of Crohn disease. *Radiographics* 2001; **21** Spec No: S161-S172
- 33 **Furukawa A**, Saotome T, Yamasaki M, Maeda K, Nitta N, Takahashi M, Tsujikawa T, Fujiyama Y, Murata K, Sakamoto T. Cross-sectional imaging in Crohn disease. *Radiographics* 2004; **24**: 689-702
- 34 **Maccioni F**, Bruni A, Viscido A, Colaiacomo MC, Cocco A, Montesani C, Caprilli R, Marini M. MR imaging in patients with Crohn disease: value of T2- versus T1-weighted gadolinium-enhanced MR sequences with use of an oral superparamagnetic contrast agent. *Radiology* 2006; **238**: 517-530
- 35 **Florie J**, Wasser MN, Arts-Cieslik K, Akkerman EM, Siersema PD, Stoker J. Dynamic contrast-enhanced MRI of the bowel wall for assessment of disease activity in Crohn's disease. *AJR Am J Roentgenol* 2006; **186**: 1384-1392
- 36 **Zhang J**, Israel GM, Hecht EM, Krinsky GA, Babb JS, Lee VS. Isotropic 3D T2-weighted MR cholangiopancreatography with parallel imaging: feasibility study. *AJR Am J Roentgenol* 2006; **187**: 1564-1570
- 37 **Lomas DJ**. Techniques for magnetic resonance imaging of the bowel. *Top Magn Reson Imaging* 2002; **13**: 379-387
- 38 **Holzkecht N**, Helmberger T, Herrmann K, Ochsenkuhn T, Goke B, Reiser M. [MRI in Crohn's disease after transduodenal contrast administration using negative oral MRI contrast media] *Radiologe* 2003; **43**: 43-50
- 39 **Gourtsoyiannis N**, Papanikolaou N, Grammatikakis J, Prassopoulos P. MR enteroclysis: technical considerations and clinical applications. *Eur Radiol* 2002; **12**: 2651-2658
- 40 **Laghi A**, Carbone I, Catalano C, Iannaccone R, Paolantonio P, Baeli I, Trenna S, Passariello R. Polyethylene glycol solution as an oral contrast agent for MR imaging of the small bowel. *AJR Am J Roentgenol* 2001; **177**: 1333-1334
- 41 **Rieber A**, Wruk D, Potthast S, Nussle K, Reinshagen M, Adler G, Brambs HJ. Diagnostic imaging in Crohn's disease: comparison of magnetic resonance imaging and conventional imaging methods. *Int J Colorectal Dis* 2000; **15**: 176-181
- 42 **Schmidt S**, Lepori D, Meuwly JY, Duvoisin B, Meuli R, Michetti P, Felley C, Schnyder P, van Melle G, Denys A. Prospective comparison of MR enteroclysis with multidetector spiral-CT enteroclysis: interobserver agreement and sensitivity by means of "sign-by-sign" correlation. *Eur Radiol* 2003; **13**: 1303-1311
- 43 **Thoeni RF**, Gould RG. Enteroclysis and small bowel series: comparison of radiation dose and examination time.

- Radiology* 1991; **178**: 659-662
- 44 **Frokjaer JB**, Larsen E, Steffensen E, Nielsen AH, Drewes AM. Magnetic resonance imaging of the small bowel in Crohn's disease. *Scand J Gastroenterol* 2005; **40**: 832-842
- 45 **Macari M**, Megibow AJ, Balthazar EJ. A pattern approach to the abnormal small bowel: observations at MDCT and CT enterography. *AJR Am J Roentgenol* 2007; **188**: 1344-1355
- 46 **Colombel JF**, Solem CA, Sandborn WJ, Booya F, Loftus EV Jr, Harmsen WS, Zinsmeister AR, Bodily KD, Fletcher JG. Quantitative measurement and visual assessment of ileal Crohn's disease activity by computed tomography enterography: correlation with endoscopic severity and C reactive protein. *Gut* 2006; **55**: 1561-1567
- 47 **Wiarda BM**, Kuipers EJ, Houdijk LP, Tuynman HA. MR enteroclysis: imaging technique of choice in diagnosis of small bowel diseases. *Dig Dis Sci* 2005; **50**: 1036-1040
- 48 **Sandborn WJ**, Feagan BG, Hanauer SB, Lochs H, Lofberg R, Modigliani R, Present DH, Rutgeerts P, Scholmerich J, Stange EF, Sutherland LR. A review of activity indices and efficacy endpoints for clinical trials of medical therapy in adults with Crohn's disease. *Gastroenterology* 2002; **122**: 512-530
- 49 **Sempere GA**, Martinez Sanjuan V, Medina Chulia E, Benages A, Tome Toyosato A, Canelles P, Bulto A, Quiles F, Puchades I, Cuquerella J, Celma J, Orti E. MRI evaluation of inflammatory activity in Crohn's disease. *AJR Am J Roentgenol* 2005; **184**: 1829-1835
- 50 **Fenoglio-Preiser CM**, Noffsinger AE, Stemmermann GN, Lantz PE, Listrom MB, Rilke FO. *Gastrointestinal Pathology*. Philadelphia: Lippincott, 1999: 154
- 51 **Trenkner SW**, Halvorsen RA Jr, Thompson WM. Neoplasms of the upper gastrointestinal tract. *Radiol Clin North Am* 1994; **32**: 15-24
- 52 **Teplick SK**, Glick SN, Keller MS. The duodenum. In: Putnam CE, Ravin CE, eds. *Textbook of Diagnostic Imaging*. Philadelphia: Saunders, 1988: 808-846
- 53 **Rubesin SE**, Gilchrist AM, Bronner M, Saul SH, Herlinger H, Grumbach K, Levine MS, Laufer I. Non-Hodgkin lymphoma of the small intestine. *Radiographics* 1990; **10**: 985-998
- 54 **Semelka RC**, John G, Kelekis NL, Burdeny DA, Ascher SM. Small bowel neoplastic disease: demonstration by MRI. *J Magn Reson Imaging* 1996; **6**: 855-860

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BASIC RESEARCH

## Effect of honey on bacterial translocation and intestinal morphology in obstructive jaundice

Cem Gencay, Sibel Serin Kilicoglu, Kemal Kismet, Bulent Kilicoglu, Serap Erel, Sabahattin Muratoglu, Asli Elif Sunay, Esra Erdemli, Mehmet Ali Akkus

Cem Gencay, Kemal Kismet, Bulent Kilicoglu, Serap Erel, Mehmet Ali Akkus, Ankara Training and Research Hospital 4th General Surgery Department, Ankara 06340, Turkey  
Sibel Serin Kilicoglu, Ufuk University School of Medicine, Department of Histology and Embryology, Ankara 06800, Turkey

Sabahattin Muratoglu, Ankara Training and Research Hospital Microbiology Department, Ankara 06340, Turkey

Asli Elif Sunay, Food Engineer, Balpamak Pazarlama, 34760, Cekmekoy, Istanbul Turkey

Esra Erdemli, Ankara University School of Medicine, Department of Histology and Embryology, Ankara 06370, Turkey

**Author contributions:** Gencay C, Kilicoglu B and Kismet K contributed equally to this work, Kilicoglu SS and Erdemli E performed histological examination, Muratoglu S performed microbiological examination, Akkus MA and Erel S analyzed data; Kismet K and Kilicoglu B wrote the paper.

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**Correspondence to:** Dr. Bulent Kilicoglu, Ankara Training and Research Hospital 4th General Surgery Department, S.B. Ankara Egitim ve Arastirma Hastanesi 4 Cerrahi Klinigi, 06340, Ulucanlar, Ankara 06370, Turkey. [kilicoglubulent@yahoo.com](mailto:kilicoglubulent@yahoo.com)

Telephone: +90-312-5953449 Fax: +90-312-3633396

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also different between these groups. Sham and honey groups had similar incidence of bacterial translocation ( $P > 0.05$ ). BDL group had significantly higher rates of bacterial translocation as compared with sham and honey groups. Bacterial translocation was predominantly detected in mesenteric lymph nodes.

**CONCLUSION:** Supplementation of honey in presence of obstructive jaundice ameliorates bacterial translocation and improves ileal morphology.

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**Key words:** Honey; Obstructive jaundice; Intestinal villus atrophy; Bacterial translocation

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### Abstract

**AIM:** To evaluate the effects of honey on bacterial translocation and intestinal villus histopathology in experimental obstructive jaundice.

**METHODS:** Thirty Wistar-Albino rats were randomly divided into three groups each including 10 animals: group I, sham-operated; group II, ligation and section of the common bile duct (BDL); group III, bile duct ligation followed by oral supplementation of honey (BDL + honey) 10 g/kg per day. Liver, blood, spleen, mesenteric lymph nodes, and ileal samples were taken for microbiological, light and transmission electron microscopic examination.

**RESULTS:** Although the number of villi per centimeter and the height of the mucosa were higher in sham group, there was no statistically significant difference between sham and BDL + honey groups ( $P > 0.05$ ). On the other hand, there was a statistically significant difference between BDL group and other groups ( $P < 0.05$ ). The electron microscopic changes were

### INTRODUCTION

Obstructive jaundice is a common clinical entity complicated by intestinal failure and endotoxemia, leading to high postoperative morbidity and mortality rates. The gastrointestinal tract performs a variety of functions in digestion, selective absorption, and secretion. However, its barrier function, which prevents spread of intraluminal bacteria and endotoxins to the organs and tissues, plays a key role<sup>[1-2]</sup>. Intestinal barrier failure is associated with an increased incidence of bacteria and toxin translocation from the intestinal lumen to the systemic circulation, causing systemic infection and multiple organ failure in the critically ill or injured patient<sup>[3]</sup>. Gut barrier failure may result from one or more of the three basic pathophysiologic conditions; disruption of the normal ecologic balance of the indigenous gut microflora, impaired host immune defenses, and physical disruption of the gut mucosal barrier<sup>[4]</sup>.

Bacterial translocation is the passage of bacteria or endotoxins from the gastrointestinal tract to extraintestinal sites, such as mesenteric lymph nodes, liver, spleen, and/or bloodstream. In a normal, healthy individual, gut-originated bacteremia and sepsis do not occur because the host has multiple defense mechanisms to prevent the bacteria and their products from crossing the mucosal barrier and spreading to systemic tissues. Under certain experimental and clinical circumstances, this intestinal barrier function becomes overwhelmed or impaired, resulting in bacterial translocation<sup>[4]</sup>. Current advances in the pathophysiology of intestinal failure in obstructive jaundice have showed that the breakage of gut barrier is multifactorial, involving disruption of the immunologic, biological, mechanical, and biochemical barrier<sup>[1]</sup>.

Honey is a supersaturated sugar solution produced by honey bees from nectar of different plants. It has a long tradition of use for wound healing since ancient times. Honey has bactericidal, bacteriostatic, antifungal, antiviral, scolical, antioxidant, antitumoral, and anti-inflammatory effects<sup>[5-12]</sup>.

In this study, we investigated the effects of honey on bacterial translocation and intestinal morphology in experimental obstructive jaundice.

## MATERIALS AND METHODS

### Animals

Thirty Wistar-Albino male rats, weighing  $250 \pm 25$  g, were housed under constant temperature ( $21 \pm 2^\circ\text{C}$ ) individually in wire cages with 12 h light-dark cycle. Twelve hours before anesthesia, animals were deprived of food, but had free access to water 2 h before anesthesia. No enteral or parenteral antibiotics were administered at any time. The rats that died during the experiment were excluded from the experiment and no new rat was included. The procedures in this experimental study were performed in accordance with the National Guidelines for The Use and Care of Laboratory Animals and approved by Animal Ethics Committee of Ankara Research and Training Hospital.

### Study groups

Rats were randomly divided into three groups each including 10 animals: group I, sham-operated; group II, ligation and section of the common bile duct (BDL); group III, BDL followed by oral supplementation of honey 10 g/kg per day (Balpamak LTD, Istanbul, Turkey), once a day, with nasogastric tube (7 Gauge feeding tube) that was inserted daily and taken off after honey supplementation. Animals were sacrificed by high-dose diethyl ether inhalation on postoperative day 7. Liver, blood, spleen, mesenteric lymph nodes, and ileal samples were taken for microbiological, light and TEM (transmission electron microscopic) examination.

There isn't a standard dose for honey in experimental studies. The dose used in previous studies ranges between 0.078 g/kg to 5 g honey/rat per day<sup>[12-15]</sup>. We gave 10 g/kg per day to each rat.

### Operative procedure

Animals were anesthetized by intramuscular injection of 30 mg/kg ketamine hydrochloride (Ketalar®; Parke-Davis, Istanbul, Turkey) and 5 mg/kg xylazine (Rompun®, Bayer, Istanbul, Turkey). Midline laparotomy was performed under sterile conditions. In the sham-operated group (group I) the common bile duct (CBD) was freed from the surrounding soft tissue and was manipulated without ligation and transection. In group II and III, CBDs of the rats were identified, double ligated with 5-0 silk, and sectioned between the ligatures. The same surgeon performed all procedures. The abdominal incisions were closed in two layers with continuous 3-0 silk sutures. Animals were allowed to feed after the operation.

### Microbiological and biochemical examination

The mesenteric lymph nodes (MNLs), spleen and liver were chopped with sterile instruments under aseptic conditions. Then the tissue samples were weighed and placed in tubes containing 1.5 mL broth (thioglycolate, Oxoid, UK) and homogenized. After that 0.01 mL tissue samples were inoculated on blood agar (Oxoid, UK) and Levine Eosine Methylene Blue (EMB) agar (Oxoid, UK). Plates were incubated at  $37^\circ\text{C}$  for examination of bacterial growth. The growth of bacteria in quantitative culture was observed at 24 h and 48 h.

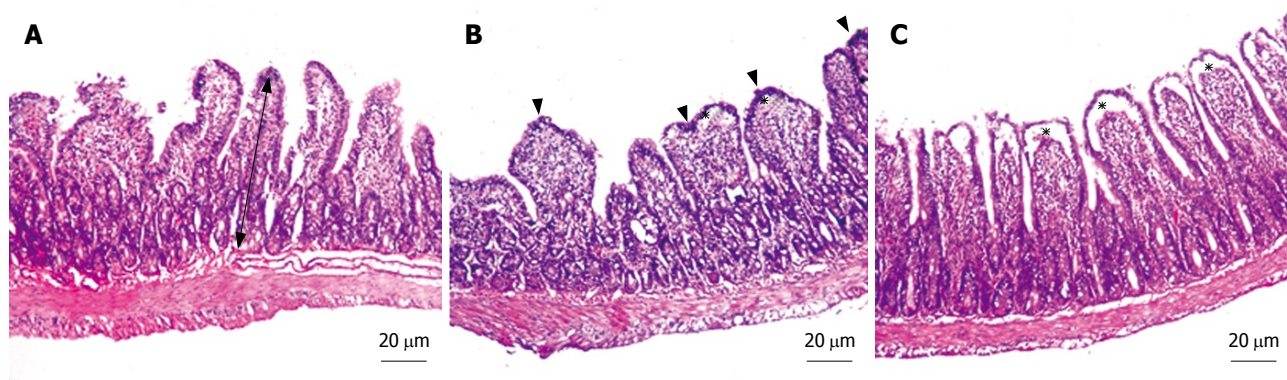
Blood samples taken from inferior vena cava of rats were inoculated on the medium of aerobic and anaerobic blood culture. The aerobic and anaerobic blood cultures were observed by incubation in BACTEC 9240 blood culture system (Becton Dickinson, USA) at  $37^\circ\text{C}$  for seven days. Samples taken from the blood culture bottle giving positive alarm were subcultured by inoculating on blood agar and EMB agar. The subcultures were inoculated at  $37^\circ\text{C}$  under aerobic and anaerobic condition and examined at 24 h and 48 h.

Total bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) levels were measured as parameters indicative of hepatic function by an autoanalyser (Olympus AU640, Japan).

### Histopathological examination

For light microscope analyses, tissue samples from the terminal ileum were obtained from all animals. In order to avoid mucosal suffering, the intestinal lumen was carefully cannulated and gently washed with normal saline solution before the sampling. The ileal samples were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at  $5\ \mu\text{m}$  by Leica RM 2125 RT, and stained with hematoxylin and eosin (HE) for routine light microscopic examination. Histopathological examinations were performed by a pathologist who was blinded to the study design and photographs were taken with Nikon Eclipse E 600. The number of villi per centimeter (V/cm) and the total mucosal thickness were assessed in all groups. The mucosal thickness was measured in a minimum of 20 well-preserved villi in each randomly selected sample from each tissue block.

For TEM (transmission electron microscopic)



**Figure 1** The micrographs of light microscope stained with haematoxylin and eosin. Micrograph (A) illustrates the typical structure of villi; (B) blunting of the villi (arrow head) and subepithelial edema (asterisk); (C) existing subepithelial edema (asterisk) and the total mucosal thickness (arrow).

analyses, samples were fixed with phosphate buffered (pH 7.3) 2.5% glutaraldehyde and 2% PFA mixture solution for 2 h at room temperature. They were washed with phosphate buffered saline solution (PBS, pH 7.3) and were fixed with 1% osmium tetroxide for 2 h as secondary fixation. After washing, they were embedded in Araldite 6005 and were cut with Leica EM FCS (Wien, Austria) ultramicrotome. One  $\mu\text{m}$  semi-thin sections were stained with Toluidin blue-Azur II to select the region of interest for the following procedures. Sixty to 70 nm thin sections were stained with uranyl acetate and lead citrate. They were examined and photographed using a LEO 906 E TEM (80 Kv, Oberkochen, Germany). The pathologist was blinded about the groups.

### Statistical analysis

Differences between the numbers of positive cultures of the groups were evaluated by chi-square test. Scores of total mucosal thickness and number of villi per centimeter were presented as mean  $\pm$  SD and compared by One-Way ANOVA or Kruskal-Wallis variance analysis. If the *P* values of the variance analyses were statistically significant, differences between groups were analyzed with the Mann-Whitney *U* test. Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS), version 13.0 for Windows (SPSS Inc., Chicago, USA). *P* < 0.05 was considered to be statistically significant.

## RESULTS

### General

All rats were sacrificed on postoperative day 7. Two rats from group II (BDL group) and one from group III (BDL + honey group), totally 3 rats, died during the early postoperative period probably due to anesthesia. The liver function tests and bilirubin levels were normal in sham group and high in BDL and BDL + honey groups.

### Intestinal morphology

In all specimens of the sham group, the histological

**Table 1** Mean number of villi per cm and mean height of mucosa ( $\mu\text{m}$ )

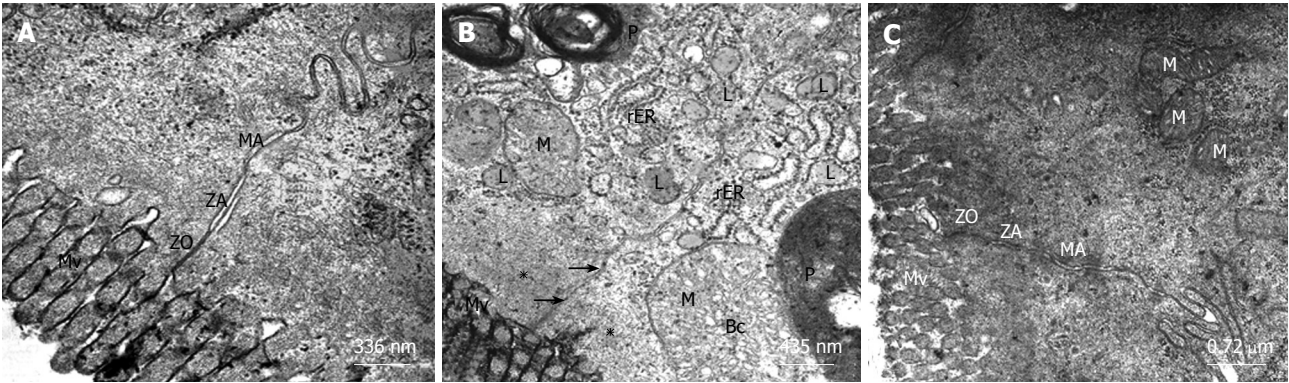
Groups	Mean number of villi per cm	Mean height of mucosa
Sham (Group I)	84.40 $\pm$ 3.75 <sup>b</sup>	640.02 $\pm$ 43.72
BDL (Group II)	73.01 $\pm$ 2.83	567.50 $\pm$ 34.54
BDL + Honey (Group III)	81.33 $\pm$ 3.46 <sup>b</sup>	625.56 $\pm$ 38.77

<sup>b</sup>*P* < 0.01 vs II, *P* values for mean height of mucosa: 0.002, I vs II; 0.005, II vs III.

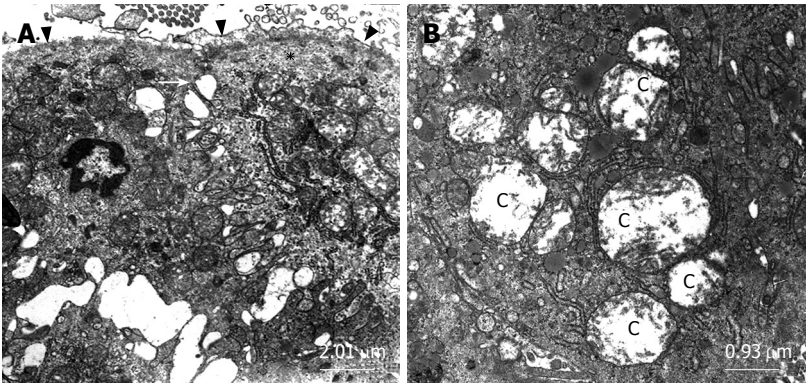
features showed regular appearance of ileal tissue. When we evaluated the specimens systematically, including assessment of villous architecture, surface and crypt epithelia, lamina propria constituents and submucosal structures, no alteration was found in sham group (Figure 1A). The specimens of the BDL group presented villous blunting associated with reduced mucosal thickness. We identified subepithelial edema mostly located at the tip of the villi, but also extended throughout the villus, with epithelial layer moderately lifted from the lamina propria. We observed that the crypts were generally preserved. The number of villi per centimeter (V/cm) (villus density) was decreased in BDL group (Figure 1B). In group III, the subepithelial edema still existed, but villous blunting was not evident. Farther, the crypts generally appeared to be preserved (Figure 1C). Although the number of villi per centimeter and the height of the mucosa were higher in sham group, there was no statistically significant difference between sham and BDL + honey groups (*P* > 0.05). On the other hand, there was a statistically significant difference between BDL group and other groups (*P* < 0.05). Mean number of villi per centimeter and mean mucosal height of the groups are given in Table 1.

The ultrastructure of intestinal epithelial junctional complexes was observed by electron microscopy. In the sham group, enterocytes were tightly bound to the luminal surface by junctional complexes. Zonulae occludens, zonulae adherentes and maculae adherentes appeared normal in the sham group. The luminal surface was covered with microvilli (Figure 2A). When we evaluated the BDL group, we





**Figure 2** These transmission electron microscope (TEM) micrographs illustrate the main ultrastructural features of enterocytes, the absorptive cells of the ileum. Micrograph (A) shows the regular structure of microvilli (Mv) and the three components of junctional complex at the luminal end of the lateral plasma membrane, zonula occludens (ZO), zonula adherens (ZA) and macula adherens (MA); Micrograph (B) shows the disintegration of the zonula occludens and disordered structure of junctional complexes (arrows). The lipid droplets (L) and phagosomes (P) in the cytoplasm, rough endoplasmic reticulum (rER), swollen mitochondria (M) with ballooned cristae (Bc) and apical surface edema (asterisk) viewed; Micrograph (C) illustrates the regular structure of microvilli (Mv) and junctional complexes (ZO, ZA, MA).



**Figure 3** Micrograph (A) shows the disintegration of the zonula occludens (arrow), apical surface edema (asterisk) and the desquamation of the epithelial tissue (arrow head); Micrograph (B) illustrates the swollen mitochondria with cavitations of matrix (C).

observed desquamated epithelial tissue, cytoplasmic vacuoles, phagosomes and disrupted structure of the tight junction between epithelial cells possibly due to apical surface edema. Zonulae occludens located within the plasma membranes of adjacent epithelial cells diverged (Figures 2B and 3A) and the mitochondria were swollen with electrolucent matrix and ballooned cristae. Markedly swollen mitochondria with peripherally placed, disoriented and disintegrating cristae and cavitations of the matrix were also observed in BDL group (Figures 2B and 3B). The structure of the microvilli and mitochondria were regular in the BDL + honey group. The junctional complexes had normal appearance (Figure 2C).

**Bacterial translocation**

The rates of bacterial translocation (BT) in all groups are summarized in Table 2. Sham and BDL + honey groups had similar incidence of BT. BDL group had significantly higher rates of BT as compared with sham and BDL + honey groups. Only BT to spleen was not significantly different between the BDL and BDL + honey groups. BT was predominantly detected in MLNs.

The most commonly isolated bacteria was *Escherichia coli*. The other isolated microorganisms were *Enterococcus spp.*, *Staphylococcus spp.*, *Proteus spp.*, *Staphylococcus aureus* and *Enterobacter cloacae*.

Table 2 Bacterial translocation rates of the groups				
Groups	Liver	Spleen	MLNs	Blood
Sham (Group I )	0/10 (0.0%)	0/10 (0.0%)	1/10 (10.0%)	0/10 (0.0%)
BDL (Group II)	6/8 (75.0%)	4/8 (50.0%)	7/8 (87.5%)	4/8 (50.0%)
BDL + Honey (Group III)	1/9 (11.1%)	2/9 (22.2%)	2/9 (22.2%)	0/9 (0.0%)
P values				
I vs II	0.002	0.023	0.002	0.023
II vs III	0.013	> 0.05	0.012	0.029
I vs III	> 0.05	> 0.05	> 0.05	> 0.05

**DISCUSSION**

Bacterial translocation is the migration of bacteria or bacterial products from the intestinal lumen to mesenteric lymph nodes or other extraintestinal organs and sites. In addition to nutrient absorption, the gut functions as a barrier to prevent the spread of intraluminal bacteria and endotoxin to systemic organs and tissues<sup>[4,16,17]</sup>.

Bile inhibits bacterial overgrowth, has a trophic effect on the intestinal mucosa, decreases epithelial internalization of enteric bacteria, exerts detergent actions with anti-adherence effects, and binds endotoxins. Therefore, the absence of bile in the intestine facilitates BT and enhances endotoxin-induced BT<sup>[16]</sup>. Obstructive jaundice is almost universally believed to promote bacterial translocation. Absence of bile from the lumen



of gut is also associated with a quantitative increase in small intestinal microflora<sup>[18]</sup>.

Translocation from the intestine is most commonly detected by measuring the presence of viable bacteria in the tissues. This can reflect not only the integrity of the intrinsic barrier function of the mucosa but also the numbers and types of microbes in the lumen and the ability of the host to kill the bacteria that translocate<sup>[19]</sup>.

Honey is a supersaturated sugar solution produced by honey bees from the nectar of plants. Some of the components of the honey are added by the bees during the maturation process or are derived from the plants<sup>[20]</sup>. The antimicrobial properties of honey are well documented<sup>[5-9]</sup>. The antibacterial activity of honey lies partially in its high osmolality due to its high sugar content, and in its acidity due mostly to the presence of gluconic acid. Although hydrogen peroxide is thought to be the main antibacterial factor in honey, the presence of non-peroxide activity was also notable. This activity is usually attributed to the presence of organic components such as syringic acid, methyl syringate, pinocembrin, pinobanksin, caffeic acid, ferulic acid, vanillic acid, cinnamic acid, and benzoic acid<sup>[21]</sup>.

The physicochemical properties of honey not only contribute to its antibacterial properties but also to its wound healing capabilities. The anti-inflammatory action of honey has been investigated, but no definite mechanism has been identified. Honey provides glucose supply for leucocytes. It also provides substrate for glycolysis, which is the major mechanism for energy production in the macrophages. Honey may modulate the activation state of immunocompetent cells (e.g. monocytes) within the wound. These data suggest that honey may have a number of effects on the molecular mechanisms of wound healing<sup>[20]</sup>.

As we mentioned before, the physical barrier function of the mucosa appears to have primary importance for preventing or limiting bacterial translocation, especially in a host with a normal gut flora, whereas the immune system appears to serve a secondary or supportive role to the intestinal mucosal barrier<sup>[4]</sup>. In our study, mean number of villi per centimeter, mean mucosal height, and electron microscopic changes of the honey group were significantly better than the data observed in the BDL group. In the sham and BDL + honey groups, enterocytes were tightly bound to the luminal surface by junctional complexes. Zonulae occludens, zonulae adherentes and maculae adherentes appeared normal in these groups. The luminal surface was covered with microvilli. When we evaluated the BDL group, we observed desquamated epithelial tissue, cytoplasmic vacuoles, phagosomes and disrupted structure of the tight junction between epithelial cells possibly due to apical surface edema. Zonulae occludens located within the plasma membranes of adjacent epithelial cells were diverging and the mitochondria were swollen with electrolucent matrix and ballooned cristae. Reduced bacterial translocation rates in honey group could be explained by decreased atrophy of intestinal mucosal villi and somewhat regular structure of enterocytes and microvilli. We concluded that

wound healing properties and cytoprotective effects of honey might be the reason of the decreased atrophy of intestinal mucosal villi. On the other hand, antimicrobial effects of honey on enteric bacteria could also decrease the overgrowth of these bacteria and reduce bacterial translocation.

Assimakopoulos *et al*<sup>[22]</sup> investigated the oxidative alterations in the intestinal mucosa of patients with obstructive jaundice and found that obstructive jaundice in humans induced intestinal oxidative stress, which might be a key factor contributing to intestinal barrier failure and the development of septic complications in this patient population. In another study, these authors showed that intestinal mucosal atrophy in obstructive jaundice was based on inhibition of proliferation and promotion of apoptotic death of enterocytes, and reactive oxygen species might be responsible for this effect<sup>[23]</sup>.

The antioxidant properties of honey have been well-documented in recent studies<sup>[24-27]</sup>. Schramm *et al*<sup>[25]</sup> found that phenolic antioxidants from processed honey were bioavailable, and these antioxidants increased antioxidant activity of plasma. Gheldof *et al*<sup>[11]</sup> also showed that the *in vivo* serum antioxidant capacity increased significantly following consumption of buckwheat honey in human. These studies showed that the antioxidant effect of honey was not only local, but also a systemic effect. According to the results of studies about antioxidative effects of honey and intestinal oxidative stress in obstructive jaundice, we concluded that the protective effect of honey on intestinal villi and mucosal structure might be attributable to antioxidative effects of honey in our study. Since we investigated only the effects of honey on bacterial translocation and intestinal villus atrophy, not the mechanism of this effect, we did not evaluate oxidative stress parameters. These parameters should be analyzed in further studies that investigate the mechanism of this effect of honey.

Since a normal functioning immune system is another important factor for adequate gut barrier function<sup>[4]</sup>, honey may also reduce bacterial translocation by its modulatory effects on immunocompetent cells<sup>[28-29]</sup>.

In this study, we demonstrated that honey reduced bacterial translocation rates and protected intestinal villus structure in experimental obstructive jaundice model. These effects of honey might be attributable to its antibacterial, antioxidant, anti-inflammatory, and immunomodulatory activities. Further studies are needed for evaluation of the exact mechanism of this effect. After the results of these studies, honey might be used for preventing harmful effects of obstructive jaundice in clinical settings.

## COMMENTS

### Background

Spontaneous bacterial infection and septicemia due to increased bacterial translocation in patients with obstructive jaundice result in significant morbidity and mortality.

### Research frontiers

The present study investigated the effects of honey on bacterial translocation

and intestinal morphology in experimental obstructive jaundice.

### Innovations and breakthroughs

Obstructive jaundice is a common clinical entity complicated by intestinal failure and endotoxemia, leading to high postoperative morbidity and mortality. Our experience from the present study shows that honey can be used safely in this situation.

### Applications

This study demonstrated that honey reduced bacterial translocation rates and protected intestinal villus structure in experimental obstructive jaundice model. Honey might be used for preventing harmful effects of obstructive jaundice in clinical settings.

### Peer review

The rationale behind this study is that the authors have previously shown that honey has reduced bacterial translocation rates and protected intestinal villus structure in experimental obstructive jaundice.

## REFERENCES

- 1 **Assimakopoulos SF**, Vagianos CE, Charonis A, Nikolopoulou VN, Scopa CD. Intestinal failure in obstructive jaundice. *World J Gastroenterol* 2005; **11**: 3806-3807
- 2 **Kayama S**, Mitsuyama M, Sato N, Hatakeyama K. Overgrowth and translocation of *Escherichia coli* from intestine during prolonged enteral feeding in rats. *J Gastroenterol* 2000; **35**: 15-19
- 3 **De-Souza DA**, Greene LJ. Intestinal permeability and systemic infections in critically ill patients: effect of glutamine. *Crit Care Med* 2005; **33**: 1125-1135
- 4 **Magnotti LJ**, Deitch EA. Burns, bacterial translocation, gut barrier function, and failure. *J Burn Care Rehabil* 2005; **26**: 383-391
- 5 **Molan PC**. Honey as an antimicrobial agent. In: Mizrahi A, Lensky Y, editors. *Bee products: Properties, Application and Apitherapy*. New York: Plenum Press, 1996: 27-37
- 6 **Jeddar A**, Kharsany A, Ramsaroop UG, Bhamjee A, Haffjee IE, Moosa A. The antibacterial action of honey. An in vitro study. *S Afr Med J* 1985; **67**: 257-258
- 7 **Efem SE**, Udoh KT, Iwara CI. The antimicrobial spectrum of honey and its clinical significance. *Infection* 1992; **20**: 227-229
- 8 **Irish J**, Carter DA, Shokohi T, Blair SE. Honey has an antifungal effect against *Candida* species. *Med Mycol* 2006; **44**: 289-291
- 9 **Al-Waili NS**. Topical honey application vs. acyclovir for the treatment of recurrent herpes simplex lesions. *Med Sci Monit* 2004; **10**: MT94-MT98
- 10 **Kilicoglu B**, Kismet K, Koru O, Tanyuksel M, Oruc MT, Sorkun K, Akkus MA. The scolicidal effects of honey. *Adv Ther* 2006; **23**: 1077-1083
- 11 **Gheldof N**, Wang XH, Engeseth NJ. Buckwheat honey increases serum antioxidant capacity in humans. *J Agric Food Chem* 2003; **51**: 1500-1505
- 12 **Mabrouk GM**, Moselhy SS, Zohny SF, Ali EM, Helal TE, Amin AA, Khalifa AA. Inhibition of methylnitrosourea (MNU) induced oxidative stress and carcinogenesis by orally administered bee honey and *Nigella* grains in Sprague Dawley rats. *J Exp Clin Cancer Res* 2002; **21**: 341-346
- 13 **Gharzouli K**, Amira S, Gharzouli A, Khennouf S. Gastroprotective effects of honey and glucose-fructose-sucrose-maltose mixture against ethanol-, indomethacin-, and acidified aspirin-induced lesions in the rat. *Exp Toxicol Pathol* 2002; **54**: 217-221
- 14 **Ali AT**, al-Swayeh OA, al-Humayyd MS, Mustafa AA, al-Rashed RS, al-Tuwaijiri AS. Natural honey prevents ischaemia-reperfusion-induced gastric mucosal lesions and increased vascular permeability in rats. *Eur J Gastroenterol Hepatol* 1997; **9**: 1101-1107
- 15 **Onat F**, Yegen BC, Lawrence R, Oktay A, Oktay S. Site of action of grayanotoxins in mad honey in rats. *J Appl Toxicol* 1991; **11**: 199-201
- 16 **Wiest R**, Garcia-Tsao G. Bacterial translocation (BT) in cirrhosis. *Hepatology* 2005; **41**: 422-433
- 17 **Balzan S**, de Almeida Quadros C, de Cleva R, Zilberstein B, Cecconello I. Bacterial translocation: overview of mechanisms and clinical impact. *J Gastroenterol Hepatol* 2007; **22**: 464-471
- 18 **Gatt M**, Reddy BS, MacFie J. Review article: bacterial translocation in the critically ill--evidence and methods of prevention. *Aliment Pharmacol Ther* 2007; **25**: 741-757
- 19 **Alexander JW**. Bacterial translocation during enteral and parenteral nutrition. *Proc Nutr Soc* 1998; **57**: 389-393
- 20 **Lusby PE**, Coombes A, Wilkinson JM. Honey: a potent agent for wound healing? *J Wound Ostomy Continence Nurs* 2002; **29**: 295-300
- 21 **Aljadi AM**, Yusoff KM. Isolation and identification of phenolic acids in Malaysian honey with antibacterial properties. *Turk J Med Sci* 2003; **33**: 229-236
- 22 **Assimakopoulos SF**, Thomopoulos KC, Patsoukis N, Georgiou CD, Scopa CD, Nikolopoulou VN, Vagianos CE. Evidence for intestinal oxidative stress in patients with obstructive jaundice. *Eur J Clin Invest* 2006; **36**: 181-187
- 23 **Assimakopoulos SF**, Scopa CD, Zervoudakis G, Mylonas PG, Georgiou C, Nikolopoulou V, Vagianos CE. Bombesin and neurotensin reduce endotoxemia, intestinal oxidative stress, and apoptosis in experimental obstructive jaundice. *Ann Surg* 2005; **241**: 159-167
- 24 **Gheldof N**, Wang XH, Engeseth NJ. Identification and quantification of antioxidant components of honeys from various floral sources. *J Agric Food Chem* 2002; **50**: 5870-5877
- 25 **Schramm DD**, Karim M, Schrader HR, Holt RR, Cardetti M, Keen CL. Honey with high levels of antioxidants can provide protection to healthy human subjects. *J Agric Food Chem* 2003; **51**: 1732-1735
- 26 **Henriques A**, Jackson S, Cooper R, Burton N. Free radical production and quenching in honeys with wound healing potential. *J Antimicrob Chemother* 2006; **58**: 773-777
- 27 **Gheldof N**, Engeseth NJ. Antioxidant capacity of honeys from various floral sources based on the determination of oxygen radical absorbance capacity and inhibition of in vitro lipoprotein oxidation in human serum samples. *J Agric Food Chem* 2002; **50**: 3050-3055
- 28 **Abuharfeil N**, Al-Oran R, Abo-Shehada M. The Effect of Bee Honey on the Proliferative Activity of Human B-and T-Lymphocytes and the Activity of Phagocytes. *Food and Agricultural Immunology* 1999; **11**: 169-177
- 29 **Watanabe K**, Shinmoto H, Kobori M, Tsushida T, Shinohara K, Kanaeda J, Yonekura M. Stimulation of cell growth in the U-937 human myeloid cell line by honey royal jelly protein. *Cytotechnology* 1998; **26**: 23-27

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RAPID COMMUNICATION

## Treatment responses in Asians and Caucasians with chronic hepatitis C infection

Kenneth K Yan, Marianne Guirgis, Thuy Dinh, Jacob George, Anouk Dev, Alice Lee, Amany Zekry

Kenneth K Yan, Marianne Guirgis, Amany Zekry, Department of Hepatology, St George Hospital, Kogarah, Sydney 2217, NSW, Australia

Thuy Dinh, Anouk Dev, Department of Gastroenterology, Monash Medical Centre, Melbourne 3168, Victoria, Australia

Jacob George, Storr Liver Unit, Westmead Millennium Institute, University of Sydney and Westmead Hospital, Sydney 2217, NSW, Australia

Alice Lee, Department of Gastroenterology and Hepatology, Concord Repatriation Hospital, Concord, Sydney 2217, NSW, Australia

**Author contributions:** Yan KK designed research; Yan KK, Guirgis M and Dinh T performed research; George J, Dev A and Lee A advised on research design; Zekry A originated research topic and supervised the project; Yan KK and Zekry A analysed results and wrote the paper.

**Correspondence to:** Dr. Amany Zekry, Department of Medicine, St. George Hospital, Kogarah, Sydney 2217, NSW, Australia. [a.zekry@unsw.edu.au](mailto:a.zekry@unsw.edu.au)

Telephone: +61-2-91132019 Fax: +61-2-91133993

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**CONCLUSION:** Genotype 1 CHC in Asian subjects is associated with higher rates of virological response compared to that in Caucasians.

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**Key words:** Hepatitis C; Treatment; Asians; Retrospective studies; Comparative study; Interferon; Ribavirin; Statistical data analysis

**Peer reviewers:** Vasily I Reshetnyak, MD, PhD, Professor, Scientist Secretary of the Scientific Research Institute of General Reanimation, 25-2, Petrovka str., Moscow 107031, Russia; Heitor Rosa, Professor, Department of Gastroenterology and Hepatology, Federal University School of Medicine, Rua 126 n.21, Goiania-GO 74093-080, Brazil

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### Abstract

**AIM:** To conduct a multicentre retrospective review of virological response rates in Asians infected with genotype 1 chronic hepatitis C (CHC) treated with combination interferon and ribavirin and then to compare their responses to that among Caucasians.

**METHODS:** Asian patients infected with genotype 1 CHC treated at 4 Australian centres between 2001 to 2005 were identified through hospital databases. Baseline demographic characteristics, biochemical, virological and histological data and details of treatment were collected. Sustained virological responses (SVR) in this cohort were then compared to that in Caucasian subjects, matched by genotype, age, gender and the stage of hepatic fibrosis.

**RESULTS:** A total of 108 Asians with genotype 1 CHC were identified. The end of treatment response (ETR) for the cohort was 79% while the SVR was 67%. Due to the relatively advanced age of the Asian cohort, only sixty-four subjects could be matched with Caucasians. The ETR among matched Asians and Caucasians was 81% and 56% respectively ( $P = 0.003$ ), while the SVR rates were 73% and 36% ( $P < 0.001$ ) respectively. This difference remained significant after adjusting for other predictive variables.

### INTRODUCTION

Chronic hepatitis C (CHC) virus infection is the leading cause of chronic liver disease worldwide. The prevalence of hepatitis C virus (HCV) infection in western countries including Australia and the United States approximates 1%<sup>[1-2]</sup>, while it is more common in most Asian countries<sup>[3-4]</sup>. Combination therapy with pegylated interferon and ribavirin given for 24 wk or 48 wk remains the most effective antiviral treatment, achieving sustained virological response (SVR) rates ranging from 50% to 80%<sup>[5-9]</sup>.

Various factors have been identified that influence response rates, including HCV genotype, body mass index and co-existent liver disease. Ethnicity was recently noted to impact on treatment responses. Studies conducted in African Americans suggest that these individuals have lower SVR rates when compared to Caucasians, even after adjusting for confounders that could potentially influence treatment response rates<sup>[10-13]</sup>. More recently, comparative studies between Asians and Caucasians have suggested a higher SVR to antiviral therapy among Asians<sup>[14-16]</sup>.

In Asians, HCV prevalence rates are approximately

6%<sup>[3]</sup>. Often subjects acquire the infection at a younger age and are therefore at increased risk of developing advanced liver disease and hepatocellular carcinoma. Despite this, Asians seem to be under-represented in clinical trials evaluating SVRs, with the largest available study comprising only 52 individuals<sup>[16]</sup>. In addition, there has been no head-to-head comparative study evaluating responses to antiviral therapy between Asians and Caucasians. The aims of the present study were therefore to: (1) assess the overall SVR rates in Asians infected with genotype 1 CHC receiving combination antiviral therapy; and (2) to undertake a case-control study comparing SVR rates in Asians compared to that in Caucasians matched by infecting virus genotype, age, gender and the extent of hepatic fibrosis.

## MATERIALS AND METHODS

Clinical databases of HCV infected patients who received combination interferon and ribavirin therapy between 2001 to 2005 at four Australian centres were reviewed and all Asian patients identified. Individual patient files were retrieved and their clinical status confirmed. Asian and Caucasian patients over the age of 18 years who received antiviral therapy for genotype 1 CHC were subsequently identified.

Exclusion criteria included patients who were HBsAg-positive, co-infection with HIV, liver transplant recipients or those receiving dialysis for chronic renal failure. Patient demographic characteristics, baseline biochemical, virological and histological data prior to commencing anti-viral therapy were recorded. Those with bridging fibrosis or cirrhosis were considered to have advanced liver disease. While baseline viral loads of individual patients were recorded, multiple different assays and units were used at the various study centres over time, making this data not reportable, or comparable. For alcohol intake, we defined significant intake as either documented daily consumption of more than 30 grams, or medical record documentation that alcoholism was an issue. Details of antiviral therapy were recorded. These included a history of previous anti-viral treatment, treatment regimen (interferon and ribavirin or pegylated interferon and ribavirin), adverse effects on therapy and any treatment dose reductions or interruptions due to either adverse effects or non-compliance. As a standard of care, all patients with HCV genotype 1 were scheduled for 48 wk of treatment.

The primary end point of this study was the proportion of patients achieving an SVR, defined as a documented non-detectable HCV RNA at least 24 wk after treatment. The end of treatment response (ETR) was defined as non-detectable HCV RNA at the end of treatment. Patients who received at least one dose of interferon but did not complete 48 wk of treatment for any reason were defined as non-responders. Asians and Caucasians infected with HCV genotype 1 were then matched by three criteria: age (within 5 years), gender and the extent of hepatic fibrosis, and their ETR and SVR rates were compared.

**Table 1** Demographic characteristics, treatment details and treatment responses of 108 HCV genotype 1-infected Asian patients

Variables	Frequency
Countries of origin	
Vietnam	43
China	34
Cambodia	19
Korea	5
Burma	3
Others	4
Gender (%)	
Male	69%
Age (yr, range)	51 (22-76)
Median weight (kg, range)	64 (33-104)
Median ALT (IU/mL, range)	94 (11-558)
Extend of fibrosis (%)	
Bridging fibrosis or cirrhosis	21%
Treatment regimen (%)	
Pegylated interferon + Ribavirin	89%
Interferon + Ribavirin	11%
Dose reduction of either drug	33%
Dose interruption of either drug	11%
Treatment responses (%)	
End of treatment virological response	77%
SVR	67%

All statistical analyses were performed by SAS software v11 (SAS Institute Inc., Cary, NC). Continuous variables are reported as median (range). Comparison of baseline demographics was performed by the paired Student *t*-test, Mann-Whitney test or  $\chi^2$  test as appropriate. Univariate analysis was performed with SVR as the dependent variable.

## RESULTS

### Baseline features of the Asian cohort

A total of 108 HCV genotype 1 infected Asian patients were identified. Their demographic characteristics, treatment details and treatment outcomes are shown in Table 1. The majority of patients were born in Vietnam, China or Cambodia; 69% were male. The cohort had a median age of 51 (22-76) and a median body weight of 64 kg (33-104 kg). The baseline alanine aminotransaminase (ALT) was 94 IU/mL (11-558 IU/mL). 21% of those who had liver biopsies had histological evidence of bridging fibrosis or cirrhosis. Eighty-nine percent received pegylated interferon and ribavirin while the remainder received standard interferon and ribavirin. Dose reduction was required in 33%, while 11% required dose interruption, for either adverse effects or non-compliance.

### End of treatment and SVR rates in Asians

An ETR occurred in 77% of the Asian cohort while 67% achieved an SVR. Factors influencing SVR rates including treatment regimen, fibrosis stage, age, gender and weight were examined by univariate analysis (Table 2). None of these factors were found to be predictive of an SVR in the Asian cohort. In particular, we did not observe a difference in ETR or SVR between those who received pegylated interferon versus standard interferon.



**Table 2** Univariate analysis of 108 HCV genotype 1-infected Asian patients: Predictors of SVR

Variables	OR (95% CI)	P-value
Gender (male)	0.77 (0.32-1.85)	0.55
Age (yr)	1.02 (0.99-1.06)	0.20
Weight (kg)	0.98 (0.95-1.06)	0.41
Pegylated interferon <i>vs</i> standard interferon	1.00 (0.28-3.57)	0.99
Treatment naïve	0.49 (0.22-1.12)	0.09
Dose reduction not required	1.45 (0.65-3.34)	0.39
Dose interruption not required	1.50 (0.44-5.10)	0.52
Absence of bridging fibrosis or cirrhosis	1.57 (0.61-4.07)	0.35

**Table 3** Comparison of the demographic characteristics and treatment details of the matched Asian and Caucasian patients

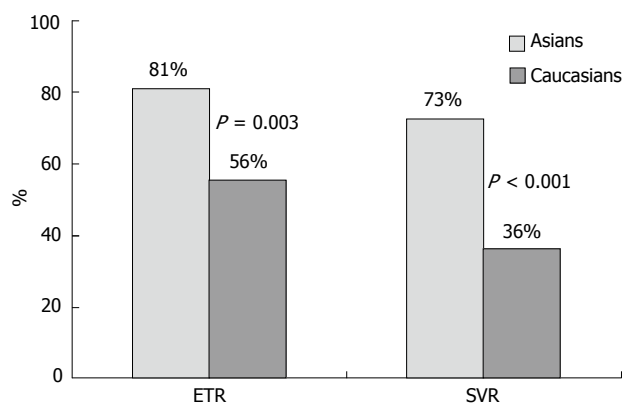
Variables	Asians	Caucasians	P-value
Age (yr, range)	47 (28-64)	46 (30-61)	Matched
Gender	70% male	70% male	Matched
Weight (kg)			
< 75	82%	42%	< 0.01
> 75	18%	58%	
Alcohol intake			
Minimal intake	86%	71%	0.12
Significant intake	14%	29%	
Liver fibrosis			
Minimal injury	48 (75%)	48 (75%)	Matched
Bridging fibrosis/Cirrhosis	16 (25%)	16 (25%)	
Treatment naïve	80%	91%	NS
Peginterferon + Ribavirin	83%	88%	NS
Dose modification	30%	28%	NS
Dose interruption	8%	10%	NS

### Baseline features of the matched cohort

Due to the more advanced age and extent of hepatic fibrosis among Asians, we were only able to match 64 Asian subjects with Caucasians by the set criteria. Comparison of the baseline demographics between the two groups is shown in Table 3. Only 18% of Asian patients weighed more than 75 kg while 58% of their Caucasian counterparts weighed more than 75 kg ( $P < 0.01$ ). Alcohol intake was less in Asian subjects with 14% of Asians and 29% of Caucasians reporting a significant alcohol intake history ( $P = 0.12$ ). There was no difference between the two groups in terms of previous therapy, type of treatment received or in the extent of dose reduction or interruptions from treatment adverse effects or non-compliance.

### End of treatment and SVR rates in the matched cohort

Comparison of the ETR and SVR between the 64 Asian patients and the matched Caucasian cohort is illustrated in Figure 1. Compared to Caucasians, Asian subjects had a significantly better ETR, 81% *vs* 56% ( $P = 0.003$ ) and SVR, 73% *vs* 36% ( $P < 0.001$ ). On univariate analysis (Table 4), Asian ethnicity was the most predictive factor for a SVR. Other factors that were associated with an SVR in this cohort were body weight < 75 kg, and minimal alcohol intake. Due to the large number of variables and the limited number of patients, we were unable to perform a multiple logistic regression analysis with SVR as the dependent variable. Hence the effect

**Figure 1** Comparison of ETR and SVR rates between HCV genotype 1-infected Asians and Caucasians matched for age, gender and fibrosis stage.**Table 4** Univariate analysis of predictors of SVR on matched HCV genotype 1-infected Asians and Caucasians

Variables	OR (95% CI)	P-value
Ethnicity (Asians)	4.92 (2.32-10.50)	< 0.001
Gender (male)	0.83 (0.39-1.79)	0.64
Age (per year)	1.04 (1.00-1.96)	0.061
Weight (< 75 kg)	2.59 (1.20-5.61)	0.016
Minimal alcohol intake	3.20 (1.17-8.73)	0.023
Peginterferon <i>vs</i> standard interferon	1.10 (0.42-2.92)	0.84
Treatment naïve	0.77 (0.29-2.04)	0.60
Dose reduction not required	1.04 (0.48-2.23)	0.93
Dose interruption not required	2.31 (0.64-8.33)	0.20
Absence of bridging fibrosis or cirrhosis	1.80 (0.80-4.04)	0.15

**Table 5** Effects of ethnicity on SVR after adjusting for individual unmatched variables

	Adjusted OR (95% CI)	P-value
Weight (< 75 kg)	4.64 (1.97-10.94)	< 0.001
Minimal alcohol intake	2.74 (0.97-7.75)	0.057
Pegylated interferon <i>vs</i> standard interferon	5.01 (2.35-10.71)	< 0.001
Treatment naïve	5.01 (2.32-10.81)	< 0.001
Dose reduction not required	4.94 (2.32-10.50)	< 0.001
Dose interruption not required	4.84 (2.26-10.38)	< 0.001

of ethnicity on SVR was only adjusted for individual variables that were not matched. In this analysis (Table 5), Asian ethnicity remained a significant predictor of SVR after allowing for other variables that were not matched.

## DISCUSSION

In this retrospective study, we confirmed that in CHC, ethnicity is an important variable influencing response to antiviral therapy. Our study of Asians infected with HCV genotype 1 has permitted several important observations to be made. Firstly, the overall SVR among Asians approached 70%. Secondly, Asians with genotype 1 CHC were more likely to respond favourably to antiviral therapy compared to matched Caucasians. Finally, excess alcohol intake and increased body weight adversely affected treatment outcomes.

The observation of a favourable response rate among Asians is in accordance with other reports<sup>[14-16]</sup>. Importantly, the effect of ethnicity on SVR rates remained significant after adjusting for other confounders including age, gender, treatment regimen and the extent of hepatic fibrosis. The present study also observed that lower body weight and minimal alcohol intake were predictive of an SVR.

Body mass index<sup>[17-18]</sup>, insulin resistance<sup>[19-21]</sup> and hepatic steatosis<sup>[22-24]</sup> are now known to play a major role in the pathogenesis of HCV infection. Insulin resistance in genotype 1 CHC is most likely related to host factors, in particular obesity, rather than virological factors, and is associated with reduced treatment response rates<sup>[25]</sup>. It was therefore interesting to observe in our study that the matched Asian cohort overall had lower body weights than Caucasians. Similar findings were reported in the studies by Hepburn *et al.*<sup>[15]</sup> and Missiha *et al.*<sup>[16]</sup>. Although in these previous studies, as well as in the current report, the effect of ethnicity remained significant after allowing for body weight, central adiposity or underlying insulin resistance were not measured. This is a limitation of the retrospective nature of this study. There are no published data on the impact of hepatic steatosis on CHC infection among Asians. It is therefore important to examine these factors in future studies as they might potentially explain the reasons why Asians having better treatment responses.

Not surprisingly, we observed that Asian subjects consumed less alcohol, possibly a reflection of cultural influences. Our study was the first to adjust for this variable. We noted that the effect of ethnicity on SVR was modified after allowing for alcohol consumption and did not reach statistical significance ( $P = 0.057$ ). Although the difference in SVR was not significant (probably related to patient numbers), this observation highlights the importance of taking alcohol intake into account in studies comparing response rates stratified for ethnicity<sup>[26-27]</sup>.

The biological basis for the difference in SVR rates between Asians and Caucasians has not been examined previously. Studies into the effects of ethnicity on CHC treatment has to date focussed entirely on African Americans. It was found that African Americans had different class II human-leukocyte antigen alleles from Caucasians<sup>[28]</sup>, which could have accounted for their worse SVR. Further, viral kinetics studies have shown that African Americans exhibit significantly lower interferon effectiveness and achieve a lower reduction in HCV RNA in the first 24 h of treatment<sup>[29]</sup>. It was also noted that African Americans had different pre-treatment cytokine profiles<sup>[30]</sup>. In addition, while they mounted a more robust HCV-specific CD4 Th1 proliferative response, it did not translate into a higher rate of IFN-gamma production, potentially secondary to their dysfunctional nature, which was associated with a failure of interferon therapy<sup>[31-32]</sup>. The significance of these studies is that the impact of ethnicity, on treatment response is more likely to be related to host factors, particularly to genetic differences in immune regulation rather than environmental factors.

Our study suffered the usual limitations of retrospective observational reports. In particular, we now know that a proportion of Asians who were initially genotyped by INNO-LiPA as 1b, were in fact genotypes 7, 8 or 9 if direct sequencing of the core region is performed<sup>[14]</sup>. Direct core sequencing was not performed in our study. It is arguable, however, if inclusion of genotypes 7, 8 or 9 would have altered the better SVR rates achieved by the Asian cohort since the original article noted that SVR rates were identical among Asians infected with genotypes 7, 8 or 9 and those infected with genotype 1 HCV infection<sup>[14]</sup>. Similar findings were noted in another study where it was shown that there was no difference in response rates between genotype 1b infected Asians and the rest of the genotype 1 infected Asians<sup>[15]</sup>. We therefore believe that the difference in SVR rates noted in our study was more likely related to ethnicity than a bias from a potentially small group of patients who might have been mistyped. It is clear however that prospective trials on larger patient cohorts including all genotypes are needed to clarify this issue.

The other limitation of this type of retrospective comparative study, as pointed out by both Hepburn *et al.*<sup>[15]</sup> and Missiha *et al.*<sup>[16]</sup>, is that it failed to recognise the wide genetic heterogeneity and different environmental factors that might exist within the same ethnic group. While we defined Asians as those who migrated from East Asia including China, Japan, Korea and South East Asia, and of parents of those origin, we do not, and could not, know if they essentially represent the same group of patients genetically.

In conclusion, Asians infected with HCV genotype 1 achieved a higher SVR rate when compared to a cohort of matched Caucasians. Future studies should focus on confirming our observations in large prospective cohorts and on characterizing the immunogenetic basis for these observations.

## COMMENTS

### Background

Treatment with interferon and ribavirin combination therapy remains currently the most effective treatment for chronic hepatitis C (CHC) virus infection, but treatment response can only be achieved in 50% to 80% of patients. Various factors including genotype, viral load, extent of liver injury on liver biopsy, age and gender of patients were known to impact on treatment responses. Identification of these factors aids selection of patients for treatment and determination of duration of therapy.

### Research frontiers

Recent studies on African Americans suggested that ethnicity might also impact on treatment responses. Data from studies on one ethnic group may not be extrapolated to other ethnic groups, and different ethnic groups may require different treatment regimens.

### Innovations and breakthroughs

This study found that Asian patients infected with hepatitis C genotype 1 had better treatment responses than Caucasian patients, even after adjusting for other predictive factors.

### Applications

The implication of the results of this study is twofold. Firstly, it prompts further basic scientific research into difference in immune response to CHC among different ethnic groups to better understand the pathogenesis of CHC. Secondly, it suggests that clinically, different treatment regimen should be studied and compared among different ethnic groups.

**Peer review**

The results provide sufficient evidence, which allow authors to make firm conclusion that ethnicity is an important factor variable influencing response to antiviral therapy in patients with CHC. The further studies to confirm the results of this observational study for patients from different ethnic backgrounds are needed. The references are appropriate, relevant and updated.

**REFERENCES**

- 1 **Armstrong GL**, Wasley A, Simard EP, McQuillan GM, Kuhnert WL, Alter MJ. The prevalence of hepatitis C virus infection in the United States, 1999 through 2002. *Ann Intern Med* 2006; **144**: 705-714
- 2 **Law MG**, Dore GJ, Bath N, Thompson S, Crofts N, Dolan K, Giles W, Gow P, Kaldor J, Loveday S, Powell E, Spencer J, Wodak A. Modelling hepatitis C virus incidence, prevalence and long-term sequelae in Australia, 2001. *Int J Epidemiol* 2003; **32**: 717-724
- 3 **Shepard CW**, Finelli L, Alter MJ. Global epidemiology of hepatitis C virus infection. *Lancet Infect Dis* 2005; **5**: 558-567
- 4 **Fung KT**, Fung J, Lai CL, Yuen MF. Etiologies of chronic liver diseases in Hong Kong. *Eur J Gastroenterol Hepatol* 2007; **19**: 659-664
- 5 **Fried MW**, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncalves FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982
- 6 **Manns MP**, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965
- 7 **Marcellin P**, Heathcote EJ, Craxi A. Which patients with genotype 1 chronic hepatitis C can benefit from prolonged treatment with the 'accordion' regimen? *J Hepatol* 2007; **47**: 580-587
- 8 **Pearlman BL**, Ehleben C, Saifee S. Treatment extension to 72 weeks of peginterferon and ribavirin in hepatitis c genotype 1-infected slow responders. *Hepatology* 2007; **46**: 1688-1694
- 9 **Shiffman ML**, Suter F, Bacon BR, Nelson D, Harley H, Sola R, Shafran SD, Barange K, Lin A, Soman A, Zeuzem S. Peginterferon alfa-2a and ribavirin for 16 or 24 weeks in HCV genotype 2 or 3. *N Engl J Med* 2007; **357**: 124-134
- 10 **Conjeevaram HS**, Fried MW, Jeffers LJ, Terrault NA, Wiley-Lucas TE, Afdhal N, Brown RS, Belle SH, Hoofnagle JH, Kleiner DE, Howell CD. Peginterferon and ribavirin treatment in African American and Caucasian American patients with hepatitis C genotype 1. *Gastroenterology* 2006; **131**: 470-477
- 11 **Jeffers LJ**, Cassidy W, Howell CD, Hu S, Reddy KR. Peginterferon alfa-2a (40 kd) and ribavirin for black American patients with chronic HCV genotype 1. *Hepatology* 2004; **39**: 1702-1708
- 12 **Muir AJ**, Bornstein JD, Killenberg PG. Peginterferon alfa-2b and ribavirin for the treatment of chronic hepatitis C in blacks and non-Hispanic whites. *N Engl J Med* 2004; **350**: 2265-2271
- 13 **Jacobson IM**, Brown RS Jr, McCone J, Black M, Albert C, Dragutsky MS, Siddiqui FA, Hargrave T, Kwo PY, Lambiase L, Galler GW, Araya V, Freilich B, Harvey J, Griffel LH, Brass CA. Impact of weight-based ribavirin with peginterferon alfa-2b in African Americans with hepatitis C virus genotype 1. *Hepatology* 2007; **46**: 982-990
- 14 **Dev AT**, McCaw R, Sundararajan V, Bowden S, Sievert W. Southeast Asian patients with chronic hepatitis C: the impact of novel genotypes and race on treatment outcome. *Hepatology* 2002; **36**: 1259-1265
- 15 **Hepburn MJ**, Hepburn LM, Cantu NS, Lapeer MG, Lawitz EJ. Differences in treatment outcome for hepatitis C among ethnic groups. *Am J Med* 2004; **117**: 163-168
- 16 **Missiha S**, Heathcote J, Arenovich T, Khan K. Impact of asian race on response to combination therapy with peginterferon alfa-2a and ribavirin in chronic hepatitis C. *Am J Gastroenterol* 2007; **102**: 2181-2188
- 17 **Bressler BL**, Guindi M, Tomlinson G, Heathcote J. High body mass index is an independent risk factor for nonresponse to antiviral treatment in chronic hepatitis C. *Hepatology* 2003; **38**: 639-644
- 18 **Walsh MJ**, Jonsson JR, Richardson MM, Lipka GM, Purdie DM, Clouston AD, Powell EE. Non-response to antiviral therapy is associated with obesity and increased hepatic expression of suppressor of cytokine signalling 3 (SOCS-3) in patients with chronic hepatitis C, viral genotype 1. *Gut* 2006; **55**: 529-535
- 19 **D'Souza R**, Sabin CA, Foster GR. Insulin resistance plays a significant role in liver fibrosis in chronic hepatitis C and in the response to antiviral therapy. *Am J Gastroenterol* 2005; **100**: 1509-1515
- 20 **Poustchi H**, Negro F, Hui J, Cua IH, Brandt LR, Kench JG, George J. Insulin resistance and response to therapy in patients infected with chronic hepatitis C virus genotypes 2 and 3. *J Hepatol* 2008; **48**: 28-34
- 21 **Bugianesi E**, Marchesini G, Gentilecore E, Cua IH, Vanni E, Rizzetto M, George J. Fibrosis in genotype 3 chronic hepatitis C and nonalcoholic fatty liver disease: Role of insulin resistance and hepatic steatosis. *Hepatology* 2006; **44**: 1648-1655
- 22 **Charlton MR**, Pockros PJ, Harrison SA. Impact of obesity on treatment of chronic hepatitis C. *Hepatology* 2006; **43**: 1177-1186
- 23 **Narita R**, Abe S, Tabaru A, Otsuki M. Impact of steatosis on insulin secretion in chronic hepatitis C patients. *Am J Gastroenterol* 2007; **102**: 2173-2180
- 24 **Bedossa P**, Moucari R, Chelbi E, Asselah T, Paradis V, Vidaud M, Cazals-Hatem D, Boyer N, Valla D, Marcellin P. Evidence for a role of nonalcoholic steatohepatitis in hepatitis C: a prospective study. *Hepatology* 2007; **46**: 380-387
- 25 **Zekry A**, McHutchison JG, Diehl AM. Insulin resistance and steatosis in hepatitis C virus infection. *Gut* 2005; **54**: 903-906
- 26 **Boccatto S**, Pistis R, Noventa F, Guido M, Benvegno L, Alberti A. Fibrosis progression in initially mild chronic hepatitis C. *J Viral Hepat* 2006; **13**: 297-302
- 27 **Poynard T**, Ratziu V, Charlotte F, Goodman Z, McHutchison J, Albrecht J. Rates and risk factors of liver fibrosis progression in patients with chronic hepatitis c. *J Hepatol* 2001; **34**: 730-739
- 28 **Thio CL**, Thomas DL, Goedert JJ, Vlahov D, Nelson KE, Hilgartner MW, O'Brien SJ, Karacki P, Marti D, Astemborski J, Carrington M. Racial differences in HLA class II associations with hepatitis C virus outcomes. *J Infect Dis* 2001; **184**: 16-21
- 29 **Layden-Almer JE**, Ribeiro RM, Wiley T, Perelson AS, Layden TJ. Viral dynamics and response differences in HCV-infected African American and white patients treated with IFN and ribavirin. *Hepatology* 2003; **37**: 1343-1350
- 30 **Kimball P**, Elswick RK, Shiffman M. Ethnicity and cytokine production gauge response of patients with hepatitis C to interferon-alpha therapy. *J Med Virol* 2001; **65**: 510-516
- 31 **Sugimoto K**, Stadanlick J, Ikeda F, Brensinger C, Furth EE, Alter HJ, Chang KM. Influence of ethnicity in the outcome of hepatitis C virus infection and cellular immune response. *Hepatology* 2003; **37**: 590-599
- 32 **Rosen HR**, Weston SJ, Im K, Yang H, Burton JR Jr, Erlich H, Klarquist J, Belle SH. Selective decrease in hepatitis C virus-specific immunity among African Americans and outcome of antiviral therapy. *Hepatology* 2007; **46**: 350-358



## Hypermethylation and aberrant expression of secreted frizzled-related protein genes in pancreatic cancer

Xian-Min Bu, Cheng-Hai Zhao, Ning Zhang, Feng Gao, Shuai Lin, Xian-Wei Dai

Xian-Min Bu, Feng Gao, Shuai Lin, Xian-Wei Dai, Department of General Surgery, Shengjing Hospital, China Medical University, Shenyang 110004, Liaoning Province, China  
Cheng-Hai Zhao, Ning Zhang, Department of Pathophysiology, China Medical University, Shenyang 110001, Liaoning Province, China

**Author contributions:** Bu XM, Zhao CH and Dai XW designed the research; Bu XM, Zhao CH, Zhang N, Gao F and Lin S performed the research; Bu XM and Zhao CH analyzed the data; Bu XM wrote the paper.

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**Correspondence to:** Xian-Wei Dai, Professor of Surgery, Department of General Surgery, Shengjing Hospital of China Medical University, 36 Sanhao Street, Heping District, Shenyang 110001, Liaoning Province, China. [xianweidai@yahoo.com.cn](mailto:xianweidai@yahoo.com.cn)  
Telephone: +86-24-83956512 Fax: +86-24-23892617

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was methylated but not expressed in CFPAC-1.

**CONCLUSION:** Hypermethylation and aberrant expression of SFRP genes are common in pancreatic cancer, which may be involved in pancreatic carcinogenesis.

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**Key words:** Hypermethylation; Secreted frizzled-related protein; Pancreatic cancer

**Peer reviewer:** Kazuhiro Hanazaki, MD, Professor and Chairman, Department of Surgery, Kochi Medical School, Kochi University, Kohasu, Okohcho, Nankoku, Kochi 783-8505, Japan

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### Abstract

**AIM:** To determine the methylation status and aberrant expression of some secreted frizzled-related protein (SFRP) genes in pancreatic cancer and explore their role in pancreatic carcinogenesis.

**METHODS:** Methylation status and expression of SFRP genes were detected by methylation-specific PCR (MSPCR) and reverse-transcription PCR (RT-PCR) respectively.

**RESULTS:** The frequencies of methylation for SFRP genes 1, 2, 4, 5 were 70%, 48.3%, 60% and 76.7% in pancreatic cancer samples, and 21.7%, 20%, 10% and 36.7% in matched cancer adjacent normal tissue samples, respectively ( $\chi^2 = 28.23$ ,  $P < 0.0001$  for SFRP gene 1;  $\chi^2 = 10.71$ ,  $P = 0.001$  for SFRP gene 2;  $\chi^2 = 32.97$ ,  $P < 0.0001$  for SFRP gene 4;  $\chi^2 = 19.55$ ,  $P < 0.0001$  for SFRP gene 5). Expression loss of SFRP genes 1, 2, 4 and 5 was found in 65%, 40%, 55% and 71.7% of 60 pancreatic cancer samples, and 25%, 15%, 18.3% and 31.7% of matched cancer adjacent normal tissue samples, respectively ( $\chi^2 = 19.39$ ,  $P < 0.0001$  for SFRP gene 1;  $\chi^2 = 9.40$ ,  $P = 0.002$  for SFRP gene 2;  $\chi^2 = 17.37$ ,  $P < 0.0001$  for SFRP gene 4;  $\chi^2 = 19.22$ ,  $P < 0.0001$  for SFRP gene 5). SFRP gene 1 was methylated but not expressed in PC-3 and PANC-1, SFRP gene 2 was methylated but not expressed in PANC-1 and CFPAC-1, SFRP gene 4 was methylated but not expressed in PC-3, and SFRP gene 5

### INTRODUCTION

Secreted frizzled-related proteins (SFRPs) are a group of negative regulators of the Wnt signaling pathway<sup>[1-3]</sup>. These proteins contain a cysteine-rich domain (CRD) which shares a sequence similarity of 30%-50% with Wnt receptor frizzled proteins. Through the CRD, SFRPs can antagonize Wnt signaling by interacting with Wnt ligand. As the Wnt signaling pathway plays an important role in cell proliferation, differentiation and apoptosis in adult tissues, aberrant activation of the Wnt pathway caused by down-regulation of SFRPs may induce tumorigenesis. It was recently reported that some members of the SFRP family are down-regulated by hypermethylation in a series of human cancers<sup>[4-9]</sup>.

The prognosis of pancreatic cancer, one of the most malignant tumors, is usually very poor. The pathogenesis of pancreatic cancer is still not very clear. It has been found that hypermethylation and subsequent expression loss of some tumor suppressor genes and tumor-related genes, such as p16<sup>[10]</sup>, RASSF1A<sup>[11]</sup>, SOCS-1<sup>[12]</sup>, and hMLH1<sup>[13]</sup> occur frequently in pancreatic cancer.

This study was designed to determine the methylation status and aberrant expression of some members of the SFRP family in pancreatic cancer and explore their role in pancreatic carcinogenesis.



## MATERIALS AND METHODS

### Cell lines, cancer and matched adjacent tissue samples

Human pancreatic cancer cell lines PC-3, PANC-1 and CFPAC-1 (from KEYGEN, Nanjing, China) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 100 µg/mL penicillin, and 100 µg/mL streptomycin, at 37°C in a humid incubator containing 50 mL/L CO<sub>2</sub>. Pancreatic cancer and matched adjacent tissue samples were obtained from patients who underwent operation at the Second Affiliated Hospital of China Medical University. The samples were frozen in liquid nitrogen immediately after surgery. Haematoxylin and eosin staining was used to assure that cancer samples were consisted mostly of tumor cells with no tumor cells in the tumor adjacent tissue samples.

### DNA and RNA extraction

DNA was extracted by a standard phenol/chloroform extraction and ethanol precipitation procedure. RNA was isolated using Tri reagent (Takara, Dalian, China) according to its manufacturer's instructions.

### Reverse transcription-PCR (RT-PCR)

RT-PCR was performed using a RNA PCR 3.0 kit (Takara, Dalian, China). cDNA was synthesized from 1 µg RNA using a random 9 primer and AMV reverse transcriptase. One cycle was performed at 30°C for 10 min, at 42°C for 25 min, at 99°C for 5 min, and at 5°C for 5 min. The primer sequences used in PCR are described elsewhere<sup>[7]</sup>. PCR was performed for one cycle at 94°C for 2 min, followed by 30 cycles at 94°C for 30 s, at 60°C for 30 s and at 72°C for 2 min.

### Methylation-specific PCR (MSPCR)

Methylation of SFRP1 was detected with a MSPCR kit (GENMED, Shanghai, China) according to its manufacturer's instructions. The primer sequences are described elsewhere<sup>[7]</sup>. MSPCR was performed for one cycle at 95°C for 5 min, followed by 35 cycles at 95°C for 30 s, at 60°C for 30 s and at 72°C for 30 s.

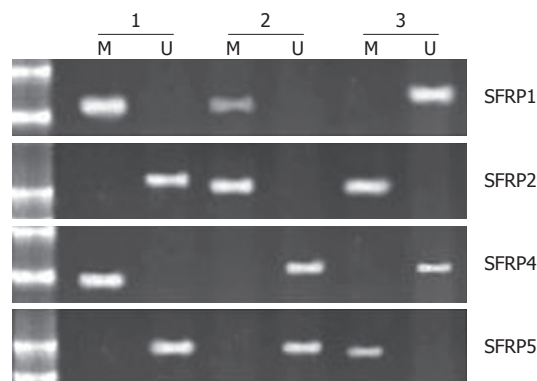
### Statistical analysis

Methylation and expression of SFRP1 in primary pancreatic cancer and its adjacent tissue samples were compared by chi-square test.  $P < 0.05$  was considered statistically significant.

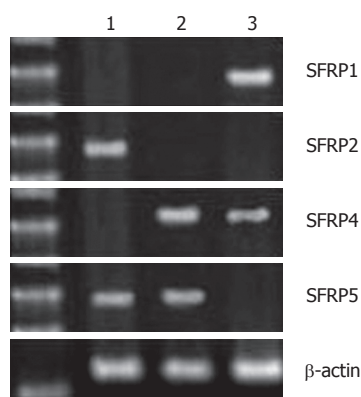
## RESULTS

### Hypermethylation and expression of SFRPs in pancreatic cancer cell lines

The methylation status of SFRPs was detected by MSPCR. SFRP1 was methylated in PC-3 and PANC-1, SFRP2 in PANC-1 and CFPAC-1, SFRP4 in PC-3 and SFRP5 was methylated in CFPAC-1, respectively (Figure 1). The mRNA expression of SFRPs was determined by RT-PCR. No expression of SFRP1, SFRP2, SFRP4 and SFRP5 was found in PC-3 and PANC-1, PANC-1 and CFPAC-1, PC-3 and in CFPAC-1, respectively (Figure 2).



**Figure 1** Hypermethylation of SFRP genes in pancreatic cancer cell lines detected by MSPCR. 1: PC-3; 2: PANC-1; 3: CFPAC-1; M: Methylated; U: Unmethylated.



**Figure 2** Expression of SFRPs in pancreatic cancer cell lines detected by RT-PCR. 1: PC-3; 2: PANC-1; 3: CFPAC-1.

The expression loss of SFRPs was correlated with the methylation status.

### Hypermethylation and expression of SFRPs in pancreatic cancer and its adjacent tissue samples

Hypermethylation of SFRP1, SFRP2, SFRP4 and SFRP5 was detected in 42 (70%), 29 (48.3%), 36 (60%) and 46 (76.7%) of 60 pancreatic cancer samples, and 13 (21.7%), 12 (20%), 6 (10%) and 22 (36.7%) of its adjacent tissue samples, respectively. The hypermethylation of each SFRP gene differed significantly in cancer and its adjacent tissue samples ( $\chi^2 = 28.23$ ,  $P < 0.0001$  for SFRP1;  $\chi^2 = 10.71$ ,  $P = 0.001$  for SFRP 2;  $\chi^2 = 32.97$ ,  $P < 0.0001$  for SFRP 4;  $\chi^2 = 19.55$ ,  $P < 0.0001$  for SFRP 5; Table 1). Expression loss of SFRP1, SFRP2, SFRP4 and SFRP5 was found in 39 (65%), 24 (40%), 33 (55%) and 43 (71.7%) of 60 pancreatic cancer samples, and 15 (25%), 9 (15%), 11 (18.3%) and 19 (31.7%) of its adjacent tissue samples, respectively. The expression loss of each SFRP gene differed significantly in cancer and its adjacent tissue samples ( $\chi^2 = 19.39$ ,  $P < 0.0001$  for SFRP1;  $\chi^2 = 9.40$ ,  $P = 0.002$  for SFRP2;  $\chi^2 = 17.37$ ,  $P < 0.0001$  for SFRP4;  $\chi^2 = 19.22$ ,  $P < 0.0001$  for SFRP5; Table 2).

## DISCUSSION

The Wnt signaling pathway plays an important role not only in development of cancer but also in cell proliferation, differentiation and apoptosis in adult tissues.

**Table 1** Hypermethylation of SFRPs in pancreatic cancer and its adjacent tissue samples

	<i>n</i>	SFRR1	SFRR2	SFRP4	SFRP5
Pancreatic cancer samples	60	42	29	36	46
Adjacent tissue samples	60	13	12	6	22
$\chi^2$		28.23	10.71	32.97	19.55
<i>P</i>		< 0.0001	0.001	< 0.0001	< 0.0001

**Table 2** Expression loss of SFRPs in pancreatic cancer and its adjacent tissue samples

	<i>n</i>	SFRR1	SFRR2	SFRP4	SFRP5
Pancreatic cancer samples	60	39	24	33	43
Adjacent tissue samples	60	15	9	11	19
$\chi^2$		19.39	9.40	17.37	19.22
<i>P</i>		< 0.0001	0.002	< 0.0001	< 0.0001

Aberrant activation of Wnt signaling in tumorigenesis has been reported frequently, and some members of the Wnt family are over-expressed in breast cancer, gastrointestinal cancer and prostate cancer<sup>[14-16]</sup>. Down-regulation of the Wnt inhibitors DKKs and SFRPs also occurs frequently in human cancers<sup>[17,18]</sup>. Most of these reports show that expression loss of these inhibitors is mainly caused by promoter hypermethylation, an important epigenetic gene silencing mechanism.

Aberrant Wnt signals are also involved in pancreatic cancer. It was reported that activated mutation of  $\beta$ -catenin on exon 3, a downstream component in the Wnt signaling pathway, plays an important role in pancreatic tumorigenesis. This kind of mutation leads to excessive accumulation of  $\beta$ -catenin and aberrant activation of the Wnt pathway<sup>[19-23]</sup>. Over-expression of many members of the Wnt family, such as Wnt1<sup>[24]</sup>, Wnt5a<sup>[25]</sup>, Wnt5b<sup>[25]</sup>, Wnt7a<sup>[26]</sup>, Wnt10b<sup>[27]</sup> in pancreatic cancer, has been reported in recent years, further suggesting that the Wnt pathway plays a role in the pathogenesis of pancreatic cancer. It has recently been shown that epigenetic inactivation of Wnt inhibitory factor 1 by hypermethylation occurs frequently in pancreatic cancer<sup>[28]</sup>.

The pathogenesis of pancreatic cancer, a very malignant carcinoma, has been poorly understood. In this study, we analyzed the hypermethylation and expression of SFRPs in pancreatic cancer and explored their role in pancreatic carcinogenesis, showing that hypermethylation and expression loss of SFRPs occur frequently in pancreatic cancer. The frequencies of hypermethylation and expression loss of SFRPs in pancreatic cancer samples were significantly higher than those in its adjacent normal tissue samples, suggesting that hypermethylation and subsequent expression loss of SFRPs occur early and play an important role in the pathogenesis of pancreatic cancer.

As we know, Wnt signaling can be divided into canonical Wnt/ $\beta$ -catenin pathway and non-canonical pathway which includes the planar cell polarity pathway and the Wnt/ $\text{Ca}^{2+}$  pathway. As we did not measure the level of  $\beta$ -catenin, we could not determine whether the pathway through which SFRP1 expression loss is involved in

the pancreatic carcinogenesis. Further study is needed to elucidate its mechanism.

## COMMENTS

### Background

Secreted frizzled-related proteins (SFRPs) are a group of negative regulators of the Wnt signaling pathway. Aberrant activation of the Wnt pathway caused by down-regulation of SFRPs may induce tumorigenesis.

### Research frontiers

The pathogenesis of pancreatic cancer, a very malignant carcinoma, is poorly understood. In this study, we found that hypermethylation status and expression of SFRPs played an important role in pancreatic carcinogenesis.

### Innovations and breakthroughs

In this study, we analyzed the hypermethylation status and expression of SFRPs in pancreatic cancer and explored their role in pancreatic carcinogenesis.

### Applications

Our study suggested that hypermethylation and subsequent expression loss of SFRPs play an important role in pancreatic carcinogenesis. For this reason, demethylated agents may be used to treat cancer in clinical practice.

### Peer review

In this study, the authors reported that the expression loss of some SFRP genes caused by hypermethylation was common in pancreatic cancer, which may play an important role in pancreatic carcinogenesis. This paper is original and informative.

## REFERENCES

- 1 **Finch PW**, He X, Kelley MJ, Uren A, Schaudies RP, Popescu NC, Rudikoff S, Aaronson SA, Varmus HE, Rubin JS. Purification and molecular cloning of a secreted, Frizzled-related antagonist of Wnt action. *Proc Natl Acad Sci USA* 1997; **94**: 6770-6775
- 2 **Melkonyan HS**, Chang WC, Shapiro JP, Mahadevappa M, Fitzpatrick PA, Kiefer MC, Tomei LD, Umansky SR. SFRPs: a family of secreted apoptosis-related proteins. *Proc Natl Acad Sci USA* 1997; **94**: 13636-13641
- 3 **Rattner A**, Hsieh JC, Smallwood PM, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J. A family of secreted proteins contains homology to the cysteine-rich ligand-binding domain of frizzled receptors. *Proc Natl Acad Sci USA* 1997; **94**: 2859-2863
- 4 **Ugolini F**, Charafe-Jauffret E, Bardou VJ, Geneix J, Adelaide J, Labat-Moleur F, Penault-Llorca F, Longy M, Jacquemier J, Birnbaum D, Pebusque MJ. WNT pathway and mammary carcinogenesis: loss of expression of candidate tumor suppressor gene SFRP1 in most invasive carcinomas except of the medullary type. *Oncogene* 2001; **20**: 5810-5817
- 5 **Caldwell GM**, Jones C, Gensberg K, Jan S, Hardy RG, Byrd P, Chughtai S, Wallis Y, Matthews GM, Morton DG. The Wnt antagonist sFRP1 in colorectal tumorigenesis. *Cancer Res* 2004; **64**: 883-888
- 6 **Takada T**, Yagi Y, Maekita T, Imura M, Nakagawa S, Tsao SW, Miyamoto K, Yoshino O, Yasugi T, Taketani Y, Ushijima T. Methylation-associated silencing of the Wnt antagonist SFRP1 gene in human ovarian cancers. *Cancer Sci* 2004; **95**: 741-744
- 7 **Zou H**, Molina JR, Harrington JJ, Osborn NK, Klatt KK, Romero Y, Burgart LJ, Ahlquist DA. Aberrant methylation of secreted frizzled-related protein genes in esophageal adenocarcinoma and Barrett's esophagus. *Int J Cancer* 2005; **116**: 584-591
- 8 **Lodygin D**, Epanchintsev A, Menssen A, Diebold J, Hermeking H. Functional epigenomics identifies genes frequently silenced in prostate cancer. *Cancer Res* 2005; **65**: 4218-4227
- 9 **Zhao CH**, Bu XM, Zhang N. Hypermethylation and aberrant expression of Wnt antagonist secreted frizzled-related protein 1 in gastric cancer. *World J Gastroenterol* 2007; **13**: 2214-2217

- 10 **Attri J**, Srinivasan R, Majumdar S, Radotra BD, Wig J. Alterations of tumor suppressor gene p16INK4a in pancreatic ductal carcinoma. *BMC Gastroenterol* 2005; **5**: 22
- 11 **Dammann R**, Schagdarsurengin U, Liu L, Otto N, Gimm O, Dralle H, Boehm BO, Pfeifer GP, Hoang-Vu C. Frequent RASSF1A promoter hypermethylation and K-ras mutations in pancreatic carcinoma. *Oncogene* 2003; **22**: 3806-3812
- 12 **Komazaki T**, Nagai H, Emi M, Terada Y, Yabe A, Jin E, Kawanami O, Konishi N, Moriyama Y, Naka T, Kishimoto T. Hypermethylation-associated inactivation of the SOCS-1 gene, a JAK/STAT inhibitor, in human pancreatic cancers. *Jpn J Clin Oncol* 2004; **34**: 191-194
- 13 **House MG**, Herman JG, Guo MZ, Hooker CM, Schulick RD, Cameron JL, Hruban RH, Maitra A, Yeo CJ. Prognostic value of hMLH1 methylation and microsatellite instability in pancreatic endocrine neoplasms. *Surgery* 2003; **134**: 902-908; discussion 909
- 14 **Blavier L**, Lazaryev A, Dorey F, Shackelford GM, DeClerck YA. Matrix metalloproteinases play an active role in Wnt1-induced mammary tumorigenesis. *Cancer Res* 2006; **66**: 2691-2699
- 15 **Katoh M**. WNT2 and human gastrointestinal cancer (review). *Int J Mol Med* 2003; **12**: 811-816
- 16 **Verras M**, Brown J, Li X, Nusse R, Sun Z. Wnt3a growth factor induces androgen receptor-mediated transcription and enhances cell growth in human prostate cancer cells. *Cancer Res* 2004; **64**: 8860-8866
- 17 **Byun T**, Karimi M, Marsh JL, Milovanovic T, Lin F, Holcombe RF. Expression of secreted Wnt antagonists in gastrointestinal tissues: potential role in stem cell homeostasis. *J Clin Pathol* 2005; **58**: 515-519
- 18 **Katoh Y**, Katoh M. Comparative genomics on DKK2 and DKK4 orthologs. *Int J Mol Med* 2005; **16**: 477-481
- 19 **Tanaka Y**, Kato K, Notohara K, Hojo H, Ijiri R, Miyake T, Nagahara N, Sasaki F, Kitagawa N, Nakatani Y, Kobayashi Y. Frequent beta-catenin mutation and cytoplasmic/nuclear accumulation in pancreatic solid-pseudopapillary neoplasm. *Cancer Res* 2001; **61**: 8401-8404
- 20 **Miao J**, Kusafuka T, Kuroda S, Yoneda A, Zhou Z, Okada A. Mutation of beta-catenin and its protein accumulation in solid and cystic tumor of the pancreas associated with metastasis. *Int J Mol Med* 2003; **11**: 461-464
- 21 **Dessimoz J**, Grapin-Botton A. Pancreas development and cancer: Wnt/beta-catenin at issue... *Cell Cycle* 2006; **5**: 7-10
- 22 **Zeng G**, Germinaro M, Micsenyi A, Monga NK, Bell A, Sood A, Malhotra V, Sood N, Midda V, Monga DK, Kokkinakis DM, Monga SP. Aberrant Wnt/beta-catenin signaling in pancreatic adenocarcinoma. *Neoplasia* 2006; **8**: 279-289
- 23 **Lowy AM**, Fenoglio-Preiser C, Kim OJ, Kordich J, Gomez A, Knight J, James L, Groden J. Dysregulation of beta-catenin expression correlates with tumor differentiation in pancreatic duct adenocarcinoma. *Ann Surg Oncol* 2003; **10**: 284-290
- 24 **Katoh M**. Expression and regulation of WNT1 in human cancer: up-regulation of WNT1 by beta-estradiol in MCF-7 cells. *Int J Oncol* 2003; **22**: 209-212
- 25 **Saitoh T**, Katoh M. Expression and regulation of WNT5A and WNT5B in human cancer: up-regulation of WNT5A by TNFalpha in MKN45 cells and up-regulation of WNT5B by beta-estradiol in MCF-7 cells. *Int J Mol Med* 2002; **10**: 345-349
- 26 **Kirikoshi H**, Katoh M. Expression of WNT7A in human normal tissues and cancer, and regulation of WNT7A and WNT7B in human cancer. *Int J Oncol* 2002; **21**: 895-900
- 27 **Kirikoshi H**, Katoh M. Expression and regulation of WNT10B in human cancer: up-regulation of WNT10B in MCF-7 cells by beta-estradiol and down-regulation of WNT10B in NT2 cells by retinoic acid. *Int J Mol Med* 2002; **10**: 507-511
- 28 **Taniguchi H**, Yamamoto H, Hirata T, Miyamoto N, Oki M, Nosho K, Adachi Y, Endo T, Imai K, Shinomura Y. Frequent epigenetic inactivation of Wnt inhibitory factor-1 in human gastrointestinal cancers. *Oncogene* 2005; **24**: 7946-7952

S- Editor Zhong XY L- Editor Wang XL E- Editor Liu Y



RAPID COMMUNICATION

## Pancreaticoduodenectomy for advanced gastric cancer with pancreaticoduodenal region involvement

Xin-Bao Wang, Li-Tao Yang, Ze-Wei Zhang, Jian-Min Guo, Xiang-Dong Cheng

Xin-Bao Wang, Li-Tao Yang, Ze-Wei Zhang, Jian-Min Guo, Xiang-Dong Cheng, Department of Hepato-Biliary-Pancreatic-Gastric Surgery, Zhejiang Cancer Hospital, Hangzhou 310022, Zhejiang Province, China

Author contributions: Wang XB performed the operation and wrote the paper; Yang LT analyzed the data; Zhang ZW followed up the patients; Guo JM and Cheng XD performed the operation.

Correspondence to: Xin-Bao Wang, Department of Hepato-Biliary-Pancreatic-Gastric Surgery, Zhejiang Cancer Hospital, Hangzhou 310022, Zhejiang Province, China. [wangxinbao1964@sohu.com](mailto:wangxinbao1964@sohu.com)

Telephone: +86-571-88122012 Fax: +86-571-88122501

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### Abstract

**AIM:** To characterize the factors of the improved survival following combined pancreaticoduodenectomy (PD) and gastrectomy for the treatment of advanced gastric cancer with pancreaticoduodenal region involvement.

**METHODS:** From 1995 to 2004, 53 patients with primary gastric cancer were diagnosed with synchronous ( $n = 44$ ) or metachronous ( $n = 9$ ) pancreaticoduodenal region involvement. Of these, 17 patients (32%) underwent total gastrectomy (TG) or distal subtotal gastrectomy (SG) combined with PD simultaneously. The preoperative demographic, clinical information, clinicopathologic features and the surgical results of these 17 patients were considered as factors influencing survival and were analyzed by the Kaplan-Meier method with log-rank comparison.

**RESULTS:** The actual 1- and 3-year survival rates of these 17 patients after resection were 77% and 34%, respectively, and three patients survived for more than 5 years after surgery. The tumor-free resection margin ( $P = 0.0174$ ) and a well-differentiated histologic type ( $P = 0.0011$ ) were significant prognostic factors on univariate analysis. No mortality occurred within one mo after operation, postoperative weight loss of different degree was present in all the patients with TG and 12 cases had other complications. There were 9 (53%) cases of recurrence in 5-48 mo after operation. The survival rate in the palliative and explorative group was significantly ( $P = 0.0064$ ) lower than in the combined PD group.

**CONCLUSION:** Judicious use of en bloc PD and gastrectomy and strictly preventing postoperative complications may improve the long-term survival for advanced gastric cancer patients with pancreaticoduodenal region involvement. Well-differentiated histology and negative resection margin are the most important predictors of long survival.

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**Key words:** Pancreaticoduodenectomy; Gastric cancer; Gastrectomy; Predictive factor; Patients

**Peer reviewer:** Harry HX Xia, PhD, MD, Novartis Pharmaceuticals Corporation, One Health Plaza, East Hanover, NJ 07936-1080, United States

Wang XB, Yang LT, Zhang ZW, Guo JM, Cheng XD. Pancreaticoduodenectomy for advanced gastric cancer with pancreaticoduodenal region involvement. *World J Gastroenterol* 2008; 14(21): 3425-3429 Available from: URL: <http://www.wjg-net.com/1007-9327/14/3425.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3425>

### INTRODUCTION

Because of earlier diagnosis, more accurate staging and safer operations, outcomes after treatment of gastric cancer are being improved, especially for early gastric cancer the results have become satisfactory<sup>[1]</sup>. And in the treatment of advanced carcinoma of the stomach, gastrectomy with extensive lymph node dissection has been reported to acquire a substantial improvement in survival<sup>[2-4]</sup>. In spite of these advances, there were still some problems as for areas of resection for advanced gastric cancer involving local organs. Arguments against enlarged resection are based on the observed increase in the morbidity and mortality rates, with little objective benefit in survival<sup>[5]</sup>. And some surgeons still consider the invasion of adjacent organs by the carcinoma of stomach as a sign of incurable disease. But others believe patients with T4 gastric cancer will benefit from extended en bloc surgical resection<sup>[6-8]</sup>.

For the anatomic reason, pancreaticoduodenal region involved in advanced gastric cancer was not scarce. But few articles about its surgical treatment were available. The aim of this study was to report our experience in undergoing pancreaticoduodenectomy



(PD) with gastrectomy for advanced gastric cancer with pancreaticoduodenal region involvement.

## MATERIALS AND METHODS

### Patients

From January 1995 to January 2004, 916 patients with gastric carcinoma underwent surgical treatment in the Department of Hepatobiliary-Pancreatic-Gastric Surgery, Zhejiang Cancer Hospital. Of the 916 cases, 44 were found to have synchronous pancreaticoduodenal region involved and 9 metachronous pancreaticoduodenal region invaded or involved. Among the 53 patients, palliative gastrectomy was performed in 6 patients, bypass through exploration performed in 14, explorations in 7 and surgical treatment was given up in 9 because of additional organs metastasis or poor physical state. And 17 patients who underwent PD with gastrectomy were selected for this study, including 11 men and 6 women with a mean age of 56 years (range from 38 to 71 years). Overall radical resectability was 32.1% (17/53) for the 53 patients, 32% (14/44) for synchronous metastases, and 33.3% (3/9) for metachronous lesions. Follow-up period ranged from 2 to 72 mo (median 38 mo).

### Methods

PD was indicated for patients with visibly synchronous pancreaticoduodenal region involved lesions who did not have peritoneal dissemination or any other distant metastasis, or for patients with metachronous pancreaticoduodenal region involved who did not have any other recurrent lesions. Three patients who had a desmoplastic reaction at the site of presumed tumor invasion were not included in this study. Confirmation of cancerous invasion of pancreaticoduodenal region was established histologically in operation. In addition to PD, TG was done in 11 (64.4%) and SG in 6 (35.6%) patients, depending on the location of the primary gastric lesion. En bloc surgical resection and D2 lymphadenectomy were performed as the standard radical gastrectomy for these 17 patients. As for reconstruction of digestive tract, binding pancreaticojejunostomy<sup>[9]</sup> and Roux-en-Y anastomosis were adopted for all the cases. In this group, two patients underwent right hemicolectomy and one patient underwent cholangiocystectomy additionally at the same time. All patients were treated with postoperative adjuvant chemotherapy using the same chemotherapy regimens (ELF regimens: etoposide + leucovorin + fluorouracil)<sup>[10,11]</sup>.

The preoperative demographic and clinical information was obtained from the patient records: age, gender, interval between gastrectomy and PD, surgical procedure and recurrence. The number and size of the tumors, extent of lymph node metastasis and surgical margin of the tumors were also recorded. The pathologic diagnosis and classification of the tumors were performed by a minimum of two pathologists.

### Data analysis

All data were treated with statistic software kit of

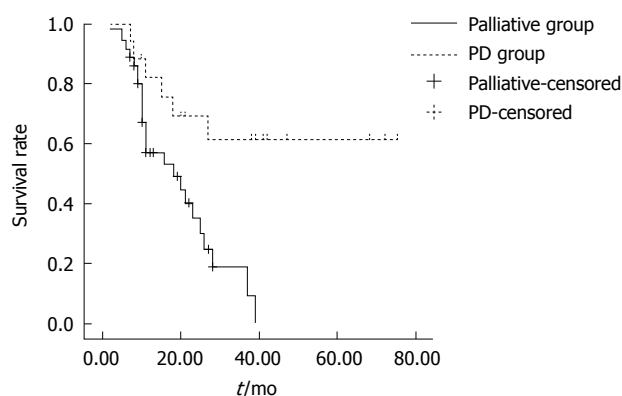
**Table 1** Characteristics and their prognostic significance for PD group

Factors	Number of patients	P
Age (yr)		0.1405
< 56	7	
≥ 56	10	
Gender		0.4412
Male	11	
Female	6	
Metastases		0.2010
Synchronous	14	
Metachronous	3	
Tumor size		0.9837
< 4 cm	8	
≥ 4 cm	9	
Histologic differentiation		0.0011
Well	4	
Moderate or poor	13	
Gastric carcinoma depth of invasion		0.0610
Borrmann III	6	
Borrmann IV	11	0.0516
Lymph node metastasis		
Positive	10	
Negative	7	0.7948
Gastrectomy pattern		
Total gastrectomy	11	
Subtotal gastrectomy	6	0.0174
Resection margins		
Positive	5	
Negative	12	0.1486
Combined other organs		
No	14	
Yes	3	

SPSS 12.0. Parameters influencing survival were compared using the Kaplan-Meier method with log-rank comparison.  $P < 0.05$  was considered significant differences.

## RESULTS

No patient died during the initial hospital stay or within 1 mo after surgery. The median diameter of the tumors was 4.0 cm (range 2.8-9.5 cm). Only four patients had well differentiated tumors and the other 13 patients had moderately or poorly differentiated tumors. According to Borrmann Type, six patients were Borrmann III and 11 were Borrmann IV. Ten patients had positive lymph node metastasis and seven had negative lymph node metastasis. Resection margins in five patients were tumor-positive and 12 were tumor-free. The actual 1- and 3-year survival rates after PD with gastrectomy were 77% and 34%, respectively. The results of the analysis of the prognostic factors are given in Table 1. Tumor-free (negative) resection margin ( $P = 0.0174$ ) was significant determinants for a favorable prognosis after PD. In terms of pathologic features, a well-differentiated type of metastases ( $P = 0.0011$ ) was a significant prognostic factor. Factors associated with the primary lesion and surgical procedures were not significant prognostic determinants. Cancer recurred in 11 (59%) of the 17 patients between 5 mo and 48 mo after PD resection. The site of initial recurrence after PD and gastrectomy was the gastric and pancreaticoduodenal



**Figure 1** Comparison of survival between patients with palliative surgery and PD. Significant difference was found between the two groups ( $P = 0.0064$ ).

bed in 6 patients, the liver in 3, and the retroperitoneal lymph nodes in 2, which were diagnosed by image methods (CT, MRI or US). Three patients survived more than 5 years after PD. No mortality occurred in one mo after operation, postoperative weight loss of different degree was present in all the patients with TG. Twelve had other complications among the 17 patients, including intra-abdominal abscesses 5 (41.7%) and anastomotic leak 3 (gastrointestinal leak 1 and bile leak 2) (25%), pneumonia 2 (16.7%), returned esophagitis 1 (8.3%) and acute renal failure 1 (8.3%). All the complications were cured by operative or conservative treatment.

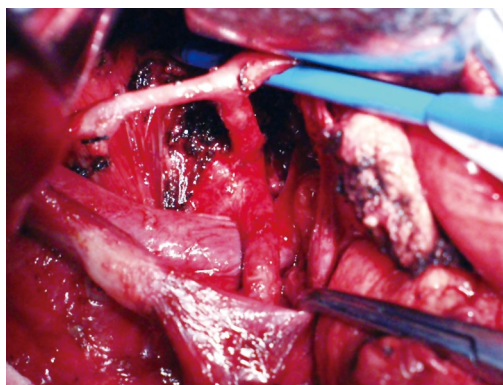
Of the 36 patients with pancreaticoduodenal region involvement who did not undergo radical resection, the actual 1- and 3-year survival rates were 41.7%(15/36) and 5.6%(2/36), respectively. The survival rate of these 36 patients (palliative group) was significantly ( $P = 0.0064$ ) lower than that of PD group (Figure 1).

## DISCUSSION

Surgery remains the only method of treatment that offers the potential for cure of gastric cancer<sup>[12]</sup>. But gastric cancer is usually diagnosed at an advanced stage because of its vague and nonspecific symptoms. And poor survival will be followed by late stage, especially when tumors invade the serosa or other organs. There is a report that with serosal involvement alone, less than 30% of the patients are living five years after surgery, and with involvement of both serosa and lymph nodes, the 5-year survival is less than 15%<sup>[13]</sup>. At the same time, opinions about extended surgical resections for advanced gastric cancer remains controversial. Takeuchi *et al*<sup>[12]</sup> retrospectively studied 65 patients without distant metastasis who underwent TG with pancreaticosplenectomy (PS) and 98 patients without distant metastasis who underwent TG alone (the TG alone group) by essentially the same technique, and concluded that combined PS with TG should never be performed as the standard surgical procedure for every stage of gastric cancer, especially stage II. But we think that in his report the PS was performed with TG to facilitate dissection of the lymph nodes around the splenic artery and splenic hilus, but not performed to

resect the involved lesions of spleen or pancreas tail.

On the other hand, some authors agreed to extend gastric resection in patients with adjacent organs involved. In the report by Kodama *et al*<sup>[14]</sup>, 77 patients with carcinoma of the stomach directly invading adjacent organs or structures were analyzed retrospectively to investigate the efficacy of en bloc resection. Forty-one patients underwent gastrectomy combined with resection of one or more invaded organs (combined resection group), while the other 36 patients underwent gastrectomy with palliative abrasion between the primary tumor and the invasion site (non-combined resection group). The results demonstrated that the five-year survival rate was 23% in the combined resection group and 0% in the non-combined resection group. They thought that an en bloc combined resection would be worth trying. Iriyama *et al*<sup>[15]</sup> reported the highest 5-year survival rate of 46% after extended gastric resection with adjacent organs in patients with T4 gastric cancer. And Korenaga *et al*<sup>[16]</sup> reported that the 5-year survival was 36.7% for those who underwent radical resection of adjacent organs and 17.4% for those who underwent palliative resection of adjacent involved organs. Ozaki *et al*<sup>[17]</sup> and others<sup>[16]</sup> have found that an aggressive approach to resection of the stomach with the body and tail of the pancreas or PD and right hemicolectomy can lead to an acceptable 5-year survival rate of 29%. Yonemura *et al*<sup>[18]</sup> have performed 26 SG with PD in combination with right hemicolectomy without any operative mortality and a 5-year survival of 33% even for patients with N3 metastases (e.g. retropancreatic nodes or superior mesenteric nodes). Cho *et al*<sup>[19]</sup> reported their 15-year experience of extended gastrectomy for advanced gastric cancer. The median survival time of the positive margin group was 34 mo. The negative margin group had a significantly longer median survival of 69 mo ( $P = 0.025$ ). When both groups of patients were stratified according to nodal stage, a positive resection margin determined a worse prognosis only in patients with node-negative disease (174 mo *vs* 37 mo,  $P = 0.0001$ ). In patients with nodal metastasis, the median survival time was similar in both groups. Their results suggested that a positive microscopic margin was associated with a worse outcome in patients with node-negative disease. Therefore, a more aggressive treatment, such as reoperation, was needed in node-negative patients with a positive microscopic disease. Our results have shown that three patients survived more than five years in radical resection group (PD group) and no patient survived more than five years in the palliative group. And the actual survival of these two groups is statistically different. Although factors associated with the primary lesion, patient demographic data and surgical procedures were not significant prognostic determinants, but negative resection margin was very important for the higher survival rate. And when pancreaticoduodenal region is involved by advanced gastric cancer, combined PD and gastrectomy will bring the chance of tumor-free resection margin. And curative (R0) resection improves prognosis<sup>[19,20]</sup>. PD with gastrectomy will benefit the lymphadenectomy of No. 7, 8, 9 and 11. Sometimes, it



**Figure 2** Lymphadenectomy of lymph nodes of No. 7, 8, 9, 11 and 16.

is helpful for lymphadenectomy of No. 16 (Figure 2), though lymph nodes of the No.16 were not resected routinely in our group. We proposed the indication of PD with gastrectomy as follows: (1) head of pancreas was invaded by gastric cancer, (2) metastasis of lymph nodes of No.6 and head of pancreas was infiltrated, (3) duodenum below pylorus 2 cm was invaded by gastric cancer, and (4) the inferior segment of common bile duct was invaded by gastric cancer. Occasionally, the above condition was not found by preoperative examination. In our group, three cases were not found till during operations. So it is necessary to check the head of pancreas, the inferior segment of common bile duct and the superior segment of duodenum below pylorus during operations when tumor was near the pancreaticoduodenal region. As for the pattern of gastrectomy, we adopted TG or SG according to the different locations of the cancer within the stomach, its pattern of growth, and the level of local spread. Subtotal gastrectomies were reserved for exophytic and small infiltrative tumors located in the lower third of the stomach. Total gastrectomy was used for tumors located in the middle and upper third of the stomach, or tumors with an infiltrative growth pattern. Our results manifested that tumor-free resection margins will benefit the survival. And we emphasize that frozen-histologic examination during operation should benefit both the definition of resection margins and the definition of tumor invasion on pancreaticoduodenal region. Large inflammatory perigastric lymph nodes or desmoplastic reaction around gastric cancer can be inaccurately presumed to be tumor invasion and adopt extended gastric resection mistakenly. Three patients who had desmoplastic reaction at the site of presumed tumor invasion were found in our hospital during operation, the pancreas and duodenum were reserved. And no inflammatory perigastric lymph nodes or desmoplastic reaction around gastric cancer were found during postoperative histologic examination in our data.

Upon decision of extended resection, as the procedure of PD and gastrectomy was relatively complicated and status of most patients was poor, a high complication rate could not be ignored. The complication rates of additional organ resection with gastrectomy have been consistently reported to be higher when compared with the patients

undergoing gastrectomy alone<sup>[21-23]</sup>. And the increasing overall complications and infectious complications have been found to be factors for the decrease in the survival of the patients. So, reinforcing perioperative management is a key point to prevent postoperative complications. Our data demonstrate that the overall complication rate was very high, and postoperative weight loss of different degree was present in all the patients with TG. Judicious use of additional PD with gastrectomy is also important for reducing postoperative complications. If a patient could not tolerate a prolonged operation, we would take simple operative method such as bypass operation. And simple gastroenterostomy or esophagojejunostomy should be adopted to reduce postoperative complications and postoperative nutritional support would improve the state of weight loss.

We adopted total parenteral nutrition (TPN) at the early stage after TG to spur positive nitrogen balance and reduce weight loss, and after an interval we adopted suitable enteral nutrition to reduce complications. A leak or fistula from the pancreatic anastomosis is the leading cause of morbidity and mortality after PD, but no pancreaticoenteral anastomosis leak occurred in our study, as we adopted binding pancreaticojejunostomy<sup>[9]</sup>, by which 3 cm of the serosa-muscular sheath of the jejunum was bound to the pancreatic remnant and could effectively prevent the development of pancreatic leak or fistula. It is a safe, simple and efficient technique.

Our study confirmed that patients with advanced gastric cancer could benefit from aggressive en bloc surgical resection and should not render unresectable when pancreaticoduodenal region was found invaded or involved. With careful patient selection, gastrectomy with PD can be performed with acceptable morbidity and minimum mortality. Well histologic differentiation and negative resection margin are the most powerful determinants of survival following an extended resection. The survival rate in the palliative and explorative group was significantly ( $P = 0.0064$ ) lower than in the combined PD group, partially because that their baseline conditions were different. And in this article, we did not analyze the survival rates of patients with TG or SG, because the number of the patients was too large to follow up. We have no complete data of these patients. So we can not compare the survival rates between patients with TG or SG and those with TG or SG combined with PD. It is the default of this study.

## COMMENTS

### Background

For advanced gastric carcinoma, gastrectomy with extensive lymph node dissection has been reported to acquire a substantial improvement in survival. But there are still some problems as for areas of resection for advanced gastric cancer involved local organs. For the anatomic reason, pancreaticoduodenal region involved in advanced gastric cancer was not scarce. But few articles about its surgical treatment have been reported. This study was to report the authors' experience in undergoing pancreaticoduodenectomy (PD) and gastrectomy for advanced gastric cancer with pancreaticoduodenal region involved.

### Research frontiers

The focus of controversy for the advanced gastric cancer involved organ



is either performing the extensive resection or giving up surgical resection. Arguments against enlarged resection are based on the observed increase in the morbidity and mortality rates, with little objective benefit in survival. And some surgeons still consider the invasion of adjacent organs by the carcinoma of stomach as a sign of incurable disease. But others believe patients with T4 gastric cancer will benefit from extended en bloc surgical resection.

### Innovations and breakthroughs

This study confirmed that patients with advanced gastric cancer could benefit from aggressive en bloc surgical resection, which should not be rendered unresectable when pancreaticoduodenal region was found invaded or involved. Negative resection margin is an important factor for the patients with extended resection.

### Applications

For the patients with advanced gastric cancer with pancreaticoduodenal region invaded, PD and extensive resection should be performed if the status of the patients permits.

### Peer review

This retrospective study assessed the performance of PD for advanced gastric cancer with pancreaticoduodenal region involvement, and observed that the 1-year and 3-year survival rates after total gastrectomy (TG) or distal subtotal gastrectomy (SG) combined with PD were 77% and 34%, which were significantly higher than in the patients with palliative treatment. It was also found that histological differentiation and negative resection margin were most important predictors.

## REFERENCES

- 1 Itoh H, Oohata Y, Nakamura K, Nagata T, Mibu R, Nakayama F. Complete ten-year postgastrectomy follow-up of early gastric cancer. *Am J Surg* 1989; **158**: 14-16
- 2 Lee JS, Douglass HO Jr. D2 dissection for gastric cancer. *Surg Oncol* 1997; **6**: 215-225
- 3 Ikeguchi M, Oka S, Gomyo Y, Tsujitani S, Maeta M, Kaibara N. Prognostic benefit of extended radical lymphadenectomy for patients with gastric cancer. *Anticancer Res* 2000; **20**: 1285-1289
- 4 Pugliese R, Maggioni D, Berardi V, Scandroglio I, Pisani D, Mariani A, Di Lernia S, Valli C, Cocotta E. Extended (D2) lymphadenectomy in gastric cancer: a five year experience. *Int Surg* 2000; **85**: 209-215
- 5 van de Velde CJ. Resection for gastric cancer in the community. *Semin Oncol* 2005; **32**: S90-S93
- 6 Shchepotin IB, Chorny VA, Nauta RJ, Shabahang M, Buras RR, Evans SR. Extended surgical resection in T4 gastric cancer. *Am J Surg* 1998; **175**: 123-126
- 7 Martin RC 2nd, Jaques DP, Brennan MF, Karpeh M. Extended local resection for advanced gastric cancer: increased survival versus increased morbidity. *Ann Surg* 2002; **236**: 159-165
- 8 Okano K, Maeba T, Ishimura K, Karasawa Y, Goda F, Wakabayashi H, Usuki H, Maeta H. Hepatic resection for metastatic tumors from gastric cancer. *Ann Surg* 2002; **235**: 86-91
- 9 Peng S, Mou Y, Cai X, Peng C. Binding pancreaticojejunostomy is a new technique to minimize leakage. *Am J Surg* 2002; **183**: 283-285
- 10 Wilke H, Preusser P, Fink U, Achterrath W, Mayer HJ, Stahl M, Lenaz L, Meyer J, Siewert JR, Gerlings H. New developments in the treatment of gastric carcinoma. *Cancer Treat Res* 1991; **55**: 363-373
- 11 Schulze-Bergkamen H, Zuna I, Teufel A, Stremmel W, Rudi J. Treatment of advanced gastric cancer with etoposide, folinic acid, and fluorouracil in the clinical setting: efficacy of therapy and value of serum tumor markers. *Med Oncol* 2002; **19**: 43-53
- 12 Takeuchi K, Tsuzuki Y, Ando T, Sekihara M, Hara T, Yoshikawa M, Ohno Y, Kuwano H. Total gastrectomy with distal pancreatectomy and splenectomy for advanced gastric cancer. *J Surg Res* 2001; **101**: 196-201
- 13 Fuchs CS, Mayer RJ. Gastric carcinoma. *N Engl J Med* 1995; **333**: 32-41
- 14 Kodama I, Takamiya H, Mizutani K, Ohta J, Aoyagi K, Kofuji K, Takeda J, Shirouzu K. Gastrectomy with combined resection of other organs for carcinoma of the stomach with invasion to adjacent organs: clinical efficacy in a retrospective study. *J Am Coll Surg* 1997; **184**: 16-22
- 15 Iriyama K, Ohsawa T, Tsuchibashi T, Noji M, Miki C, Ilunga K, Suzuki H. Results of combined resection of invaded organs in patients with potentially curable, advanced gastric cancer. *Eur J Surg* 1994; **160**: 27-30
- 16 Korenaga D, Okamura T, Baba H, Saito A, Sugimachi K. Results of resection of gastric cancer extending to adjacent organs. *Br J Surg* 1988; **75**: 12-15
- 17 Ozaki H, Kinoshita T, Kosuge T, Yamamoto J, Shimada K, Inoue K, Koyama Y, Mukai K. An aggressive therapeutic approach to carcinoma of the body and tail of the pancreas. *Cancer* 1996; **77**: 2240-2245
- 18 Yonemura Y, Ooyama S, Matumoto H, Kamata T, Kimura H, Takegawa S, Kosaka T, Yamaguchi A, Miwa K, Miyazaki I. Pancreaticoduodenectomy in combination with right hemicolectomy for surgical treatment of advanced gastric carcinoma located in the lower half of the stomach. *Int Surg* 1991; **76**: 226-229
- 19 Cho BC, Jeung HC, Choi HJ, Rha SY, Hyung WJ, Cheong JH, Noh SH, Chung HC. Prognostic impact of resection margin involvement after extended (D2/D3) gastrectomy for advanced gastric cancer: a 15-year experience at a single institute. *J Surg Oncol* 2007; **95**: 461-468
- 20 Piso P, Bellin T, Aselmann H, Bektas H, Schlitt HJ, Klempnauer J. Results of combined gastrectomy and pancreatic resection in patients with advanced primary gastric carcinoma. *Dig Surg* 2002; **19**: 281-285
- 21 Kaposztas Z, Kalmar K, Cseke L, Illenyi L, Kelemen D, Horvath OP. Prognostic factors in the surgical treatment of gastric cancer--10 years experience. *Magy Seb* 2007; **60**: 71-78
- 22 Cuschieri A, Fayers P, Fielding J, Craven J, Banciewicz J, Joypaul V, Cook P. Postoperative morbidity and mortality after D1 and D2 resections for gastric cancer: preliminary results of the MRC randomised controlled surgical trial. The Surgical Cooperative Group. *Lancet* 1996; **347**: 995-999
- 23 Bonenkamp JJ, Songun I, Hermans J, Sasako M, Welvaart K, Plukker JT, van Elk P, Obertop H, Gouma DJ, Taat CW. Randomised comparison of morbidity after D1 and D2 dissection for gastric cancer in 996 Dutch patients. *Lancet* 1995; **345**: 745-748

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## CASE REPORT

# Acute small bowel obstruction caused by endometriosis: A case report and review of the literature

Antonella De Ceglie, Claudio Bilardi, Sabrina Blanchi, Massimo Picasso, Marcello Di Muzio, Alberto Trimarchi, Massimo Conio

Antonella De Ceglie, Department of Gastroenterology, Giovanni Paolo II, Cancer Institute, Bari 70126, Italy  
Claudio Bilardi, Sabrina Blanchi, Massimo Picasso, Massimo Conio, Department of Gastroenterology, General Hospital, Sanremo 18038, Italy

Marcello Di Muzio, Department of Pathology, General Hospital, Sanremo 18038, Italy

Alberto Trimarchi, Department of Surgery, General Hospital, Sanremo 18038, Italy

**Author contributions:** De Ceglie A and Conio M contributed equally to this work; Bilardi C, Picasso M, Di Muzio M and Trimarchi A reported the case; De Ceglie A and Blanchi S researched bibliography; De Ceglie A, Bilardi C and Conio M wrote the paper.

**Correspondence to:** Massimo Conio, MD, Department of Gastroenterology, General Hospital, C.so Garibaldi 187/3, Sanremo 18038, Italy. [mxconio@tin.it](mailto:mxconio@tin.it)

Telephone: +39-184-578986 Fax: +39-184-500846

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Saga 849-8501, Japan; Amado S Peña, Professor, Department of Pathology, Immunogenetics, VU University Medical Centre, De Boelelaan 1117, PO Box 7057, Amsterdam 1007 MB, The Netherlands

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## INTRODUCTION

Endometriosis is characterized by the presence of functional endometrial tissue consisting of glands and/or stroma located outside the uterus<sup>[1]</sup>. It is a painful chronic disease occurring in 5%-15% of menstruating women<sup>[1-3]</sup>.

The reported prevalence of endometriosis is 1%-20% in asymptomatic women, 10%-25% in infertile patients and 60%-70% in women with chronic pelvic pain<sup>[4-5]</sup>.

Endometriosis can be divided into intra- and extra-peritoneal sites. In decreasing order of frequency, the intra-peritoneal locations are ovaries (30%), uterosacral and large ligaments (18%-24%), fallopian tubes (20%), pelvic peritoneum, pouch of Douglas, and gastrointestinal (GI) tract. Extra-peritoneal locations include cervical portio (0.5%), vagina and rectovaginal septum, round ligament and inguinal hernia sac (0.3%-0.6%), navel (1%), abdominal scars after gynaecological surgery (1.5%) and caesarian section (0.5%). Endometriosis rarely affects extra-abdominal organs such as the lungs, urinary system, skin and the central nervous system<sup>[6-9]</sup>.

GI involvement of endometriosis has been found in 3%-37% of women, most commonly in the sigmoid colon, rectum and terminal ileum<sup>[10-13]</sup>.

We report a case in which endometrial infiltration of the small bowel caused acute obstruction, requiring emergency surgery. Diagnosis of ileal endometriosis was made by pathological examination of the resected specimen.

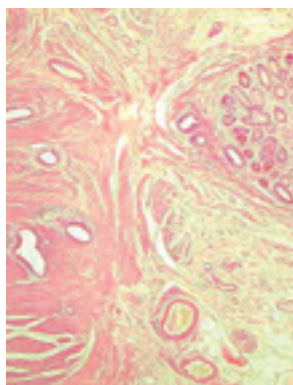
## Abstract

Gastrointestinal involvement of endometriosis has been found in 3%-37% of menstruating women and exclusive localization on the ileum is very rare (1%-7%). Endometriosis of the distal ileum is an infrequent cause of intestinal obstruction, ranging from 7% to 23% of all cases with intestinal involvement. We report a case in which endometrial infiltration of the small bowel caused acute obstruction requiring emergency surgery, in a woman whose symptoms were not related to menses. Histology of the resected specimen showed that endometriosis was mainly prevalent in the muscularis propria and submucosa and that the mucosa was not ulcerated but had inflammation and glandular alteration. Endometrial lymph node involvement, with a cystic glandular pattern was also detected.

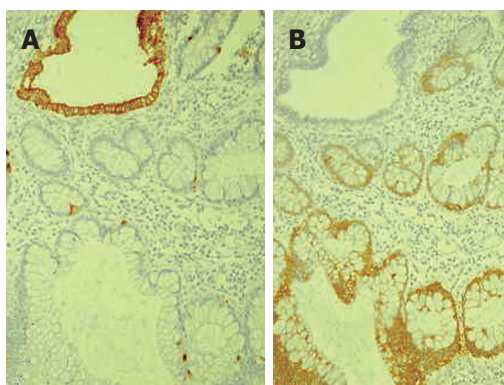
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**Key words:** Endometriosis; Small bowel; Ileum; Obstruction; Abdominal pain; Intestinal resection

**Peer reviewers:** Kazuma Fujimoto, Professor, Department of Internal Medicine, Saga Medical School, Nabeshima, Saga,



**Figure 1** Histology of ileal wall showing endometrial tissue in the muscular layer, with foci of mucosal involvement.



**Figure 2** Histopathology showing CK20 immunostaining of intestinal epithelium (A) and CK 7 immunostaining of endometrioid glands (B).

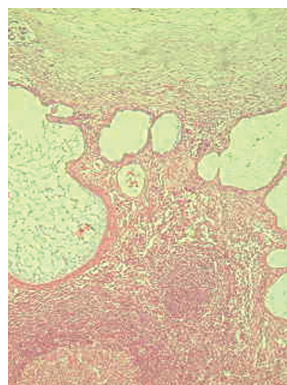
## CASE REPORT

A 44-year old woman was referred to our unit because of diffuse abdominal pain, associated with diarrhoea alternating with constipation.

The patient began complaining of mild abdominal discomfort nine months earlier. Episodes of pain relapsed irregularly, lasted a few hours and were not related to menses. She had two normal labors at the age of 30 and 32 years, regular menses and no history of dyspareunia. No other symptoms were present. Her past medical history was unremarkable.

Three months earlier, evaluation by her primary care physician revealed normal blood tests, and an abdominal ultrasound examination was unremarkable. Faecal analysis was negative for both parasites and occult blood. Antispasmodic drugs were administered, but the patient experienced a progressive worsening of the cramping abdominal pain and the onset of constipation.

Physical examination showed mild diffuse abdominal tenderness. No abdominal masses or enlarged lymph nodes were noted. Auscultation detected an increase of bowel sounds and peristaltic rushes. Colonoscopy and abdominal computed tomography (CT) scan were planned. However, 48 h later, the patient required emergency admission for small bowel occlusion. Abdominal X-ray examination showed dilated loops of the small intestine with no air in the colon, and CT scan revealed an irregular mass involving the ileum with dilation of the small intestine. A colonoscopy was performed to refine the diagnosis. The colon was



**Figure 3** Endometriosis involving lymph nodes with a cystic glandular pattern.

normal, but the ileum, at about 5 cm from the ileocecal valve, showed a tight extrinsic compression with intact mucosa.

Surgery was immediately carried out and an obstructing mass 5 cm in length involving the distal ileum was detected, with diffuse dilation of the small intestine. A right hemicolectomy with resection of 25 cm of ileum was performed. There was no evidence of macroscopic lesions in other abdominal and pelvic organs. The post-operative course was uneventful and the patient left the hospital 9 d later.

Histology of the resected specimen showed endometriosis involving the ileum and causing a stricture. The bowel wall was infiltrated, but the mucosa was not ulcerated. Endometriosis was mainly prevalent in the muscularis propria and submucosa. The mucosal involvement showed inflammation and glandular alteration (Figure 1). Immunocytochemistry with cytokeratin (CK) of different molecular weight (CK7 and CK20) was performed. Endometrioid glands and the intestinal epithelium were positive for CK7 and CK20, respectively (Figure 2). Endometrial lymph node involvement, with a cystic glandular pattern, was also detected (Figure 3).

## DISCUSSION

Endometriosis is a common disease of unknown etiology. Many theories have been proposed to explain this condition. The most widely accepted is Sampson's retrograde menstruation theory: during menstruation, endometrial tissue refluxes through the fallopian tubes, implanting and growing on the serosal surface of abdominal and pelvic organs<sup>[14-15]</sup>.

Alternatively, extrauterine growth of endometriotic tissue could occur as a result of metaplastic transformation of pluripotential peritoneal mesothelium (Minh's theory)<sup>[16]</sup>.

Another theory implies the migration of cells through the lymphatic system or via hematogenous spread<sup>[17]</sup>. Donnez *et al*<sup>[18]</sup> hypothesized that endometrial nodules may develop from metaplasia of mullerian remnants. In some cases, they could result from iatrogenic displacement of the decidua during a caesarean section<sup>[19]</sup>.

The "neurologic hypothesis" is a new concept in the pathogenesis of endometriosis: the lesions seem

to infiltrate the large bowel wall along the nerves, at a distance from the primary lesion<sup>[20]</sup>. However, other factors, immunological, genetic and familial, could be involved in the pathogenesis of this disease<sup>[21-23]</sup>.

Endometriosis usually becomes apparent in the reproductive years when the lesions are stimulated by ovarian hormones. Forty percent of the patients present symptoms in a cyclic manner, which are usually related with menses<sup>[24]</sup>. In our patient, symptoms relapsed irregularly and were not related with menses.

At present, superficial endometriosis is considered a normal phenomenon in women at the childbearing age, whereas deep infiltrative endometriosis (DIE) and endometrial ovarian cysts are the severe and painful manifestations of the condition<sup>[25]</sup>. DIE occurs in 30%-40% of the patients with endometriosis<sup>[26]</sup>.

Pelvic pain, infertility and dyspareunia are the characteristic symptoms of the disease<sup>[25]</sup>, but the clinical presentation is often non-specific.

Extra-pelvic endometriosis affects the GI tract of 5% of women with this condition<sup>[27]</sup>. The rectosigmoid is the most common site for intestinal endometriosis, accounting for 70% of all cases, while small bowel involvement, usually confined to the distal ileum, is less frequent (1%-7%) and exclusive localization on the ileum is very rare (1%-7%)<sup>[13]</sup>. Different incidence rates of endometriosis at different sites may be due to the fact that endometriosis is often an incidental finding at surgery<sup>[9]</sup>.

In a review of 1000 women who underwent laparotomy for gynecological symptoms, Jubanyik *et al*<sup>[28]</sup> described 181 (18%) cases of GI endometriosis, but only one patient had small bowel involvement. Melody *et al*<sup>[29]</sup> reported distal ileum involvement in 35 out of 36 patients. In a radiologic study, Scarmato *et al*<sup>[30]</sup> detected endometriosis of the terminal and mid-ileum in four patients and one patient, respectively. Endometriosis in the jejunum<sup>[31]</sup> and proximal ileum has also been documented<sup>[32]</sup>. Anaf *et al*<sup>[20]</sup>, considering bowel endometriosis an "infiltration or invasion phenomenon", found that there is a histological continuity between the superficial and underlying deep lesions of the large bowel wall, suggesting that lesions originating from the serosa progressively invade the muscularis propria. The mucosa is rarely involved as it is poorly innervated. Pelvic, pericolic and para-aortic lymph node involvement of endometriosis has also been reported, often coexisting with endometriosis of the bowel wall<sup>[33]</sup>. Lymph node involvement may be a consequence of lymphatic dissemination from endometrial foci in the intestinal wall<sup>[34-35]</sup>.

Symptoms are initially cyclical but may become permanent when the lesions progress.

It is difficult to establish a preoperative diagnosis of GI endometriosis, because GI tract symptoms can mimic a wide spectrum of diseases, including irritable bowel syndrome, infectious diseases, ischemic enteritis/colitis, inflammatory bowel disease and neoplasm<sup>[10,30,36-37]</sup>. GI endometriosis patients present with relapsing bouts of abdominal pain, abdominal distention, tenesmus,

constipation and diarrhoea<sup>[9]</sup>. Rectal bleeding and pain during defecation may also occur<sup>[38]</sup>.

Endometriosis infiltrating the muscularis propria may lead to localized fibrosis in the bowel wall, strictures, and small or large bowel obstruction<sup>[9-10]</sup>.

The true incidence of endometriosis causing bowel obstruction is unknown<sup>[11]</sup>, although complete obstruction of the bowel lumen occurs in less than 1% of cases<sup>[39]</sup>.

Endometriosis of the distal ileum is an infrequent cause of intestinal obstruction, ranging from 7% to 23% of all cases with intestinal involvement<sup>[31,33,40-41]</sup>.

The incidence of intestinal resection for bowel obstruction is 0.7% among patients undergone surgical treatment for abdominopelvic endometriosis<sup>[39]</sup>.

In our case, as in others previously reported in the literature, it was impossible to establish a timely and accurate preoperative diagnosis for the vagueness of symptoms, similar to other cases of bowel obstruction.

However, endometriosis of the small bowel should be suspected in young, nulliparous patients with abdominal pain, in conjunction with signs of obstruction<sup>[12]</sup>. Mussa *et al*<sup>[42]</sup> reported a case of small bowel endometriosis with intestinal obstruction, protein-losing enteropathy and anasarca. Wong *et al*<sup>[43]</sup> described a case of endometriosis of the small bowel mimicking pancreatitis.

Rarely, intestinal endometriosis may occur with perforation<sup>[44-45]</sup>. Malignancy has been reported in 0.7%-1% of patients and 78.7% of the cases occur in the ovary<sup>[46]</sup>. The colorectum is involved in only 5% of patients<sup>[47]</sup>.

The differential histologic diagnosis of endometrioid adenocarcinoma (AC) and colonic AC is difficult because colonic AC has a significant mucosal component, while endometrioid AC usually involves the outer layers of the colon<sup>[45,47-48]</sup>. Immunohistochemical staining for CK7 and CK20 seems to be useful in differentiating colonic and endometrioid AC<sup>[49-50]</sup>. Approximately, 75%-95% of primary colonic AC cases have a CK7-negative and CK20-positive phenotype, whereas 80%-100% of endometrial AC cases have a CK7-positive and CK20-negative phenotype<sup>[50]</sup>. Although endoscopic diagnosis of colonic endometriosis has been reported<sup>[51]</sup>, the mucosa is usually normal or shows minimal mucosal abnormalities<sup>[45,47-48]</sup>, friability<sup>[52]</sup>, extrinsic process or fibrosed stenoses<sup>[53]</sup>.

Rectal bleeding may be caused by mucosal injury during the passage of stools through a stenosed colon with the intramural endometriotic tissue increased at the time of menses if it occurs. Colonic mucosa heals rapidly and no signs are detectable at endoscopy<sup>[54]</sup>.

Endoscopic biopsies usually yield insufficient tissue for a definitive pathologic diagnosis as endometriosis involves the deep layers of the bowel wall. Endometriosis can induce mucosal changes without any specific pattern, which mimic findings of other diseases such as inflammatory bowel disease, ischemic colitis or neoplasm<sup>[55]</sup>.

Radiologically, lesions of endometriosis are either of



constricting and polypoid type or both<sup>[54]</sup>. On barium studies, radiographic findings caused by implants in the ileum are similar to those in the colon. Rectosigmoid or cecal endometriosis on double contrast barium enema studies is seen as an extrinsic mass with spiculation and tethering of folds<sup>[30,56]</sup>.

The diagnosis of endometriosis may be suspected on the basis of the clinical history<sup>[30]</sup>. Less than 50% of patients have concurrent pelvic endometriosis<sup>[57]</sup>.

CT is not the primary imaging modality for evaluation of bowel endometriosis, although it can occasionally demonstrate a stenosing rectosigmoid mass<sup>[58]</sup>.

Multislice CT (MSCT) has a great potential for detecting alterations in the intestinal wall, especially if it is combined with enteroclysis (MSCTe). Biscaldi *et al*<sup>[59]</sup> carried out a study on 98 women with symptoms suggestive of colorectal endometriosis and MSCTe identified 94.8% of bowel endometriotic nodules.

Magnetic resonance imaging (MRI) has a high sensitivity (77%-93%) in the diagnosis of bowel endometriosis<sup>[60-61]</sup>.

The depth of rectal wall infiltration by endometriosis is poorly defined by MRI. A combination of MRI and rectal endoscopic ultrasonography (EUS) has recently been proposed<sup>[62]</sup>. When retroperitoneal infiltration is present, it is mandatory to know if the bowel wall is involved in order to identify patients requiring bowel resection.

Both rectal EUS sensitivity and negative predictive value range from 92% to 100%. The specificity and positive predictive value are rather poor, which are 66% and 64%, 83% and 94%, respectively, as reported in two different studies<sup>[63-64]</sup>.

There is a great interest in the use of serum markers to diagnose endometriosis, but they are not sufficiently accurate for use in clinical practice<sup>[65]</sup>.

Cancer antigen CA-125 has been used to monitor the progress of endometriosis<sup>[66]</sup>. CA19-9 has a lower sensitivity than CA-125, and cytokine interleukin-6 may be more sensitive and specific than CA-125.

Surgery is the choice of treatment for intestinal endometriosis in most cases. For the accidental finding without symptoms of obstruction, hormone therapy with danazol or gonadotrophin-releasing hormone (GnRH) analogs may be considered<sup>[9]</sup>. Surgical treatment should be indicated for women with pain, bleeding, changes in bowel habits and intestinal obstruction<sup>[51]</sup>. In the small bowel, the treatment of endometriosis is surgical resection of the involved bowel, while medical therapy is only a temporary treatment<sup>[12]</sup>.

Intestinal endometriosis may be active in the peri- and post- menopausal years and even surgery may be necessary for these patients<sup>[54]</sup>.

In this paper, we report an unusual presentation of endometriosis characterized by abrupt onset of small bowel occlusion. The present report points out that endometriosis remains a challenging condition for clinicians, especially, as in our case, when the symptoms are not related to menses. Intestinal endometriosis should be considered in patients with epigastric,

abdominal and/or pelvic pain, in conjunction with signs of obstruction.

## REFERENCES

- 1 Olive DL, Schwartz LB. Endometriosis. *N Engl J Med* 1993; **328**: 1759-1769
- 2 Lu PY, Ory SJ. Endometriosis: current management. *Mayo Clin Proc* 1995; **70**: 453-463
- 3 Keane TE, Peel AL. Endometrioma. An intra-abdominal troublemaker. *Dis Colon Rectum* 1990; **33**: 963-965
- 4 Abrao MS, Podgaec S, Filho BM, Ramos LO, Pinotti JA, de Oliveira RM. The use of biochemical markers in the diagnosis of pelvic endometriosis. *Hum Reprod* 1997; **12**: 2523-2527
- 5 Pritts EA, Taylor RN. An evidence-based evaluation of endometriosis-associated infertility. *Endocrinol Metab Clin North Am* 2003; **32**: 653-667
- 6 Bergqvist A. Different types of extragenital endometriosis: a review. *Gynecol Endocrinol* 1993; **7**: 207-221
- 7 Frackiewicz EJ, Zarotsky V. Diagnosis and treatment of endometriosis. *Expert Opin Pharmacother* 2003; **4**: 67-82
- 8 Fox H, Buckley CH. Current concepts of endometriosis. *Clin Obstet Gynaecol* 1984; **11**: 279-287
- 9 Lin YH, Kuo LJ, Chuang AY, Cheng TI, Hung CF. Extrapelvic endometriosis complicated with colonic obstruction. *J Chin Med Assoc* 2006; **69**: 47-50
- 10 Yantiss RK, Clement PB, Young RH. Endometriosis of the intestinal tract: a study of 44 cases of a disease that may cause diverse challenges in clinical and pathologic evaluation. *Am J Surg Pathol* 2001; **25**: 445-454
- 11 Paksoy M, Karabicak I, Ayan F, Aydogan F. Intestinal obstruction due to rectal endometriosis. *Mt Sinai J Med* 2005; **72**: 405-408
- 12 Orbuch IK, Reich H, Orbuch M, Orbuch L. Laparoscopic treatment of recurrent small bowel obstruction secondary to ileal endometriosis. *J Minim Invasive Gynecol* 2007; **14**: 113-115
- 13 Macafee CH, Greer HL. Intestinal endometriosis. A report of 29 cases and a survey of the literature. *J Obstet Gynaecol Br Emp* 1960; **67**: 539-555
- 14 Quinn M. Endometriosis: the consequence of neurological dysfunction? *Med Hypotheses* 2004; **63**: 602-608
- 15 Witz CA. Current concepts in the pathogenesis of endometriosis. *Clin Obstet Gynecol* 1999; **42**: 566-585
- 16 Minh HN, Smadja A, Orcel L. [An integrated histogenetic concept of internal and external endometriosis] *J Gynecol Obstet Biol Reprod (Paris)* 1986; **15**: 29-35
- 17 Audebert AJ. [External endometriosis: histogenesis, etiology and natural course] *Rev Prat* 1990; **40**: 1077-1081
- 18 Donnez J, Spada F, Squifflet J, Nisolle M. Bladder endometriosis must be considered as bladder adenomyosis. *Fertil Steril* 2000; **74**: 1175-1181
- 19 Luisi S, Gabbanini M, Sollazzi S, Calonaci F, Razzi S, Petraglia F. Surgical scar endometriosis after Cesarean section: a case report. *Gynecol Endocrinol* 2006; **22**: 284-285
- 20 Anaf V, El Nakadi I, Simon P, Van de Stadt J, Fayt I, Simonart T, Noel JC. Preferential infiltration of large bowel endometriosis along the nerves of the colon. *Hum Reprod* 2004; **19**: 996-1002
- 21 Dmowski WP, Gebel HM, Rawlins RG. Immunologic aspects of endometriosis. *Obstet Gynecol Clin North Am* 1989; **16**: 93-103
- 22 De Falco M, Ragusa M, Oliva G, Miranda A, Parmeggiani D, Sperlongano P, Accardo M, Calzolari F, Misso C, Monacelli M, Avenia N. [Is extrauterine endometriosis confined to the gynecological sphere? A critical review of the experience in a general surgery unit] *G Chir* 2007; **28**: 83-92
- 23 Moen MH, Magnus P. The familial risk of endometriosis. *Acta Obstet Gynecol Scand* 1993; **72**: 560-564
- 24 Badawy SZ, Freedman L, Numann P, Bonaventura M, Kim



- S. Diagnosis and management of intestinal endometriosis. A report of five cases. *J Reprod Med* 1988; **33**: 851-855
- 25 **Amaral VF**, Ferriani RA, Sa MF, Nogueira AA, Rosa e Silva JC, Rosa e Silva AC, Moura MD. Positive correlation between serum and peritoneal fluid CA-125 levels in women with pelvic endometriosis. *Sao Paulo Med J* 2006; **124**: 223-227
  - 26 **Cornillie FJ**, Oosterlynck D, Lauweryns JM, Koninckx PR. Deeply infiltrating pelvic endometriosis: histology and clinical significance. *Fertil Steril* 1990; **53**: 978-983
  - 27 **Prystowsky JB**, Stryker SJ, Ujiki GT, Poticha SM. Gastrointestinal endometriosis. Incidence and indications for resection. *Arch Surg* 1988; **123**: 855-858
  - 28 **Jubanyik KJ**, Comite F. Extrapelvic endometriosis. *Obstet Gynecol Clin North Am* 1997; **24**: 411-440
  - 29 **Melody GF**. Endometriosis causing obstruction of the ileum. *Obstet Gynecol* 1956; **8**: 468-472
  - 30 **Scarmato VJ**, Levine MS, Herlinger H, Wickstrom M, Furth EE, Tureck RW. Ileal endometriosis: radiographic findings in five cases. *Radiology* 2000; **214**: 509-512
  - 31 **Martimbeau PW**, Pratt JH, Gaffey TA. Small-bowel obstruction secondary to endometriosis. *Mayo Clin Proc* 1975; **50**: 239-243
  - 32 **Rio FW**, Edwards DL, Regan JF, Schmutzer KJ. Endometriosis of the small bowel. *Arch Surg* 1970; **101**: 403-405
  - 33 **Cameron IC**, Rogers S, Collins MC, Reed MW. Intestinal endometriosis: presentation, investigation, and surgical management. *Int J Colorectal Dis* 1995; **10**: 83-86
  - 34 **Lorente Poyatos R**, Palacios Perez A, Bravo Bravo F, Lopez Caballero FJ, Bouhmid A, Huertas Nadal C, Ruiz Escolano E. [Rectosigmoid endometriosis with lymph node involvement] *Gastroenterol Hepatol* 2003; **26**: 23-25
  - 35 **Sheikh HA**, Krishnamurti U, Bhat Y, Rajendiran S. A 42-year-old woman with a 7-month history of abdominal pain. A, endometriosis involving ileocecal junction and 2 pericolic lymph nodes; B, intranodal benign glandular inclusions. *Arch Pathol Lab Med* 2005; **129**: e218-e221
  - 36 **Cappell MS**, Friedman D, Mikhail N. Endometriosis of the terminal ileum simulating the clinical, roentgenographic, and surgical findings in Crohn's disease. *Am J Gastroenterol* 1991; **86**: 1057-1062
  - 37 **Dimoulis P**, Koutroubakis IE, Tzardi M, Antoniou P, Matalliotakis IM, Kouroumalis EA. A case of sigmoid endometriosis difficult to differentiate from colon cancer. *BMC Gastroenterol* 2003; **3**: 18
  - 38 **Conio M**, Buscarini E, Bianchi S, Lapertosa G, Zambelli A. Sigmoid endometriosis. *Gastrointest Endosc* 2004; **60**: 434-435
  - 39 **de Bree E**, Schoretsanitis G, Melissas J, Christodoulakis M, Tsiftsis D. Acute intestinal obstruction caused by endometriosis mimicking sigmoid carcinoma. *Acta Gastroenterol Belg* 1998; **61**: 376-378
  - 40 **Riaz N**, Khurshaidi N. Acute small bowel obstruction secondary to ileal endometrioma. *J Coll Physicians Surg Pak* 2007; **17**: 228-229
  - 41 **Ridha JR**, Cassaro S. Acute small bowel obstruction secondary to ileal endometriosis: report of a case. *Surg Today* 2003; **33**: 944-947
  - 42 **Mussa FF**, Younes Z, Tihan T, Lacy BE. Anasarca and small bowel obstruction secondary to endometriosis. *J Clin Gastroenterol* 2001; **32**: 167-171
  - 43 **Wong LS**, Mahendrakumar R, Bullen BR. Small-bowel endometriosis masquerading as pancreatitis. *J R Soc Med* 1999; **92**: 17-18
  - 44 **Varras M**, Kostopanagiotou E, Katis K, Farantos CH, Angelidou-Manika Z, Antoniou S. Endometriosis causing extensive intestinal obstruction simulating carcinoma of the sigmoid colon: a case report and review of the literature. *Eur J Gynaecol Oncol* 2002; **23**: 353-357
  - 45 **Yantiss RK**, Clement PB, Young RH. Endometriosis of the intestinal tract: a study of 44 cases of a disease that may cause diverse challenges in clinical and pathologic evaluation. *Am J Surg Pathol* 2001; **25**: 445-454
  - 46 **Heaps JM**, Nieberg RK, Berek JS. Malignant neoplasms arising in endometriosis. *Obstet Gynecol* 1990; **75**: 1023-1028
  - 47 **Chen KT**. Endometrioid adenocarcinoma arising from colonic endometriosis mimicking primary colonic carcinoma. *Int J Gynecol Pathol* 2002; **21**: 285-288
  - 48 **Zanetta GM**, Webb MJ, Li H, Keeney GL. Hyperestrogenism: a relevant risk factor for the development of cancer from endometriosis. *Gynecol-Oncol* 2000; **79**: 18-22
  - 49 **Han AC**, Hovenden S, Rosenblum NG, Salazar H. Adenocarcinoma arising in extragonadal endometriosis: an immunohistochemical study. *Cancer* 1998; **83**: 1163-1169
  - 50 **Chu P**, Wu E, Weiss LM. Cytokeratin 7 and cytokeratin 20 expression in epithelial neoplasms: a survey of 435 cases. *Mod Pathol* 2000; **13**: 962-972
  - 51 **Bozdech JM**. Endoscopic diagnosis of colonic endometriosis. *Gastrointest Endosc* 1992; **38**: 568-570
  - 52 **Averbach M**, Abrao MS, Podgaec S, Correa P. Rectal endometriosis. *Gastrointest Endosc* 2005; **62**: 978-979; discussion 979
  - 53 **Korber J**, Grammel S, Lobeck H, Weidemann H. [Stenosis of the terminal ileum. Endometriosis as the differential diagnosis of Crohn's disease] *Dtsch Med Wochenschr* 1997; **122**: 926-929
  - 54 **Bartkowiak R**, Zieniewicz K, Kaminski P, Krawczyk M, Marianowski L, Szymanska K. Diagnosis and treatment of sigmoidal endometriosis--a case report. *Med Sci Monit* 2000; **6**: 787-790
  - 55 **Langlois NE**, Park KG, Keenan RA. Mucosal changes in the large bowel with endometriosis: a possible cause of misdiagnosis of colitis? *Hum Pathol* 1994; **25**: 1030-1034
  - 56 **Gordon RL**, Evers K, Kressel HY, Laufer I, Herlinger H, Thompson JJ. Double-contrast enema in pelvic endometriosis. *AJR Am J Roentgenol* 1982; **138**: 549-552
  - 57 **Croom RD 3rd**, Donovan ML, Schwesinger WH. Intestinal endometriosis. *Am J Surg* 1984; **148**: 660-667
  - 58 **Fishman EK**, Scatarige JC, Saksouk FA, Rosenshein NB, Siegelman SS. Computed tomography of endometriosis. *J Comput Assist Tomogr* 1983; **7**: 257-264
  - 59 **Biscaldi E**, Ferrero S, Fulcheri E, Ragni N, Remorgida V, Rollandi GA. Multislice CT enteroclysis in the diagnosis of bowel endometriosis. *Eur Radiol* 2007; **17**: 211-219
  - 60 **Bazot M**, Darai E, Hourani R, Thomassin I, Cortez A, Uzan S, Buy JN. Deep pelvic endometriosis: MR imaging for diagnosis and prediction of extension of disease. *Radiology* 2004; **232**: 379-389
  - 61 **Takeuchi H**, Kuwatsuru R, Kitade M, Sakurai A, Kikuchi I, Shimanuki H, Kinoshita K. A novel technique using magnetic resonance imaging jelly for evaluation of rectovaginal endometriosis. *Fertil Steril* 2005; **83**: 442-447
  - 62 **Roseau G**, Dumontier I, Palazzo L, Chapron C, Dousset B, Chaussade S, Dubuisson JB, Couturier D. Rectosigmoid endometriosis: endoscopic ultrasound features and clinical implications. *Endoscopy* 2000; **32**: 525-530
  - 63 **Delpy R**, Barthet M, Gasmi M, Berdah S, Shojai R, Desjeux A, Boubli L, Grimaud JC. Value of endorectal ultrasonography for diagnosing rectovaginal septal endometriosis infiltrating the rectum. *Endoscopy* 2005; **37**: 357-361
  - 64 **Camagna O**, Dhainaut C, Dupuis O, Soncini E, Martin B, Palazzo L, Chosidow D, Madelenat P. [Surgical management of rectovaginal septum endometriosis from a continuous series of 50 cases] *Gynecol Obstet Fertil* 2004; **32**: 199-209
  - 65 **Mounsey AL**, Wilgus A, Slawson DC. Diagnosis and management of endometriosis. *Am Fam Physician* 2006; **74**: 594-600
  - 66 **Bedaiwy MA**, Falcone T. Laboratory testing for endometriosis. *Clin Chim Acta* 2004; **340**: 41-56



# Simultaneous laparoscopy-assisted low anterior resection and distal gastrectomy for synchronous carcinoma of rectum and stomach

Qian-Lin Zhu, Min-Hua Zheng, Bo Feng, Ai-Guo Lu, Min-Liang Wang, Jian-Wen Li, Wei-Guo Hu, Lu Zang, Zhi-Hai Mao, Feng Dong, Jun-Jun Ma, Ya-Ping Zong

Qian-Lin Zhu, Min-Hua Zheng, Bo Feng, Ai-Guo Lu, Min-Liang Wang, Jian-Wen Li, Wei-Guo Hu, Lu Zang, Zhi-Hai Mao, Feng Dong, Jun-Jun Ma, Ya-Ping Zong, Department of General Surgery, Ruijin Hospital Affiliated to Shanghai Jiaotong University; Shanghai Minimally Invasive Surgery Center, Shanghai 200025, China

**Author contributions:** Zheng MH and Zhu QL contributed equally to this work; Zheng MH, Lu AG, Wang ML, Li JW and HU WG performed the operation; Feng B, Zang L, Mao ZH, Dong F, Ma JJ and Zong YP assisted in the reference research; Zhu QL, Feng B and Ma JJ wrote the paper.

**Correspondence to:** Min-Hua Zheng, Department of General Surgery, Ruijin Hospital Affiliated to Shanghai Jiaotong University, Shanghai Minimally Invasive Surgery Center, Shanghai 200025, China. [zqlalani@163.com](mailto:zqlalani@163.com)

Telephone: +86-21-64458887 Fax: +86-21-64458887

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## Abstract

Laparoscopic resection of rectal cancer or gastric cancer has been advocated for the benefits of a reduced morbidity, a shorter treatment time, and similar outcomes. However, simultaneous laparoscopy-assisted low anterior resection and distal gastrectomy for synchronous carcinoma of rectum and stomach are rarely documented in literature. Endoscopic examination revealed a synchronous carcinoma of rectum and stomach in a 55-year-old male patient with rectal bleeding and epigastric discomfort. He underwent a simultaneous laparoscopy-assisted low anterior resection and distal gastrectomy with regional lymph nodes dissected. The operation time was 270 min and the estimated blood loss was 120 mL. The patient required parenteral analgesia for less than 24 h. Flatus was passed on postoperative day 3, and a solid diet was resumed on postoperative day 7. He was discharged on postoperative day 13. With the advances in laparoscopic technology and experience, simultaneous resection is an attractive alternative to a synchronous gastrointestinal cancer.

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**Key words:** Laparoscopy; Gastric cancer; Rectal cancer;

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**Peer reviewer:** Dr. Shawn David Safford, Department of Surgery, Duke University Medical Center, 994 West Ocean View Avenue, Norfolk VA23503, United States

Zhu QL, Zheng MH, Feng B, Lu AG, Wang ML, Li JW, Hu WG, Zang L, Mao ZH, Dong F, Ma JJ, Zong YP. Simultaneous laparoscopy-assisted low anterior resection and distal gastrectomy for synchronous carcinoma of rectum and stomach. *World J Gastroenterol* 2008; 14(21): 3435-3437 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3435.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3435>

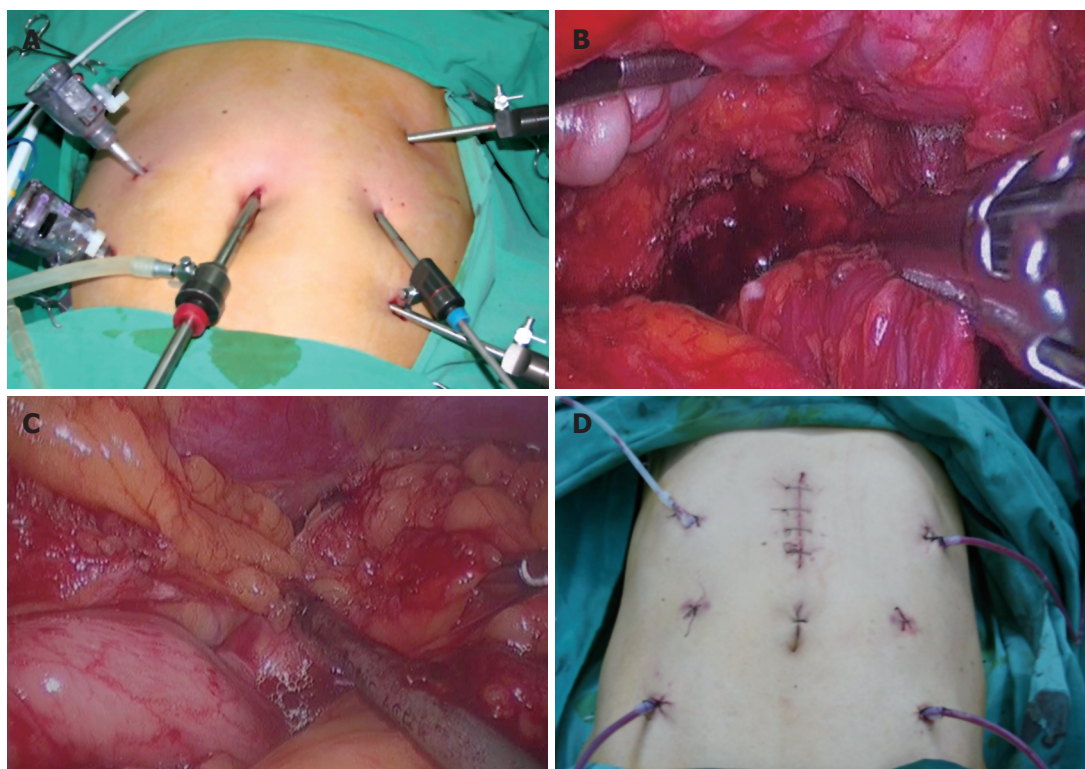
## INTRODUCTION

Rectal and gastric cancers are sometimes diagnosed simultaneously as a synchronous carcinoma of rectum and stomach in a single patient. An enlarged open procedure combining both rectal resection and gastrectomy with a curable intention is normally performed for such cases, but a long operation time and severe surgical trauma often result in a reluctant recovery.

Laparoscopic resection is feasible and safe for both rectal and gastric cancers nowadays. It has been widely advocated for the benefits of a reduced morbidity and a shorter hospitalization time without sacrificing the oncological outcome. As this novel technique permits a multiple segmental resection, simultaneous laparoscopy-assisted low anterior resection and distal gastrectomy for a single patient have become attractive to a synchronous carcinoma of rectum and stomach. We present such a case.

## CASE REPORT

A 55-year-old male patient presented with rectal bleeding and epigastric discomfort with alternation in bowel habit, mucus, and weight loss for 6 mo. Colonoscopy revealed a circumferential mass 8 cm from the anal verge, which was confirmed to be a rectal adenocarcinoma by biopsy. Meanwhile, gastroscopy revealed a 3 cm × 3 cm ulcerative lesion located at the lesser curvature near the



**Figure 1** A: Position of the working ports; B: Transection of the rectum with a linear stapler; C: Dissection of short gastric vessels with harmonic scalpel; D: Placement of drainage tubes.

pylorus, which was confirmed to be a signet-ring cell adenocarcinoma by biopsy. Computed tomography (CT) of the abdomen and thorax demonstrated no significant metastatic lesions. Various treatment strategies were discussed and the patient underwent simultaneous laparoscopy-assisted low anterior resection and distal gastrectomy (LADG) and D2 lymphadenectomy.

The operation was performed under general anesthesia. The patient was first at a Lloyd-Davis position to accomplish the low anterior resection, and then changed into a Trendelenburg position to complete the distal gastrectomy. We used the Veress method to establish pneumoperitoneum and maintain the intra-abdominal pressure at around 15 mmHg. Trocars for low anterior resection were placed. In brief, an umbilical port (10 mm) was used for a video scope (usually a 30-degree scope), and two ports were created for working in the right and left low quadrants of abdomen, with the lower port in the right quadrant for main working (12 mm) and the rest three for assistance (5 mm). When gastrectomy was performed, another 5 mm assistant port was made in the left subcostal region (Figure 1A). The main surgeon stood on the right of the patient and then between the split legs of the patient after completion of the low anterior resection.

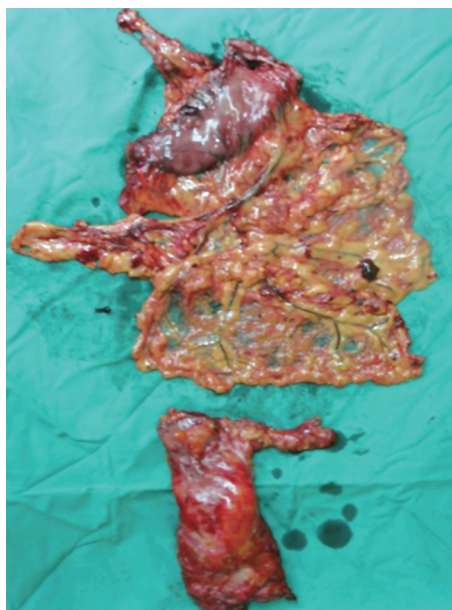
The right peritoneum was dissected along the iliac vessels with a harmonic scalpel, then the sigmoid colon and rectum as well as their mesenteries were mobilized down to the pelvic floor. The lymphovascular pedicle was ligated at the radical site of the inferior mesenteric vessel with a polymer plastic clip, while the distal rectum was transected intracorporeally with a laparoscopic linear stapler (Figure 1B). The first part of the operation was

completed within 75 min.

The gastrocolic ligament was divided from the hepatic flexure towards the splenic flexure for gastric mobilization, while the gastroepiploic vessels and short gastric vessels were ligated simultaneously (Figure 1C). The gastrohepatic and duodenohepatic ligaments were then divided to allow ligation of the right and left gastric vessels. After completion of the whole procedure, the proximal stomach was entirely mobilized with an adequate perigastric lymphadenectomy. This part of the operation was accomplished within 2 h and 30 min, and the estimated blood loss was 120 mL.

A 5 cm median epigastric incision was made and protected with a plastic bag to ensure the transection of the two specimens. The bowels were then anastomosed intra-corporeally with a double stapler. The first portion of the duodenum and stomach body was transected with the gastrointestinal continuity performed in a Billroth-I fashion. Four protective drainage tubes were placed near the two anastomotic junctions (Figure 1D). The total operation time was 4 h and 30 min. The patient received parenteral analgesia for less than one day. Flatus was passed on postoperative day 3, the patient resumed a liquid diet on postoperative day 5 and a solid diet on postoperative day 7. No clinically significant complication occurred postoperatively and all the drainage tubes were pulled out consecutively. The patient was discharged 13 d after the operation. Pathologic examination of the rectum showed a moderately differentiated adenocarcinoma invading the subserosal tissue without breaching the serosa. The distal margin measured 2 cm, and no involvement of tumor was found in the mesenteric or paracolic lymph nodes. Pathological examination of the





**Figure 2** Specimens obtained at distal gastrectomy, regional lymphadenectomy and low anterior resection from their anatomical sites.

stomach showed a poorly differentiated adenocarcinoma invading the muscular layer, involvement of tumor was observed in 8 of the 20 lymph nodes (Figure 2).

## DISCUSSION

Synchronous gastrointestinal cancers are normally treated with conventional surgical approaches. In brief, a long medial incision from the xiphoid to the pubic symphysis is made to ensure the upper-abdominal and pelvic procedures, with a dissection of regional lymph nodes. These maneuvers might cause surgical traumas, and result in a slow postoperative recovery, with a negative influence on the prognosis of tumors. Laparoscopic technique provides a simultaneous resection of the rectal and gastric lesions and minimizes the surgical influence on human body. For the rectal procedure, the amplifying effect of laparoscopy helps the surgeons to identify and protect the ureter and automatic nerve plexus in a narrow pelvic cavity in spite of a bulky tumor and thickened mesentery. A 30-degree camera offers a multi-angle image, which greatly assists the surgeon to complete a TME approach as well as the intra-corporeal transection and anastomosis of the bowels<sup>[1]</sup>. For the gastric procedure, advanced laparoscopic instruments and materials, such as harmonic scalpel, multi-sized titanium or polymer clips significantly

reduce the intra-operative hemorrhage. A broad view of the intra-peritoneal cavity under laparoscope facilitates the multi-plane manipulation of gastrectomy, especially during the dissection of the upper branches of perigastric vessels like short gastric vessels<sup>[2]</sup>. We transected the two specimens and reconstructed the upper gastrointestinal duct extra-corporeally with a manual method, which could obviously reduce the operation time and cost. To summarize, laparoscopic colorectal and gastric surgery is feasible and safe in clinical trials with the benefit of avoiding a second operation, thus reducing morbidity, and shortening the hospital stay without sacrificing the oncological outcome<sup>[3,4]</sup>. As some other radical surgeries for synchronous gastrointestinal cancers have also been reported, simultaneous laparoscopic approach obviously reduces the morbidity and recurrence rate<sup>[5,6]</sup>.

To the best of our knowledge, this is the first report of simultaneous laparoscopy-assisted low anterior resection and distal gastrectomy for synchronous carcinoma of the rectum and stomach. A good recovery of the patient reveals that this technique is advantageous over the conventional approaches in treating gastrointestinal malignancies and broadens the indications for laparoscopic approaches. This minimally invasive technique may have a bright future.

## REFERENCES

- 1 **Zhou ZG**, Hu M, Li Y, Lei WZ, Yu YY, Cheng Z, Li L, Shu Y, Wang TC. Laparoscopic versus open total mesorectal excision with anal sphincter preservation for low rectal cancer. *Surg Endosc* 2004; **18**: 1211-1215
- 2 **Kitano S**, Shiraishi N. Minimally invasive surgery for gastric tumors. *Surg Clin North Am* 2005; **85**: 151-164, xi
- 3 **Guillou PJ**, Quirke P, Thorpe H, Walker J, Jayne DG, Smith AM, Heath RM, Brown JM. Short-term endpoints of conventional versus laparoscopic-assisted surgery in patients with colorectal cancer (MRC CLASICC trial): multicentre, randomised controlled trial. *Lancet* 2005; **365**: 1718-1726
- 4 **Kitano S**, Shiraishi N, Kakisako K, Yasuda K, Inomata M, Adachi Y. Laparoscopy-assisted Billroth-I gastrectomy (LADG) for cancer: our 10 years' experience. *Surg Laparosc Endosc Percutan Tech* 2002; **12**: 204-207
- 5 **Leung KL**, Lee JF, Yiu RY, Ng SS, Li JC. Simultaneous laparoscopic resection of rectal cancer and liver metastasis. *J Laparoendosc Adv Surg Tech A* 2006; **16**: 486-488
- 6 **Jafari Giv M**, Ho YH. Concurrent laparoscopic right hemicolectomy and ultra-low anterior resection with colonic J-pouch anal anastomosis for synchronous carcinoma. *Tech Coloproctol* 2007; **11**: 55-57

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LETTERS TO THE EDITOR

## Hepatic encephalopathy in patients with liver cirrhosis: Is there a role of malnutrition?

Evangelos Kalaitzakis, Einar Björnsson

Evangelos Kalaitzakis, Einar Björnsson, Section of Gastroenterology and Hepatology, Department of Internal Medicine, Sahlgrenska University Hospital, Gothenburg 41345, Sweden

**Author contributions:** Kalaitzakis E and Björnsson E contributed equally in idea conception, writing of manuscript and approval of final manuscript.

**Correspondence to:** Evangelos Kalaitzakis, MD, PhD, Section of Gastroenterology and Hepatology, Department of Internal Medicine, Sahlgrenska University Hospital, Gothenburg 41345, Sweden. [evangelos.kalaitzakis@vgregion.se](mailto:evangelos.kalaitzakis@vgregion.se)

Telephone: +46-31-3421000 Fax: +46-31-822152

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### TO THE EDITOR

The pathogenesis of hepatic encephalopathy (HE), a common complication of liver cirrhosis, remains incompletely understood but it is probably multifactorial in most cases<sup>[1]</sup>. Malnutrition is also commonly encountered in patients with cirrhosis and it has been reported to have an effect on health related quality of life<sup>[2]</sup>. Although experimental studies suggest that low energy intake and poor nutritional status may facilitate the development of HE<sup>[3]</sup>, there are scarce data on the potential role of malnutrition in HE in patients with liver cirrhosis.

Recently Soros *et al*<sup>[4]</sup> performed a study investigating the potential role of malnutrition and hypermetabolism in HE in which 223 patients with non-alcoholic cirrhosis were enrolled. They were evaluated for the presence of HE according to the West Haven criteria and for malnutrition by means of body mass index (BMI), anthropometric measurements, and bioelectrical impedance analysis. Energy metabolism was also assessed by means of indirect calorimetry. Eighty-five (38%) out of 223 patients had no clinically evident HE, 123/223 (55%) had HE grade 1 and 15/223 (7%) had HE grade 2 or 3. Neither metabolic variables or BMI nor fat free mass or muscle mass differed significantly in patients with HE grade 1-3 from those without HE. In multivariate analysis none of these parameters was found to be independently related to HE. The authors concluded that malnutrition or catabolism does not seem to be independent risk factors for the presence of HE in patients with liver cirrhosis<sup>[4]</sup>.

Recently, we performed a prospective study evaluating HE in 128 patients with liver cirrhosis of various etiologies<sup>[5]</sup>. HE was evaluated by means of the West Haven criteria and two psychometric tests (number connection test A and B). HE was defined as overt HE according to the West Haven criteria and/or number connection test A and/or B > 3 standard deviations of the general population. Nutritional status was evaluated with BMI and anthropometric measurement as well as estimation of recent weight change. Malnutrition was defined as anthropometric measurement below the 5th

### Abstract

Hepatic encephalopathy (HE) is a common complication in patients with liver cirrhosis but its pathogenesis remains incompletely understood. Malnutrition is commonly encountered in patients with liver cirrhosis and it has been reported to affect the quality of life of this group of patients. Experimental studies suggest that low energy intake and poor nutritional status may facilitate the development of HE but there are scarce data on the potential role of malnutrition in HE in patients with liver cirrhosis. Two recently published studies have evaluated the potential role of malnutrition in the development of HE in cirrhotic patients with conflicting results. In this letter to the editor we briefly present the results of the two studies as well as potential reasons for the conflicting results reported.

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**Key words:** Hepatic encephalopathy; Liver cirrhosis; Malnutrition

**Peer reviewers:** Gary A Abrams, Associate Professor, Department of Medicine, University of Alabama at Birmingham, 1530 3rd Ave South, Birmingham 35294, United States; Paul J Pockros, MD, Gastroenterology/Hepatology, Scripps Clinic, 10666 N Torrey Pines Road, La Jolla 92037, United States

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percentile according to standard values for the general population and/or BMI < 20 kg/m<sup>2</sup> and/or weight loss ≥ 5%-10% in the previous 3-6 mo. The presence of diabetes mellitus was also assessed with fasting glucose measurement. Forty percent of our patients were malnourished, 26% had diabetes, and 34% had HE. Patients with malnutrition suffered more frequently from HE compared to those without malnutrition (46% *vs* 27%, *P* = 0.031), but there was no difference in age, etiology, or severity of liver cirrhosis. In multivariate analysis, the time needed to perform number connection test A was independently correlated to age, severity of cirrhosis expressed as the Child-Pugh score, diabetes and malnutrition<sup>[5]</sup>. This is in agreement with a previous study showing that diabetes mellitus is associated with HE in patients with hepatitis C cirrhosis<sup>[6]</sup>.

In the paper of Soros *et al*<sup>[4]</sup>, they did not report how many patients had diabetes mellitus. However, the risk of diabetes mellitus has been reported to be increased in patients with cirrhosis due to hepatitis C<sup>[7]</sup> and the majority of patients enrolled in the study of Soros *et al*<sup>[4]</sup> (56%) had viral cirrhosis<sup>[4]</sup>. It is therefore unknown whether the patients with HE had a higher proportion of diabetes compared with the patients without HE. This might have had an effect on the median BMI in the two groups as diabetes is more prevalent in patients with increased BMI, thus accounting for the lack of difference in median BMI between patients with HE and those without HE<sup>[4]</sup>. In fact, the BMI of patients with HE ranged from 14.5 to 36.3 kg/m<sup>2</sup> as compared to 17.5-28.4 kg/m<sup>2</sup> in those without HE<sup>[4]</sup>. Furthermore, in our study, recent weight change was included in the definition of malnutrition<sup>[4]</sup> whereas in the study of Sörös *et al* no definition of malnutrition was provided<sup>[4]</sup>. Interestingly we found that although patients with and without low fat or muscle mass did not differ in number connection A performance times, a recent weight loss was related to longer performance times [81 s (51) *vs* 54 s (32), *P* = 0.001]<sup>[5]</sup>. It is therefore conceivable that deterioration in nutritional status, rather than nutritional

status itself, may be of great importance for cognitive dysfunction in patients with liver cirrhosis. Finally, another factor that may, at least in part, explain the differences between the results of the two studies<sup>[4,5]</sup> is that we also included patients with minimal HE in our analyses<sup>[5]</sup> whereas as Soros *et al*<sup>[4]</sup> included only patients with clinically overt HE in their study.

In conclusion, methodological differences regarding the definitions of HE and malnutrition as well as the assessment of the role of diabetes mellitus in cognitive dysfunction may explain the differences in the results of the two studies<sup>[4,5]</sup>. As both studies had limitations mentioned by their authors<sup>[4,5]</sup> and the pathophysiology of HE is complex, it is clear that further studies are warranted to fully delineate the potential role of malnutrition in cognitive dysfunction in patients with liver cirrhosis.

## REFERENCES

- 1 **Butterworth RF.** Hepatic encephalopathy: a neuropsychiatric disorder involving multiple neurotransmitter systems. *Curr Opin Neurol* 2000; **13**: 721-727
- 2 **Norman K,** Kirchner H, Lochs H, Pirlich M. Malnutrition affects quality of life in gastroenterology patients. *World J Gastroenterol* 2006; **12**: 3380-3385
- 3 **Thompson JS,** Schafer DF, Haun J, Schafer GJ. Adequate diet prevents hepatic coma in dogs with Eck fistulas. *Surg Gynecol Obstet* 1986; **162**: 126-130
- 4 **Soros P,** Bottcher J, Weissenborn K, Selberg O, Muller MJ. Malnutrition and hypermetabolism are not risk factors for the presence of hepatic encephalopathy: a cross-sectional study. *J Gastroenterol Hepatol* 2008; **23**: 606-610
- 5 **Kalaitzakis E,** Olsson R, Henfridsson P, Hugosson I, Bengtsson M, Jalan R, Bjornsson E. Malnutrition and diabetes mellitus are related to hepatic encephalopathy in patients with liver cirrhosis. *Liver Int* 2007; **27**: 1194-1201
- 6 **Sigal SH,** Stanca CM, Kontorinis N, Bodian C, Ryan E. Diabetes mellitus is associated with hepatic encephalopathy in patients with HCV cirrhosis. *Am J Gastroenterol* 2006; **101**: 1490-1496
- 7 **Zein NN,** Abdulkarim AS, Wiesner RH, Egan KS, Persing DH. Prevalence of diabetes mellitus in patients with end-stage liver cirrhosis due to hepatitis C, alcohol, or cholestatic disease. *J Hepatol* 2000; **32**: 209-217

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### Akira Andoh, MD

Department of Internal Medicine, Shiga University of Medical Science, Seta Tukinowa, Otsu 520-2192, Japan

### Claudio Bassi, MD, Professor

Department of Surgery and Gastroenterology, Hospital GB Rossi, University of Verona, Piazza LA Scuro 37134 Verona, Italy

### Marc Basson, MD, PhD, MBA, Chief of Surgery

John D Dingell VA Medical Center, 4646 John R. Street, Detroit, MI 48301, United States

### Reinhard Buettner, Professor

Institute of Pathology, University Hospital Bonn, Sigmund-Freud-Str. 25, D-53127 Bonn, Germany

### Andrew D Clouston, Associate Professor

Histopath Laboratories, Suite 4, Level 9, Strathfield Plaza, Strathfield, Sydney, 2135, Australia

### Dario Conte, Professor

GI Unit-IRCCS Osp. Maggiore, Chair of Gastroenterology, Via F. Sforza, 35, Milano 20122, Italy

### George N Dalekos, MD, PhD, Associate Professor Medicine

Department of Medicine, Academic Liver Unit & Research Lab of Internal Medicine, Medical School, University of Thessaly, University Hospital of Larissa, PO Box 1425, 41110, Larissa, Greece

### Dr. William Dickey

Altnagelvin Hospital, Londonderry, BT47 6SB, Northern Ireland, United Kingdom

### Francesco Feo, Professor

Dipartimento di Scienze Biomediche, Sezione di Patologia Sperimentale e Oncologia, Università di Sassari, Via P. Manzella 4, 07100 Sassari, Italy

### Nikolaus Gassler, Professor

Institute of Pathology, University Hospital RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany

### Peter Raymond Gibson, Professor

Department of Medicine, Box Hill Hospital, Box Hill, Victoria 3128, Australia

### Mark D Gorrell, PhD, Professor

Centenary Institute of Cancer Medicine and Cell Biology, Locked bag No. 6, Newtown, NSW 2042, Australia

### Dr. Pietro Invernizzi

Division of Internal Medicine, Department of Medicine, Surgery, Dentistry, San Paolo School of Medicine, University of Milan, Via Di Rudinfi 8, 20142 Milan, Italy

### Tom H Karlsen, MD

Institute of Immunology, Rikshospitalet University Hospital, N-0027 Oslo, Norway

### Serhan Karvar, MD, Assistant Professor of Medicine

University of Southern California, Keck School of Medicine, Division of Gastrointestinal & Liver Diseases, 2011 Zonal Avenue, HMR 101, Los Angeles, CA 90089, United States

### Leonidas G Koniaris, Professor

Alan Livingstone Chair in Surgical Oncology, 3550 Sylvester Comprehensive Cancer Center (310T), 1475 NW 12th Ave., Miami, FL 33136, United States

### Dr. Limas Kupcinskas, Professor

Gastroenterology of Kaunas University of Medicine, Mickeviciaus 9, Kaunas LT 44307, Lithuania

### Yuk-Tong Lee, MD

Department of Medicine and Therapeutics, Prince of Wales Hospital, Shatin, New Territories, Hong Kong, China

### Finlay A Macrae, MD, Professor

Royal Melbourne Hospital, Po Box 2010, Victoria 3050, Australia

### Hanns-Ulrich Marschall, Associate Professor

Karolinska Institutet, Department of Medicine, Division of Gastroenterology and Hepatology, Karolinska University Hospital Huddinge, Stockholm S-14188, Sweden

### Giuseppe Mazzella, Professor

Dipartimento di Medicina Interna e Gastroenterologia, Università di Bologna, Policlinico S Orsola-Malpighi, Via Massarenti 9, 40138 Bologna, Italy

### Hiroaki Nagano, MD, PhD, Associate Professor

Division of Hepato-Biliary-Pancreatic Surgery and Transplantation, Department of Surgery, Graduate School of Medicine, Osaka University, 2-2 Yamadaoka E-2, Suita 565-0871 Osaka, Japan

### Ramesh Roop Rai, MD, DM, Professor & Head

Department of Gastroenterology & Hepatology, S.M.S. Medical College & Hospital, Jaipur 302019, (Rajasthan), India

### Dr. Markus Reiser, Professor

Gastroenterology-Hepatology, Ruhr-Universität Bochum, Bürkle-de-la-Camp-Platz 1, Bochum 44789, Germany

### Ian C Roberts-Thomson, Professor

Department of Gastroenterology and Hepatology, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South 5011, Australia

### Damian Casadesus Rodriguez, MD, PhD

Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

### Francis Seow-Choen, Professor

Seow-Choen Colorectal Centre, Mt Elizabeth Medical Centre, Singapore, 3 Mt Elizabeth Medical Centre #09-10, 228510, Singapore

### Paul E Sijens, PhD, Associate Professor

Radiology, UMCG, Hanzplein 1, 9713GZ Groningen, The Netherlands

### Christian D Stone, MD, MPH, Director

Inflammatory Bowel Disease Program, Assistant Professor of Medicine, Division of Gastroenterology, Washington University School of Medicine, 660 South Euclid Avenue, Campus Box 8124, Saint Louis, MO 63110, United States

### Andrew Ukleja, MD, Assistant Professor

Clinical Assistant Professor of Medicine, Director of Nutrition Support Team, Director of Esophageal Motility Laboratory, Cleveland Clinic Florida, Department of Gastroenterology, 2950 Cleveland Clinic Blvd., Weston, FL 33331, United States

### Marie-Catherine Vozenin-brottons, PhD

UPRES EA 27-10, IRSN/IGR, 39 rue C. Desmoulins, Villejuif Cedex 94305, France

### Eddie Wisse, Professor

Irisweg 16, Keerbergen 3140, Belgium

### Harry HX Xia, PhD, MD

Novartis Pharmaceuticals Corporation, One Health Plaza, East Hanover, NJ 07936-1080, United States

### Kentaro Yoshika, Associate Professor

Division of Gastroenterology, Department of I, Fujita Health University School of Medicine, 1-98 Dengakugakubo, Kutsukade, Toyoake 470-1190, Japan



## Meetings

### Events Calendar 2008-2009

**FALK SYMPOSIA 2008**  
 January 24-25, Frankfurt, Germany  
 Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008  
 February 14-16, Paris, France  
 EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
 8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
 3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation  
 Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

February 29, Québec, Canada  
 Canadian Association of Gastroenterology  
 E-mail: [general@cag-acg.org](mailto:general@cag-acg.org)

March 10-13, Birmingham, UK  
 British Society of Gastroenterology Annual Meeting  
 E-mail: [BSG@mailbox.ulcc.ac.uk](mailto:BSG@mailbox.ulcc.ac.uk)

March 14-15, HangZhou, China  
 Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
 Asian Pacific Association for the Study of the Liver  
 18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
 Shanghai-Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
 OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas  
 E-mail: [robert.giuli@oeso.org](mailto:robert.giuli@oeso.org)

April 9-12, Los Angeles, USA  
 SAGES 2008 Annual Meeting - part of Surgical Spring Week  
[www.sages.org/08program/html/](http://www.sages.org/08program/html/)

April 18-22, Buenos Aires, Argentina  
 9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
 Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
 43<sup>rd</sup> Annual Meeting of the European Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary

Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA  
 Digestive Disease Week 2008

May 21-22, California, USA  
 ASGE Annual Postgraduate Course  
 Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion  
 E-mail: [education@fsg.org](mailto:education@fsg.org)

June 4-7, Helsinki, Finland  
 The 39<sup>th</sup> Nordic Meeting of Gastroenterology  
[www.congex.com/ngc2008](http://www.congex.com/ngc2008)

June 5-8, Sitges (Barcelona), Spain  
 Semana de las Enfermedades Digestivas  
 E-mail: [sepd@sepd.es](mailto:sepd@sepd.es)

June 6-8, Prague, Czech Republic  
 3<sup>rd</sup> Annual European Meeting: Perspectives in Inflammatory Bowel Diseases  
 E-mail: [meetings@imedex.com](mailto:meetings@imedex.com)

June 10-13, Istanbul, Turkey  
 ESGAR 2008 19<sup>th</sup> Annual Meeting and Postgraduate Course  
 E-mail: [fca@netvisao.pt](mailto:fca@netvisao.pt)

June 11-13, Stockholm, Sweden  
 16<sup>th</sup> International Congress of the European Association for Endoscopic Surgery  
 E-mail: [info@aes-eur.org](mailto:info@aes-eur.org)

June 13-14, Amsterdam, Netherlands  
 Falk Symposium 165: XX International Bile Acid Meeting. Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic  
 Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management  
 E-mail: [idla2008@guarant.cz](mailto:idla2008@guarant.cz)

June 25-28, Barcelona, Spain  
 10<sup>th</sup> World Congress on Gastrointestinal Cancer  
 Imedex and ESMO  
 E-mail: [meetings@imedex.com](mailto:meetings@imedex.com)

June 25-28, Lodz, Poland  
 Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatologists (IAP)  
 E-mail: [office@epc-iap2008.org](mailto:office@epc-iap2008.org)  
[www.e-p-c.org](http://www.e-p-c.org)  
[www.pancreatology.org](http://www.pancreatology.org)

June 26-28, Bratislava, Slovakia  
 5<sup>th</sup> Central European Gastroenterology Meeting  
[www.ceurgem2008.cz](http://www.ceurgem2008.cz)

July 9-12, Paris, France  
 ILTS 14<sup>th</sup> Annual International Congress  
[www.iltis.org](http://www.iltis.org)

September 10-13, Budapest, Hungary  
 11<sup>th</sup> World Congress of the International Society for Diseases of the Esophagus  
 E-mail: [isde@isde.net](mailto:isde@isde.net)

September 13-16, New Delhi, India  
 Asia Pacific Digestive Week  
 E-mail: [apdw@apdw2008.net](mailto:apdw@apdw2008.net)

III FALK GASTRO-CONFERENCE  
 September 17, Mainz, Germany

Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany  
 Falk Symposium 166:  
 GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic  
 Prague Hepatology Meeting 2008  
[www.czech-hepatology.cz/phm2008](http://www.czech-hepatology.cz/phm2008)

September 20-21, Mainz, Germany  
 Falk Symposium 167:  
 Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France  
 Third Annual Meeting  
 European Society of Coloproctology  
[www.escp.eu.com](http://www.escp.eu.com)



October 8-11, Istanbul, Turkey  
 18<sup>th</sup> World Congress of the International Association of Surgeons,  
 Gastroenterologists and Oncologists  
 E-mail: [orkun.sahin@serenas.com.tr](mailto:orkun.sahin@serenas.com.tr)

October 18-22, Vienna, Austria  
 16<sup>th</sup> United European Gastroenterology Week  
[www.negf.org](http://www.negf.org)  
[www.acv.at](http://www.acv.at)

October 22-25, Minnesota, USA  
 Anstralian Gastroenterology Week 2008  
 E-mail: [gesa@gesa.org.au](mailto:gesa@gesa.org.au)

October 22-25, Brisbane, Australia  
 71<sup>st</sup> Annual Colon and Rectal Surgery Conference  
 E-mail: [info@colonrectalcourse.org](mailto:info@colonrectalcourse.org)

October 31-November 4, Moscone West Convention Center, San Francisco, CA  
 59<sup>th</sup> AASLD Annual Meeting and Postgraduate Course  
 The Liver Meeting  
 Information: [www.aasld.org](http://www.aasld.org)

November 6-9, Lucerne, Switzerland  
 Neurogastroenterology & Motility Joint International Meeting 2008  
 E-mail: [ngm2008@mci-group.com](mailto:ngm2008@mci-group.com)  
[www.ngm2008.com](http://www.ngm2008.com)

November 12, Santiago de Chile, Chile  
 Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt  
 1<sup>st</sup> Hepatology and Gastroenterology Post Graduate Course  
[www.egyptgastrohep.com](http://www.egyptgastrohep.com)

December 7-9, Seoul, Korea  
 6<sup>th</sup> International Meeting  
 Hepatocellular Carcinoma: Eastern and Western Experiences  
 E-mail: [sglee@amc.seoul.kr](mailto:sglee@amc.seoul.kr)

INFORMATION FOR ALL  
 FALK FOUNDATION e.V.  
 E-mail: [symposia@falkfoundation.de](mailto:symposia@falkfoundation.de)  
[www.falkfoundation.de](http://www.falkfoundation.de)

Advanced Courses - European

Institute of Telesurgery EITS - 2008  
 Strasbourg, France  
 January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S  
 April 3-5, November 27-29  
 Laparoscopic Digestive Surgery

June 27-28, November 7-8  
 Laparoscopic Colorectal Surgery

July 3-5  
 Interventional GI Endoscopy Techniques  
 Contact address for all courses:  
 E-mail: [info@eits.fr](mailto:info@eits.fr)

International Gastroenterological Congresses 2009  
 March 23-26, Glasgow, Scotland  
 Meeting of the British Society of Gastroenterology (BSG)  
 E-mail: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

May 17-20, Denver, Colorado, USA  
 Digestive Disease Week 2009

November 21-25, London, UK  
 Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.





## Instructions to authors

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*World Journal of Gastroenterology* (World J Gastroenterol ISSN 1007-9327 CN 14-1219/R) is a weekly open access peer-reviewed journal supported by an editorial board consisting of 1215 experts in gastroenterology and hepatology from 60 countries. The aim of the journal is to deliver the most clinically relevant original and commentary articles to readers, and to make the full text publicly available to all clinicians, scientists, patients and biomedical students on an unrestricted platform, so that they can access and learn about the most recent key advances in the field.

In addition to the open access nature, another key characteristic of *WJG* is its reading guidance for each article which includes background, research frontier, related reports, breakthroughs, applications, terminology, and comments of peer reviewers for the general readers.

*WJG* publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidemiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

The main goal of *WJG* is to publish high quality commentary articles contributed by leading experts in gastroenterology and hepatology and original articles that combine the clinical practice and advanced basic research, to provide an interactive platform for clinicians and researchers in internal medicine, surgery, infectious diseases, traditional Chinese medicine, oncology, integrated Chinese and Western medicine, imaging, endoscopy, interventional therapy, pathology and other basic medical specialties, and thus eventually improving the clinical practice and healthcare for patients.

### Indexed and abstracted in

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### Published by

The WJG Press

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**Author contributions:** The format of this section should be like this: Author contributions: Wang CL and Liang L contributed equally to this work; Wang CL, Liang L, Fu JF, Zou CC, Hong F and Wu XM designed research; Wang CL, Zou CC, Hong F and Wu XM performed research; Xue JZ and Lu JR contributed new reagents/analytic tools; Wang CL, Liang L and Fu JF analyzed data; and Wang CL, Liang L and Fu JF wrote the paper.

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The format for structured abstracts can be found at: <http://www.wjgnet.com/wjg/help/11.doc>.

#### Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

#### Text

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and case reports, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the body text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, should be found at: <http://www.wjgnet.com/wjg/help/instructions.jsp>.

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Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: <http://www.wjgnet.com/1007-9327/13/4520.pdf>; <http://www.wjgnet.com/1007-9327/13/4554.pdf>; <http://www.wjgnet.com/1007-9327/13/4891.pdf>; <http://www.wjgnet.com/1007-9327/13/4986.pdf>; <http://www.wjgnet.com/1007-9327/13/4498.pdf>. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ... *etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

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Three-line tables should be numbered 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each table. Detailed legends should not be included under tables, but rather added into the text where applicable. The information should complement but not duplicate the text. Use one horizontal line under the title, a second under column heads, and a third below the Table, above any footnotes. Vertical and italic lines should be omitted.

### Notes in tables and illustrations

Data that are not statistically significant should not be noted. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 should be noted (*P* > 0.05 should not be noted). If there are other series of *P* values, <sup>c</sup>*P* < 0.05 and <sup>d</sup>*P* < 0.01 are used. A third series of *P* values can be expressed as <sup>e</sup>*P* < 0.05 and <sup>f</sup>*P* < 0.01. Other notes in tables or under illustrations should be expressed as <sup>1</sup>F, <sup>2</sup>F, <sup>3</sup>F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

### Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

## REFERENCES

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The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability<sup>[1,2]</sup>". If references are cited directly in the text, they should be put together within the text, for example, "From references<sup>[19,22-24]</sup>, we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

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Authors: the name of the first author should be typed in bold-faced letters. The family name of all authors should be typed with the initial letter capitalized, followed by their abbreviated first and middle initials. (For example, Lian-Sheng Ma is abbreviated as Ma LS, Bo-Rong Pan as Pan BR). The title of the cited article and italicized journal title (journal title should be in its abbreviated form as shown in PubMed), publication date, volume number (in black), start page, and end page [PMID: 11819634 DOI: 10.3748/wjg.13.5396].

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### Format

#### Journals

*English journal article (list all authors and include the PMID where applicable)*

- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

*Chinese journal article (list all authors and include the PMID where applicable)*

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

*In press*

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

*Organization as author*

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

*No author given*

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

*Volume with supplement*

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment

of migraine and in comparison with sumatriptan. *Headache* 2002; 42 Suppl 2: S93-99 [PMID: 12028325]

*Issue with no volume*

- 8 Banit DM, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (401): 230-238 [PMID: 12151900]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS/A Careaction* 2002; 1-6 [PMID: 12154804]

## Books

*Personal author(s)*

- 10 Sherlock S, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 Lam SK. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 Breedlove GK, Schorffheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 Harnden P, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

*Conference paper*

- 14 Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

## Electronic journal (list all authors)

Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

## Patent (list all authors)

- 16 Pagedas AC, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

## Inappropriate references

Authors should always cite references that are relevant to their article, and avoid any inappropriate references. Inappropriate references include those linked with a hyphen when the difference between the two numbers is greater than five. For example, [1-6], [2-14] and [1, 3, 4-10, 22] are all considered inappropriate references. Authors should not cite their own unrelated published articles.

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## Statistical expression

Express *t* test as *t* (in italics), *F* test as *F* (in italics), chi square test as  $\chi^2$  (in Greek), related coefficient as *r* (in italics), degree of freedom as *v* (in Greek), sample number as *n* (in italics), and probability as *P* (in italics).

## Units

Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose)  $6.4 \pm 2.1$  mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6  $24.5 \mu\text{g/L}$ ; CO<sub>2</sub> volume fraction, 50 mL/L CO<sub>2</sub>, not 5% CO<sub>2</sub>; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

The format for how to accurately write common units and quantum numbers can be found at: <http://www.wjgnet.com/wjg/help/15.doc>.

## Abbreviations

Standard abbreviations should be defined in the abstract and on first mention in the text. In general, terms should not be abbreviated unless they are used repeatedly and the abbreviation is helpful to the reader. Permissible abbreviations are listed in Units, Symbols and Abbreviations: A Guide for Biological and Medical Editors and Authors (Ed. Baron DN, 1988) published by The Royal Society of

Medicine, London. Certain commonly used abbreviations, such as DNA, RNA, HIV, LD50, PCR, HBV, ECG, WBC, RBC, CT, ESR, CSF, IgG, ELISA, PBS, ATP, EDTA, mAb, can be used directly without further explanation.

## Italics

Quantities: *t* time or temperature, *c* concentration, *A* area, *l* length, *m* mass, *V* volume.

Genotypes: *gyrA*, *arg 1*, *c myc*, *c fos*, etc.

Restriction enzymes: *EcoRI*, *HindIII*, *BamHI*, *Kpn I*, *Kpn I*, etc.

Biology: *H pylori*, *E coli*, etc.

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<sup>[1]</sup>Passed away on October 20, 2007

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## Cardiac evaluation of liver transplant candidates

Mercedes Susan Mandell, JoAnn Lindenfeld, Mei-Yung Tsou, Michael Zimmerman

Mercedes Susan Mandell, Department of Anesthesiology, University of Colorado Health Sciences Center, Aurora, Colorado 80045, United States

JoAnn Lindenfeld, Department of Cardiology, University of Colorado Health Sciences Center, Aurora, Colorado 80045, United States

Mei-Yung Tsou, Department of Anesthesiology, Taipei Veterans General Hospital and National Yang-Ming University School of Medicine, Taipei, Taiwan

Michael Zimmerman, Department of Surgery, University of Colorado Health Sciences Center, Aurora, Colorado 80045, United States

**Author contributions:** Mandell MS, Lindenfeld J, Tsou MY, Zimmerman M contributed equally to this paper.

**Correspondence to:** Mercedes Susan Mandell, MD, PhD, University of Colorado Health Sciences Center, Leprino Building 7th Floor, 12401 East 17th Ave B113, Aurora, Colorado 80045, United States. [susan.mandell@uchsc.edu](mailto:susan.mandell@uchsc.edu)

Telephone: +1-720-8486709 Fax: +1-720-8487375

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### Abstract

Physicians previously thought that heart disease was rare in patients with end stage liver disease. However, recent evidence shows that the prevalence of ischemic heart disease and cardiomyopathy is increased in transplant candidates compared to most other surgical candidates. Investigators estimate that up to 26% of all liver transplant candidates have at least one critical coronary artery stenosis and that at least half of these patients will die perioperatively of cardiac complications. Cardiomyopathy also occurs in greater frequency. While all patients with advanced cardiac disease have defects in cardiac performance, a larger than expected number of patients have classical findings of dilated, restrictive and hypertrophic cardiomyopathy. This may explain why up to 56% of patients suffer from hypoxemia due to pulmonary edema following transplant surgery. There is considerable controversy on how to screen transplant candidates for the presence of heart disease. Questions focus upon, which patients should be screened and what tests should be used. This review examines screening strategies for transplant candidates and details the prognostic value of common tests used to identify ischemic heart disease. We also review the physiological consequences of cardiomyopathy in transplant candidates and explore the specific syndrome of "cirrhotic cardiomyopathy".

### INTRODUCTION

Physicians previously thought that heart disease was rare in patients with cirrhosis<sup>[1]</sup>. Postoperative mortality was previously due to operative complications and poor donor graft function. Better surgical technique and donor organ management have significantly improved patient survival. However, as long term patient survival increased, cardiac complications emerged as a more common cause of early morbidity and mortality. Recent studies report a high incidence of post-transplant cardiovascular complications with arrhythmias and overt congestive heart failure in as many as 25% and 56% of all transplant recipients respectively<sup>[2,3]</sup>.

It is now clear that patients with end stage liver disease are at increased risk of acute coronary occlusion, myocardial failure, arrhythmia and complete cardiovascular collapse following transplantation compared to other major surgical procedures. However, there is no consensus on how to efficiently detect cardiovascular disease in asymptomatic patients prior to transplantation or to determine what risk a transplant candidate with heart disease has of suffering from a serious perioperative adverse event. Without this information it is difficult to determine what type or severity of heart disease should exclude a patient from transplantation.

### CARDIOVASCULAR DISEASES IN CANDIDATES FOR LIVER TRANSPLANTATION

The most common cardiovascular diseases in

transplant candidates are ischemic coronary artery disease (CAD) and cardiomyopathy. These diseases are independent negative predictors of outcome following transplantation. However, the poor exercise capacity in patients with advanced liver disease makes it difficult to identify cardiac disease. These patients may not experience common symptoms brought on by exercise such as chest pain or shortness of breath. Further, it may be impossible to determine if some symptoms such as shortness of breath are caused by cardiac or liver disease. Thus, screening asymptomatic patients for underlying cardiac disease is an essential step in the evaluation of transplant candidates.

### Ischemic heart disease

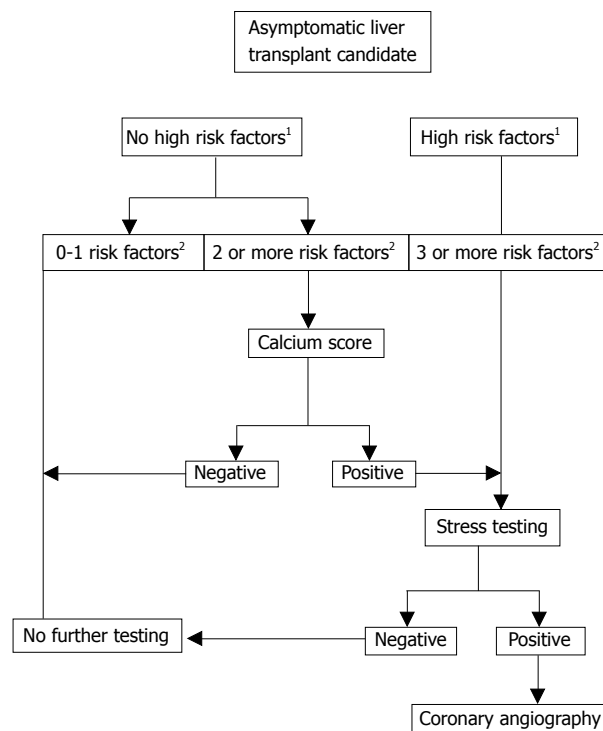
Patients can have substantial atherosclerosis, but remain asymptomatic. Symptoms only develop once the amount of coronary blood flow is insufficient to meet the oxygen needs of the myocardial tissue. This usually occurs when the degree of occlusion exceeds 50%<sup>[4]</sup>. However, lesion size alone does not always predict the onset of symptoms. Rather, symptoms are influenced by myocardial tissue demands. Vigorous exercise increases myocardial oxygen demand and may cause symptoms with lesions that are less than 50%. Figure 1 shows the algorithm for the diagnosis of ischemic heart disease.

Myocardial infarction can occur in patients who have lesions that occupy less than 50% of the coronary lumen<sup>[4,5]</sup>. This is caused by plaque rupture, resulting in acute vessel thrombosis. Liver disease may increase the risk of coronary complications in patients with non-occlusive disease. Chronic inflammation coupled with increased blood flow can predispose to plaque rupture. These adverse conditions are worsened by the high metabolic demand that occurs during liver transplantation.

Evidence suggests that CAD is more prevalent in transplant candidates. Studies show that at least one critical coronary artery lesion occurs in 5% to 26% of all liver transplant candidates who are asymptomatic<sup>[6-9]</sup>. Up to 50% of patients with significant CAD will die perioperatively from cardiac complications<sup>[7]</sup>. This is substantially greater than the one year mortality rate (10%) for all liver transplant recipients<sup>[10]</sup>, or the mortality rate from cardiac complications for other study populations<sup>[11,12]</sup>. A previous history or classic symptoms of CAD places patients in well-tested protocols that direct further evaluation<sup>[13,14]</sup>. However, the challenge remains in identifying those patients who are asymptomatic, but have significant CAD.

### RISK FACTORS FOR CAD IN CIRRHOSIS

Specific patient attributes are correlated with CAD<sup>[15]</sup>. The actual demographic profile of a patient with CAD is remarkably similar throughout the world<sup>[16,17]</sup>. High blood pressure, elevated cholesterol, diabetes and obesity are the most prevalent attributes. Risk factors for CAD are also prevalent in liver transplant candidates<sup>[9]</sup>. Age > 50 years, male gender, diabetes and obesity are



**Figure 1** Algorithm for the diagnosis of ischemic heart disease. There is no consensus on how to evaluate asymptomatic liver transplant recipients for the presence of CAD. However, a logical algorithm can be constructed using the current evidence from a limited number of outcome studies. The utility of this algorithm will require outcome testing to determine its sensitivity and specificity. In this approach, asymptomatic liver transplant recipients are divided according to the presence risk factors for CAD. Patients with 0-1 risk factor require no further evaluation. Those with 2 risk factors are first assessed by calcium scoring. If the score is zero, no further testing is required. If the test is not zero patients are referred for stress testing. Patients referred directly for stress testing include those with 3 or more risk factors and those with high risk factors. A positive stress test is an indication for coronary catheterization. <sup>1</sup>High Risk Factors for CAD include diabetes, NASH, previous CAD, peripheral vascular disease; <sup>2</sup>Risk Factors for CAD include age > 50 yr, hypertension, elevated cholesterol and obesity.

common. In addition, altered lipid metabolism and other unknown factors may make cirrhosis an independent risk factor. Cirrhotic patients with 0-1 risk factors have a low likelihood for CAD. However, the presence of two or more factors (other than age) places patients at moderate to severe risk of CAD<sup>[9]</sup>. A diagnosis of non-alcoholic steatohepatitis (NASH) independently increases the risk of CAD<sup>[18]</sup>. Critical CAD occurs in approximately 23% of patient with NASH<sup>[18]</sup>. Even though NASH patients have additional risk factors including obesity and diabetes, the prevalence of CAD is greater than the sum of associated risk factors<sup>[19,20]</sup>. Overall transplant candidates with diabetes, peripheral vascular disease including carotid atherosclerosis, abdominal aneurysm, chronic renal failure or high risk-Framington Score are likely to have CAD and need further evaluation<sup>[14]</sup>.

### SCREENING TESTS FOR CAD IN CIRRHOSIS

The prognostic value of any screening test is related to the prevalence of the disease in the population under

study<sup>[14]</sup>. Some investigators argue that the prevalence of CAD in transplant candidates is insufficient to warrant testing all asymptomatic patients greater than 50 years of age<sup>[21]</sup>. However, this opinion does not take into account the high perioperative mortality associated with CAD in these patients. Therefore, it is reasonable to err on the side of caution by optimizing sensitivity at the expense of lowering specificity. While few patients with only one risk factor have positive findings, 2 risk factors significantly increases the chance of critical CAD in this population<sup>[6,21]</sup>. However, not all risk factors confer an equal chance of CAD. Diabetes and NASH are more predictive of CAD than other attributes such as age or gender<sup>[21,22]</sup>. Therefore, the decision to proceed with noninvasive testing must be tempered by the relative predictive strength of each risk factor rather than just the number of factors present.

### Calcium scores

Calcium crystals are commonly deposited in coronary atherosclerotic lesions and the degree of calcification correlates with the severity of occlusive coronary atherosclerosis<sup>[23]</sup>. Computerized tomography is a non-invasive and rapid way to measure calcium deposits within the coronary vasculature. The amount of cardiac calcium is summated into scores (CACS) that are reported as a percentile according to age and gender. Higher scores suggest a greater degree of coronary artery stenosis<sup>[24]</sup>. Calcium scores are, therefore, used to detect and grade the severity of CAD<sup>[25]</sup>. However, the CACS has limited predictive value as a single screening study for CAD. The CACS is relatively insensitive with a weighted average of only 40% for detecting critical lesions<sup>[25]</sup>. A comparison of CACS with echocardiography also suggests a lack of specificity<sup>[26-30]</sup>. Despite these limitations, CACS does provide incremental prognostic information to assess cardiac risk with respect to cardiac death, myocardial revascularization or myocardial infarction<sup>[31]</sup>. Patients with a CACS > 100 are 5 times more likely to have ischemic coronary events compared to those with a CACS < 100 and significant coronary disease is rare with a score of zero.

Non-calcified coronary plaque can also cause myocardial infarction and death. However, non-calcified lesions are uncommon in patients with a CACS of zero<sup>[32]</sup>. When present, stenosis is usually less than 50% of the luminal diameter. This finding reinforces the strong predictive value of a CACS of zero. However, non-calcified lesions and their degree of stenosis increase significantly as the CACS increases. Current evidence suggest that CACS as a single test has limited value except to separate patients with minimal risk of CAD (CACS = zero) from those who have more advanced disease.

### Stress testing

Stress testing is used to increase myocardial oxygen demand in order to identify critically obstructed coronary vessels. Graded exercise and the intravenous administration of drugs that increase oxygen consumption are the two most efficient ways to test for reversible ischemia. Ischemic changes are captured using common imaging techniques such as echocardiographic wall motion abnormalities and

radionuclide myocardial perfusion defects. A stress test cannot identify disease caused by atherosclerotic plaques that are too small to limit coronary blood flow, even if these lesions are at risk of rupture.

More severe disease (more vessels involved and/or more severe stenosis) improves the predictive power of the test. In general, graded exercise stress tests coupled with echocardiography or myocardial perfusion imaging have similar sensitivity and specificity<sup>[33]</sup> but, the predictive values of the tests diverge when pharmacologic agents are used. Echocardiography is less sensitive but more specific than myocardial perfusion with dobutamine<sup>[34]</sup> and dobutamine is better than vasodilators such as adenosine or dipyridamole for inducing ischemia with either technique<sup>[35]</sup>.

Dobutamine myocardial perfusion imaging is more sensitive than dobutamine echocardiography for detecting myocardial ischemia in patients with liver disease<sup>[36]</sup>. In transplant candidates these tests have a better negative rather than positive predictive value<sup>[37]</sup>. Thus, a negative test confers a minimal chance of CAD. There is a greater chance that no lesion will be identified angiographically if the test is positive. However, the unique physiological stress associated with liver transplantation places patients at greater risk of plaque rupture, even if less than 50% of the vessel is occluded. There are more false positive stress tests in transplant candidates. However, there are also more postoperative deaths in transplant recipients who had abnormal myocardial perfusion studies but no angiographic lesions<sup>[36]</sup>. These patients die of perioperative cardiovascular complications, sepsis and donor graft failure<sup>[38]</sup>. Investigators suggest that myocardial perfusion defects with a normal angiogram are not necessarily benign findings and could be caused by reduced microvascular coronary blood flow<sup>[38]</sup>.

Questions remain as to what degree of lesion requires intervention and what type of treatment is best. Most subcritical coronary stenoses in surgical patients can be medically managed. However, investigators report a perioperative death rate of more than 50% in transplant recipients with CAD who were managed medically<sup>[7]</sup>. Further myocardial infarction during transplant surgery has occurred with as little as 30% vessel occlusion<sup>[36]</sup>. To date there is no consensus on what degree of stenosis needs treatment in liver transplant candidates because there are very few reports on the outcomes of coronary interventions in transplant candidates.

## CARDIOMYOPATHY

The prevalence of cardiomyopathy is greater in patients with end stage liver disease than the general population<sup>[39-41]</sup>. Conditions such as hepatitis C can cause immune-mediated myocarditis and fibrosis resulting in restrictive cardiomyopathy<sup>[41]</sup>. Hemochromatosis and amyloidosis also cause restrictive cardiomyopathy due to the infiltration of iron and protein respectively. Findings similar to dilated cardiomyopathy are also commonly reported from routine echocardiographic screening of

transplant candidates<sup>[40]</sup>. Further, an increased reporting of hypertrophic cardiomyopathy in transplant candidates suggests that this disorder may also be more prevalent in cirrhotic patients<sup>[42,43]</sup>.

Symptoms of liver disease are often similar to those of heart failure. Therefore, it is difficult to determine if fatigue, shortness of breath and adventitious heart sounds are due to liver disease alone or if patients have cardiac failure. Routine screening of transplant candidates with echocardiography is an effective way to identify comorbid cardiac disease. Even though cardiac disease can be readily identified, there is no agreement on how disease influences transplant candidacy. Currently, there is no outcome data using cardiomyopathy as a primary study variable. However, investigators have reported that patients with dilated cardiomyopathy seem to improve after successful transplantation<sup>[40]</sup>. In contrast, the prognosis for infiltrative processes such as Hepatitis C, amyloidosis and hemochromatosis does not seem as good and cardiac disease is reported to progress to overt heart failure despite successful transplantation<sup>[41,44]</sup>.

### **Cirrhotic cardiomyopathy**

Some cirrhotic patients have obvious features of cardiomyopathy, however most have more subtle defects in myocardial function that are not apparent on cursory examination. Early cardiac decompensation is often missed because the cardiac workload is reduced by peripheral vasodilation caused by liver failure<sup>[45]</sup>. These patients are dismissed as having normal heart function. However, when these patients are subject to physiological or pharmacological stress, they develop clinical signs of suboptimal perfusion including renal failure and acidosis. Investigators have concluded that exercise uncovers an intrinsic defect in myocardial function that predisposes to heart failure<sup>[46,47]</sup>. This condition is called "cirrhotic cardiomyopathy" and although the clinical presentation can be variable, all patients have four common features. These are: (1) Baseline increased cardiac output; (2) Attenuated systolic contraction and diastolic relaxation; (3) Electrophysiological abnormalities including repolarization change; and (4) A reduced response of the heart to direct beta stimulation ( $\beta$ -incompetence)<sup>[48]</sup>. These changes occur in the absence of overt congestive failure.

### **Myocardial function in cirrhotic cardiomyopathy**

Indices of left ventricular contractility such as the stroke index, mean systolic ejection rate, left ventricular stroke-work and left ventricular stroke-power are greater than expected in the cirrhotic patient at rest. The only clue early that the heart may not be normal is a blunted response to exercise<sup>[49]</sup>. The standing position and physical or mental stress have minimal impact on cardiac output in cirrhotic patients. But, the absolute and relative increase in cardiac output in response to exercise is reduced compared to controls<sup>[49]</sup>. Thus, aerobic exercise capacity and maximal heart rate are lower than expected<sup>[49-51]</sup>.

Early histological changes include myocardial hypertrophy, interstitial and cellular edema and signs of cellular injury<sup>[52]</sup>. This causes thickening of the left

ventricle with the septum affected more than the free wall<sup>[49]</sup>. Overall, these effects are more pronounced in patient with ascites compared to those without<sup>[46]</sup>. As wall thickness increases so does the degree of diastolic dysfunction. Impaired diastolic relaxation prolongs isovolumetric relaxation and the ventricular pressure is greater than normal for any given end diastolic volume. The left atrium dilates in response to the higher impedance to left ventricular filling. When these patients experience circulatory changes that rapidly increase filling pressure, congestive heart failure develops. This likely explains why patients experience an increased incidence of congestive heart failure after procedures such as transjugular intrahepatic shunts and liver transplantation<sup>[53]</sup>. In fact, diastolic disturbance is such a consistent feature of cirrhotic cardiomyopathy that many investigators suggest that some degree of diastolic dysfunction is present in all patients with liver disease<sup>[52]</sup>.

At rest, systolic function appears to be normal in most patients with liver disease. However, the mechanics of systolic contraction are commonly disturbed. This is shown by examination of the systolic time interval. The length of systole remains constant, but left ventricular ejection period takes up a larger percentage of the time interval. This in turn shortens the pre-ejection period<sup>[54]</sup>. In contrast to changes in diastole, systolic dysfunction usually only becomes evident during exercise. Ejection fraction does not increase as expected under conditions of stress. Further, an increase in filling pressures does not increase ejection fraction and the Frank Starling curve flattens<sup>[54]</sup>. As liver disease progresses systolic function can be insufficient to meet the resting tissue oxygen demands<sup>[55]</sup>. The impaired systolic response to stress is etiologic in the increased incidence of pulmonary edema and congestive heart failure following procedures that abruptly increase blood flow to the heart<sup>[54]</sup>.

### **Electromechanical disorders in cirrhotic cardiomyopathy**

The events of electrical depolarization and mechanical systole are normally tightly linked in time and exhibit little variability. Thus, the events are considered "coupled". The time interval needed for ventricular repolarization is limited and also has little variability. This is to prevent the next depolarizing current from advancing into a partially depolarized conducting system and causing re-entry arrhythmias. Patients with liver disease exhibit three common cardiac electrophysiological disturbances in cardiac function. These include: (1) Electromechanical dissociation; (2) Prolongation of ventricular repolarization (the QT interval); and (3) Chronotropic incompetence<sup>[52]</sup>.

The time period between electrical and mechanical systole is longer in patients with cirrhosis<sup>[56]</sup>. If the conducting system is still partially depolarized when the next action potential arrives, electrical depolarization cannot capture all the mechanical activity of the myocardium. Thus investigators think that defects in electromechanical coupling may contribute to impaired systolic performance in cirrhosis by failing to recruit all available myocardium for the next ventricular contraction<sup>[57]</sup>. Once electromechanical dissociation



becomes severe, it prolongs the time required for repolarization (QT interval). Prolongation and variability in the QT interval can affect cardiac rhythm and cause serious disturbances including ventricular fibrillation. The severity of electromechanical dissociation is clearly related to the severity of liver disease. This is shown by the fact that the length of the QT interval correlates directly with the Child-Pugh score<sup>[54]</sup>. Further, the QT interval in cirrhotics varies in different regions of the heart<sup>[58]</sup>. This dispersion of the QT interval is distinctly abnormal and the degree of abnormality is also related to the severity of liver disease. While patients with congenital prolonged QT appear to have defects in the sodium receptors that regulate electrical gating, the potassium gates of the conducting system are primarily affected in patients with liver disease<sup>[59]</sup>.

Normally, stimulation of  $\beta$ -adrenergic cardiac receptors in healthy subjects causes an increase in both the rate and force of cardiac contractions. However, cirrhotic patients exhibit a blunted response to both physiological and pharmacological  $\beta$  stimulation<sup>[55]</sup>. A suboptimal response of heart rate to  $\beta$  stimulation, termed chronotropic incompetence is found in patients with heart failure in addition those with cirrhosis. It is a proven poor prognostic indicator in all types of heart failure<sup>[60]</sup>. Chronotropic incompetence is likely also a predictor of mortality in patients with liver disease<sup>[47]</sup>.

Studies of experimental animals show that the progressive failure of  $\beta$ -agonists to elicit a positive chronotropic and inotropic response is caused by an acquired defect in the mechanism of  $\beta$ -receptor signaling. There is both a reduction in the number of  $\beta$ -adrenoceptors in the cell membrane and multiple defects in the pathway that link receptor stimulation to contractility<sup>[61]</sup>. These findings occur to some degree in all patients with liver disease<sup>[48]</sup>. The response to  $\beta$ -stimulation is relatively preserved early in the course of liver disease but becomes increasingly "incompetent" as liver disease progresses. In end stage liver disease, no significant increase in heart rate occurs in response to Valsalva maneuver or tilting. Further, the dose of isoproterenol required to raise heart rate is much larger than in healthy subjects<sup>[62]</sup>. Investigators think that failure of  $\beta$ -adrenergic receptors to appropriately stimulate all potential myocardial activity may also contribute to systolic dysfunction in cirrhotic cardiomyopathy<sup>[48]</sup>. This helps to explain why signs of cardiomyopathy are only observed under conditions of physical or pharmacological stress.

## CONCLUSION

Cardiac disease appears more frequently in patients with end stage liver disease. The two most common conditions are ischemic heart disease and cardiomyopathy. Some question whether it is cost effective to screen all transplant candidates for CAD. The unusually high perioperative mortality in transplant patients who do have CAD warrants a systematic evaluation in every patient which presumes a greater risk of atherosclerotic coronary disease. No single test has 100% predictive value. Therefore diagnostic protocols must account for the

variation in prevalence that occurs in subsets of transplant candidates and the limitation of each type of test.

In contrast to ischemic heart disease, most patients with advanced liver disease have myocardial defects that cause systolic and diastolic impairment that is not always evident at rest. There are also underlying electrophysiological defects that cause an uncoupling of mechanical and electrical activity. Diagnosis of "cirrhotic cardiomyopathy" is difficult since the findings can be subtle as some patients will develop frank heart failure when exposed to pharmacological or physiological stress such as liver transplantation.

## REFERENCES

- 1 **Turner TB**, Bennett VL, Hernandez H. The beneficial side of moderate alcohol use. *Johns Hopkins Med J* 1981; **148**: 53-63
- 2 **Snowden CP**, Hughes T, Rose J, Roberts DR. Pulmonary edema in patients after liver transplantation. *Liver Transpl* 2000; **6**: 466-470
- 3 **Donovan CL**, Marcovitz PA, Punch JD, Bach DS, Brown KA, Lucey MR, Armstrong WF. Two-dimensional and dobutamine stress echocardiography in the preoperative assessment of patients with end-stage liver disease prior to orthotopic liver transplantation. *Transplantation* 1996; **61**: 1180-1188
- 4 **Mann J**, Davies MJ. Mechanisms of progression in native coronary artery disease: role of healed plaque disruption. *Heart* 1999; **82**: 265-268
- 5 **Nagoshi T**, Koiwaya Y, Doi H, Eto T. Angiographic coronary morphology in patients with ischemic heart disease. *J Cardiol* 2000; **36**: 91-102
- 6 **Carey WD**, Dumot JA, Pimentel RR, Barnes DS, Hobbs RE, Henderson JM, Vogt DP, Mayes JT, Westveer MK, Easley KA. The prevalence of coronary artery disease in liver transplant candidates over age 50. *Transplantation* 1995; **59**: 859-864
- 7 **Plotkin JS**, Scott VL, Pinna A, Dobsch BP, De Wolf AM, Kang Y. Morbidity and mortality in patients with coronary artery disease undergoing orthotopic liver transplantation. *Liver Transpl Surg* 1996; **2**: 426-430
- 8 **Morris JJ**, Hellman CL, Gawey BJ, Ramsay MA, Valek TR, Gunning TC, Swygert TH, Shore-Lesserson L, Lalehzarian F, Brayman KL. Case 3-1995. Three patients requiring both coronary artery bypass surgery and orthotopic liver transplantation. *J Cardiothorac Vasc Anesth* 1995; **9**: 322-332
- 9 **Tiukinhoy-Laing SD**, Rossi JS, Bayram M, De Luca L, Gafoor S, Blei A, Flamm S, Davidson CJ, Gheorghiade M. Cardiac hemodynamic and coronary angiographic characteristics of patients being evaluated for liver transplantation. *Am J Cardiol* 2006; **98**: 178-181
- 10 **United Network for Organ Sharing Organ Procurement: Available at The U.S. Transplant Network/Scientific Registry of Transplant Recipients 2006 Annual report: Transplant data.** Available from: URL: [http://www.ustransplant.org/annual\\_reports/current/survival\\_rates.htm](http://www.ustransplant.org/annual_reports/current/survival_rates.htm)
- 11 **Yang H**, Raymer K, Butler R, Parlow J, Roberts R. The effects of perioperative beta-blockade: results of the Metoprolol after Vascular Surgery (MaVS) study, a randomized controlled trial. *Am Heart J* 2006; **152**: 983-990
- 12 **Devereaux PJ**, Goldman L, Cook DJ, Gilbert K, Leslie K, Guyatt GH. Perioperative cardiac events in patients undergoing noncardiac surgery: a review of the magnitude of the problem, the pathophysiology of the events and methods to estimate and communicate risk. *CMAJ* 2005; **173**: 627-634
- 13 **Mangano DT**, Browner WS, Hollenberg M, London MJ, Tubau JF, Tateo IM. Association of perioperative myocardial ischemia with cardiac morbidity and mortality in men

- undergoing noncardiac surgery. The Study of Perioperative Ischemia Research Group. *N Engl J Med* 1990; **323**: 1781-1788
- 14 **Eagle KA**, Berger PB, Calkins H, Chaitman BR, Ewy GA, Fleischmann KE, Fleisher LA, Froehlich JB, Gusberg RJ, Leppo JA, Ryan T, Schlant RC, Winters WL Jr, Gibbons RJ, Antman EM, Alpert JS, Faxon DP, Fuster V, Gregoratos G, Jacobs AK, Hiratzka LF, Russell RO, Smith SC Jr. ACC/AHA guideline update for perioperative cardiovascular evaluation for noncardiac surgery--executive summary: a report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Update the 1996 Guidelines on Perioperative Cardiovascular Evaluation for Noncardiac Surgery). *J Am Coll Cardiol* 2002; **39**: 542-553
  - 15 **Welten GM**, Schouten O, van Domburg RT, Feringa HH, Hoeks SE, Dunkelgrun M, van Gestel YR, Goei D, Bax JJ, Poldermans D. The influence of aging on the prognostic value of the revised cardiac risk index for postoperative cardiac complications in vascular surgery patients. *Eur J Vasc Endovasc Surg* 2007; **34**: 632-638
  - 16 **Boersma E**, Poldermans D, Bax JJ, Steyerberg EW, Thomson IR, Banga JD, van De Ven LL, van Urk H, Roelandt JR. Predictors of cardiac events after major vascular surgery: Role of clinical characteristics, dobutamine echocardiography, and beta-blocker therapy. *JAMA* 2001; **285**: 1865-1873
  - 17 **Bhatt DL**, Steg PG, Ohman EM, Hirsch AT, Ikeda Y, Mas JL, Goto S, Liau CS, Richard AJ, Rother J, Wilson PW. International prevalence, recognition, and treatment of cardiovascular risk factors in outpatients with atherothrombosis. *JAMA* 2006; **295**: 180-189
  - 18 **Targher G**, Arcaro G. Non-alcoholic fatty liver disease and increased risk of cardiovascular disease. *Atherosclerosis* 2007; **191**: 235-240
  - 19 **Targher G**, Bertolini L, Padovani R, Rodella S, Arcaro G, Day C. Differences and similarities in early atherosclerosis between patients with non-alcoholic steatohepatitis and chronic hepatitis B and C. *J Hepatol* 2007; **46**: 1126-1132
  - 20 **London RM**, George J. Pathogenesis of NASH: animal models. *Clin Liver Dis* 2007; **11**: 55-74, viii
  - 21 **Kryzhanovski VA**, Beller GA. Usefulness of preoperative noninvasive radionuclide testing for detecting coronary artery disease in candidates for liver transplantation. *Am J Cardiol* 1997; **79**: 986-988
  - 22 **Haffner S**, Taegtmeier H. Epidemic obesity and the metabolic syndrome. *Circulation* 2003; **108**: 1541-1545
  - 23 **Rumberger JA**, Simons DB, Fitzpatrick LA, Sheedy PF, Schwartz RS. Coronary artery calcium area by electron-beam computed tomography and coronary atherosclerotic plaque area. A histopathologic correlative study. *Circulation* 1995; **92**: 2157-2162
  - 24 **Shaw LJ**, Raggi P, Schisterman E, Berman DS, Callister TQ. Prognostic value of cardiac risk factors and coronary artery calcium screening for all-cause mortality. *Radiology* 2003; **228**: 826-833
  - 25 **O'Rourke RA**, Brundage BH, Froelicher VF, Greenland P, Grundy SM, Hachamovitch R, Pohost GM, Shaw LJ, Weintraub WS, Winters WL Jr. American College of Cardiology/American Heart Association Expert Consensus Document on electron-beam computed tomography for the diagnosis and prognosis of coronary artery disease. *J Am Coll Cardiol* 2000; **36**: 326-340
  - 26 **Ramakrishna G**, Breen JF, Mulvagh SL, McCully RB, Pellikka PA. Relationship between coronary artery calcification detected by electron-beam computed tomography and abnormal stress echocardiography: association and prognostic implications. *J Am Coll Cardiol* 2006; **48**: 2125-2131
  - 27 **Berman DS**, Wong ND, Gransar H, Miranda-Peats R, Dahlbeck J, Hayes SW, Friedman JD, Kang X, Polk D, Hachamovitch R, Shaw L, Rozanski A. Relationship between stress-induced myocardial ischemia and atherosclerosis measured by coronary calcium tomography. *J Am Coll Cardiol* 2004; **44**: 923-930
  - 28 **Anand DV**, Lim E, Raval U, Lipkin D, Lahiri A. Prevalence of silent myocardial ischemia in asymptomatic individuals with subclinical atherosclerosis detected by electron beam tomography. *J Nucl Cardiol* 2004; **11**: 450-457
  - 29 **Raggi P**, Shaw LJ, Berman DS, Callister TQ. Prognostic value of coronary artery calcium screening in subjects with and without diabetes. *J Am Coll Cardiol* 2004; **43**: 1663-1669
  - 30 **Nasir K**, Shaw LJ, Liu ST, Weinstein SR, Mosler TR, Flores PR, Flores FR, Raggi P, Berman DS, Blumenthal RS, Budoff MJ. Ethnic differences in the prognostic value of coronary artery calcification for all-cause mortality. *J Am Coll Cardiol* 2007; **50**: 953-960
  - 31 **Kondos GT**, Hoff JA, Sevruckov A, Daviglus ML, Garside DB, Devries SS, Chomka EV, Liu K. Electron-beam tomography coronary artery calcium and cardiac events: a 37-month follow-up of 5635 initially asymptomatic low- to intermediate-risk adults. *Circulation* 2003; **107**: 2571-2576
  - 32 **Cheng VY**, Lepor NE, Madyoon H, Eshaghian S, Naraghi AL, Shah PK. Presence and severity of noncalcified coronary plaque on 64-slice computed tomographic coronary angiography in patients with zero and low coronary artery calcium. *Am J Cardiol* 2007; **99**: 1183-1186
  - 33 **Loong CY**, Anagnostopoulos C. Diagnosis of coronary artery disease by radionuclide myocardial perfusion imaging. *Heart* 2004; **90** Suppl 5: v2-v9
  - 34 **Geleijnse ML**, Krenning BJ, Nemes A, Soliman OI, Galema TW, ten Cate FJ. Diagnostic value of dobutamine stress echocardiography in patients with normal wall motion at rest. *Echocardiography* 2007; **24**: 553-557
  - 35 **Paetsch I**, Jahnke C, Wahl A, Gebker R, Neuss M, Fleck E, Nagel E. Comparison of dobutamine stress magnetic resonance, adenosine stress magnetic resonance, and adenosine stress magnetic resonance perfusion. *Circulation* 2004; **110**: 835-842
  - 36 **Tsutsui JM**, Mukherjee S, Elhendy A, Xie F, Lyden ER, O'Leary E, McGrain AC, Porter TR. Value of dobutamine stress myocardial contrast perfusion echocardiography in patients with advanced liver disease. *Liver Transpl* 2006; **12**: 592-599
  - 37 **Zoghbi GJ**, Patel AD, Ershadi RE, Heo J, Bynon JS, Iskandrian AE. Usefulness of preoperative stress perfusion imaging in predicting prognosis after liver transplantation. *Am J Cardiol* 2003; **92**: 1066-1071
  - 38 **Guckelberger O**, Byram A, Klupp J, Neumann UP, Glanemann M, Stockmann M, Neuhaus R, Neuhaus P. Coronary event rates in liver transplant recipients reflect the increased prevalence of cardiovascular risk-factors. *Transpl Int* 2005; **18**: 967-974
  - 39 **Nagarakanti R**, Whellan D, Rubin S, Mather PJ. Reversible cardiomyopathies. *Cardiol Rev* 2007; **15**: 178-183
  - 40 **Torregrosa M**, Aguade S, Dos L, Segura R, Gonzalez A, Evangelista A, Castell J, Margarit C, Esteban R, Guardia J, Genesca J. Cardiac alterations in cirrhosis: reversibility after liver transplantation. *J Hepatol* 2005; **42**: 68-74
  - 41 **Matsumori A**. Hepatitis C virus infection and cardiomyopathies. *Circ Res* 2005; **96**: 144-147
  - 42 **Harley ID**, Jones EF, Liu G, McCall PR, McNicol PL. Orthotopic liver transplantation in two patients with hypertrophic obstructive cardiomyopathy. *Br J Anaesth* 1996; **77**: 675-677
  - 43 **Paramesh AS**, Fairchild RB, Quinn TM, Leya F, George M, Van Thiel DH. Amelioration of hypertrophic cardiomyopathy using nonsurgical septal ablation in a cirrhotic patient prior to liver transplantation. *Liver Transpl* 2005; **11**: 236-238
  - 44 **Goss JA**, Stribling R, Martin P. Adult liver transplantation for metabolic liver disease. *Clin Liver Dis* 1998; **2**: 187-210
  - 45 **Iwakiri Y**, Groszmann RJ. The hyperdynamic circulation of chronic liver diseases: from the patient to the molecule. *Hepatology* 2006; **43**: S121-S131

- 46 **Valeriano V**, Funaro S, Lionetti R, Riggio O, Pulcinelli G, Fiore P, Masini A, De Castro S, Merli M. Modification of cardiac function in cirrhotic patients with and without ascites. *Am J Gastroenterol* 2000; **95**: 3200-3205
- 47 **Ma Z**, Lee SS. Cirrhotic cardiomyopathy: getting to the heart of the matter. *Hepatology* 1996; **24**: 451-459
- 48 **Liu H**, Song D, Lee SS. Cirrhotic cardiomyopathy. *Gastroenterol Clin Biol* 2002; **26**: 842-847
- 49 **Wong F**, Girgrah N, Graba J, Allidina Y, Liu P, Blendis L. The cardiac response to exercise in cirrhosis. *Gut* 2001; **49**: 268-275
- 50 **Epstein SK**, Ciubotaru RL, Zilberberg MD, Kaplan LM, Jacoby C, Freeman R, Kaplan MM. Analysis of impaired exercise capacity in patients with cirrhosis. *Dig Dis Sci* 1998; **43**: 1701-1707
- 51 **Campillo B**, Fouet P, Bonnet JC, Atlan G. Submaximal oxygen consumption in liver cirrhosis. Evidence of severe functional aerobic impairment. *J Hepatol* 1990; **10**: 163-167
- 52 **Milani A**, Zaccaria R, Bombardieri G, Gasbarrini A, Pola P. Cirrhotic cardiomyopathy. *Dig Liver Dis* 2007; **39**: 507-515
- 53 **Schwartz JM**, Beymer C, Althaus SJ, Larson AM, Zaman A, Glickerman DJ, Kowdley KV. Cardiopulmonary consequences of transjugular intrahepatic portosystemic shunts: role of increased pulmonary artery pressure. *J Clin Gastroenterol* 2004; **38**: 590-594
- 54 **Bernardi M**, Rubboli A, Trevisani F, Cancellieri C, Ligabue A, Baraldini M, Gasbarrini G. Reduced cardiovascular responsiveness to exercise-induced sympathoadrenergic stimulation in patients with cirrhosis. *J Hepatol* 1991; **12**: 207-216
- 55 **Grose RD**, Nolan J, Dillon JF, Errington M, Hannan WJ, Bouchier IA, Hayes PC. Exercise-induced left ventricular dysfunction in alcoholic and non-alcoholic cirrhosis. *J Hepatol* 1995; **22**: 326-332
- 56 **Henriksen JH**, Fuglsang S, Bendtsen F, Christensen E, Moller S. Dyssynchronous electrical and mechanical systole in patients with cirrhosis. *J Hepatol* 2002; **36**: 513-520
- 57 **Bal JS**, Thuluvath PJ. Prolongation of QTc interval: relationship with etiology and severity of liver disease, mortality and liver transplantation. *Liver Int* 2003; **23**: 243-248
- 58 **Hansen S**, Moller S, Bendtsen F, Jensen G, Henriksen JH. Diurnal variation and dispersion in QT interval in cirrhosis: relation to haemodynamic changes. *J Hepatol* 2007; **47**: 373-380
- 59 **Roden DM**, Viswanathan PC. Genetics of acquired long QT syndrome. *J Clin Invest* 2005; **115**: 2025-2032
- 60 **Brubaker PH**, Kitzman DW. Prevalence and management of chronotropic incompetence in heart failure. *Curr Cardiol Rep* 2007; **9**: 229-235
- 61 **Laffi G**, Lagi A, Cipriani M, Barletta G, Bernardi L, Fattorini L, Melani L, Riccardi D, Bandinelli G, Mannelli M, La Villa G, Gentilini P. Impaired cardiovascular autonomic response to passive tilting in cirrhosis with ascites. *Hepatology* 1996; **24**: 1063-1067
- 62 **Ma Z**, Meddings JB, Lee SS. Membrane physical properties determine cardiac beta-adrenergic receptor function in cirrhotic rats. *Am J Physiol* 1994; **267**: G87-G93

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## EDITORIAL

# Selection criteria for liver resection in patients with hepatocellular carcinoma and chronic liver disease

Spiros G Delis, Christos Dervenis

Spiros G Delis, Christos Dervenis, 1st Surgical Clinic, Liver Surgical Unit, Kostantopouleio-Agia Olga Hospital, Athens 14233, Greece

**Author contributions:** Delis SG and Dervenis C contributed equally to this work; they both designed and performed the research; Delis SG wrote the paper and Dervenis C reviewed and revised the paper before submission.

**Correspondence to:** Spiros G Delis, MD, PhD, Liver Unit, 1st Surgical Clinic, Kostantopouleio-Agia Olga Hospital, 3-5 Agias Olgas street, Athens 14233, Greece. [sdelis55@hotmail.com](mailto:sdelis55@hotmail.com)

**Telephone:** +3-210-5012849 **Fax:** +3-210-5012849

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## INTRODUCTION

Surgery, including liver transplantation (OLT) remains the most efficient treatment of patients with hepatocellular carcinoma (HCC)<sup>[1,2]</sup>. However, less than 30% of patients are eligible for liver resection (LR) due to HCC multifocality on a background of chronic liver disease<sup>[1,2]</sup>. Over the past 10 years, there has been considerable progress in both diagnosis and surgical outcome of HCC patients<sup>[1,2]</sup>. Selective preoperative morphological assessment, preoperative use of portal vein embolization<sup>[3]</sup> and the improvement of surgical techniques<sup>[4,5]</sup> are factors that improve the safety of LR. In addition, better selection of the candidates for LR has been accomplished by both imaging advancements and preoperative accurate evaluation of liver functional reserve. We review herein the available data regarding selection criteria for LR in the setting of HCC and underlying liver disease.

## CLINICAL CLASSIFICATION

Cancer classification aims to establish prognosis and select the adequate treatment for the best candidates. The Barcelona-Clinic Liver Cancer (BCLC) classification has emerged as the standard classification for clinical management of HCC<sup>[2,6]</sup>. This system links tumor stage with treatment strategy and has been externally validated. Three stages of HCC have been reported according to BCLC group: Early stages, Intermediate-advanced HCC and End-stage HCC. At the early stages tumor status is defined by size of the main nodule and multicentricity (single < 2 cm, single 2-5 cm, 3 nodules < 3 cm). Variables related to liver function are relevant in patients not suitable for transplantation as portal hypertension and normal bilirubin in patients undergoing resection. The limitations of one-dimensional systems, such as the Okuda staging and the Child-Pugh classification have been overcome. Several proposals reported recently and sub-classify patients at advanced stages such as the CUP

## Abstract

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide with an annual occurrence of one million new cases. An etiologic association between HBV infection and the development of HCC has been established with a relative risk 200-fold greater than in non-infected individuals. Hepatitis C virus is also proving an important predisposing factor for this malignancy with an incidence rate of 7% at 5 years and 14% at 10 years. The prognosis depends on tumor stage and degree of liver function, which affect the tolerance to invasive treatments. Although surgical resection is generally accepted as the treatment of choice for HCC, new treatment strategies, such as local ablative therapies, transarterial embolization and liver transplantation, have been developed nowadays. With increasing detection of small HCCs from screening programs for cirrhotic patients, it is foreseen that locoregional therapy will play an important role in the near future.

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**Key words:** Hepatocellular carcinoma; Hepatoma; Resection; Ablation; Transplantation; ICG clearance; Remnant liver volume; Milan criteria; MELD score

**Peer reviewer:** Frank A Anania, Professor, Emory University School of Medicine, Division of Division Digestive Diseases, 615 Michael Street, Room 255 Whitehead Biomedical Research Building, Atlanta, GA 30322, United States



and the CLIP score<sup>[7,8]</sup>. The new TNM in accordance with the AJCC is based on series of patients undergoing resection. Pathological information is needed, thus representing a limitation for preoperative clinical use. Finally, the Japan Integrated Staging (JIS) includes two previous classifications: the TNM and the Japanese version of the Child-Pugh classification and offers advantages compared to CLIP score<sup>[9]</sup>.

## MILAN CRITERIA FOR LIVER TRANSPLANTATION

In the past 10 years, results of OLT have improved steadily because of careful patient selection pioneered by the introduction of the Conventional Milan Criteria (CMC)<sup>[10]</sup>. The aim of these criteria was to achieve a good outcome in patients who fulfilled the criteria and avoid a poor prognosis in patients who exceed them. These are patients with single HCC < 5 cm or up to three nodules < 3 cm who in major units achieve 70% survival at 5 years with a recurrence below 15%. The major drawback of OLT is the scarcity of donors. The increase of waiting time has led to 20% of the candidates to drop out due to progression of disease jeopardizing the outcome according to intention-to-treat analysis.

### *Pre-transplantation neoadjuvant treatments*

Several attempts have been made to prevent tumor progression during waiting time by applying adjuvant therapies, mainly percutaneous ablation and transcatheter arterial chemoembolization (TACE). These therapies have been tested only in the setting of observational studies, case series and cohort studies that provide limited information because of heterogeneity of patient and tumor characteristics, variable waiting times, use of different treatment modalities, variable evaluation of response and lack of consensus about criteria of drop-out<sup>[1,2,9]</sup>. Among the case series and cohort studies, some investigators suggest a favorable impact of treatment in decreasing the dropout rate. Mazzaferro reported no dropouts in 50 patients within Milan criteria treated with RFA<sup>[11]</sup>. Some studies reported no dropouts in patients within Milan treated by TACE and short waiting time (178 d) while others documented a probability of dropout of 15% at 6 mo and 25% at 12 mo<sup>[9]</sup>. Cumulative results show that RFA achieves the highest rates of complete necrosis (12%-55%) compared with TACE (22%-29%). Complete necrosis is best achieved with percutaneous ablation in tumors < 3 cm in diameter. It is estimated that drop-out rates will increase with the expansion of selection criteria as demonstrated by Roayaie<sup>[12]</sup>. Recurrence rates are majorly related to tumor stage than to neo-adjuvant therapies. It is recognized that recurrence rates are low when applying the Milan criteria, compared to a wide selection of candidates.

### *Milan versus expanded criteria for OLT*

The growing experience and success of OLT for HCC

have fuelled controversies related to expansion of Milan criteria. Among the proposed expanded criteria the UCSF criteria (single tumor nodule up to 6.5 cm; or three or fewer tumors, the largest of which is  $\leq$  4.5 cm with the sum of the tumor diameters  $\leq$  8 cm) reflect a modest expansion of tumor size limits<sup>[13]</sup>. However, there are limitations in applicability of the UCSF criteria in the pre-transplant setting, considering that most of the patients adhering to the UCSF were also within the Milan criteria. More to the point, the overlapping population of patients adhering to the UCSF, but not the Milan criteria is often negligible and estimated to be less than 10% of the total transplanted population<sup>[1,2,9]</sup>. In addition, the limitations of pre-transplant imaging studies, exemplified by tumor under-staging in 20% of patients, have been a major concern for liberalizing the existing criteria for OLT.

Down-staging refers to a change as a result of treatment so that disease will reach the Milan criteria, as assessed by imaging techniques. In the seminal study from Majno *et al*<sup>[14]</sup>, TACE induced down-staging of HCC > 3 cm and resulted in good survival rates after OLT, but the benefit was not confirmed in other investigations that studied TACE or RFA. The UCSF group reported a cohort of 30 patients treated heterogeneously, so that disease would reach the Milan criteria. Half the patients with down-staged tumors were effectively transplanted, although some bias exists and control studies required. Therefore, nowadays down-staging should be assessed in the setting of clinical research.

## LIVER RESECTION

Although surgery remains the only treatment for HCC in patients with or without cirrhosis, most individuals with HCC are ineligible for surgical intervention. In eligible patients, the methods of surgical therapy are partial hepatectomy and liver transplantation. In addition to resection and liver transplantation, percutaneous ablation is considered as a treatment option that offers a high rate of complete response and thus a potential for cure. In selected patients, a 5-year survival rate of 60% to 75% can be achieved after surgery<sup>[1,2,9]</sup>. However, in those with advanced HCC, the consequent improvement in long-term survival is poor because of the high rate of recurrence or the development of intra-hepatic metastases that disseminate *via* the portal vein or spread to other parts of the liver. Nevertheless, the management of HCC has undergone major changes over the last few decades. Earlier detection enabled by screening methods that use ultrasonographic evaluation and AFP analysis in high-risk populations, more accurate patient assessment, advances in imaging, improved surgical techniques, and the availability of local treatment options have improved outcomes.

### *HCC in patients with a non-cirrhotic liver*

Only 5% of the cases of HCC in Western countries (as opposed to 40% in Asia) develop in a non-cirrhotic liver<sup>[1]</sup>. When HCC occurs in a non-cirrhotic liver,

solitary tumor nodes that are limited to one liver lobe and lack satellite foci are frequently present. Without predisposing cirrhosis, HCC is often not diagnosed, until the tumor causes symptoms because of its size. Sometimes HCC is an incidental finding revealed by ultrasonographic studies<sup>[1]</sup>.

The treatment approach for patients with HCC without cirrhosis should be based on factors such as extra-hepatic tumor manifestation, tumor size and the number and distribution of nodules. In such patients, curative resection should be considered whenever possible.

Major hepatectomy defined as resection of more than three liver segments is feasible if the remnant liver volume is adequate. The evolution of transection devices and postoperative care had a major impact in both morbidity and mortality after LR. Most centers documented a less than 5% mortality rate recently<sup>[1,2,4,5]</sup> compared to a higher incidence reported 10 years ago. Blood transfusion requirements have also been restricted from 80% to 20% in major reference centres. This was accomplished by bloodless techniques with intermittent inflow occlusion and better selection of candidates with single lesion and absence of portal hypertension<sup>[1,2,4,5,15,16]</sup>.

Pre-treatment imaging studies such as high-resolution triple-phase computed tomography (CT) and nuclear magnetic resonance imaging (MRI), either with or without angiography, can be used to match patients and their most appropriate treatment. Positron emission tomography (PET) is also useful in the identification of extra-hepatic metastases that considerably influence clinical decision-making. Knowledge about the relation of the tumor to regional anatomic structures such as large vessels is crucial because it provides valuable information about resectability. Furthermore, volumetric studies can be used to define the residual parenchyma exactly. If there is any suspicion of lymph node metastasis or peritoneal dissemination, diagnostic laparoscopy with intra-operative ultrasonography is useful, and if multiple metastases are confirmed, explorative laparotomy can be prevented.

The determination of hepatic reserve is also significant when resection is considered. The healthy liver has a great capability for regeneration and adjusts to the metabolic requirements of the host after LR due to hypertrophy of the residual liver. Therefore, even in patients with a large tumor, extensive resection is possible. In an otherwise healthy liver, up to 75% of the parenchyma can be resected.

Patients with a localized unilobar tumor in a non-cirrhotic liver or Child class A cirrhosis with adequate remnant liver parenchyma may be considered for partial hepatectomy (lobectomy). Partial hepatectomy usually ensures a safety margin of at least 1 cm and is associated with an operative mortality rate of less than 5%. From an oncologic perspective, anatomic resection that may include satellite lesions is more effective than limited resection without a surrounding margin. For patients with inadequate or borderline remnant parenchyma, hypertrophy of the prospective liver remnant can be induced by preoperative portal vein embolization (PVE).

In certain circumstances, an unfavorable location of the tumor and involvement of the confluence of the three hepatic veins and either the caval vein or the retro-hepatic caval vein can render resection by conventional techniques impossible. In these rare cases, special techniques such as *in situ* or ante situm resection can be used.

The overall long-term results after resection are favourable. However, only 20% to 30% of patients with HCC are eligible for resection because of advanced or multifocal disease or inadequate functional hepatic reserve. In patients with solitary lesions of less than 5 cm, no vascular invasion, and a negative surgical margin of at least 1 cm, the 5-year survival rate after resection is reported to be greater than 70%<sup>[17]</sup>. Despite earlier detection, safer surgical procedures, and more aggressive treatment of HCC, recurrence (because of multicentric carcinogenesis or intrahepatic metastases from the primary tumor) is likely. In selected patients, repeated resection provides good long-term benefits and is an option for those with solitary peripheral tumors that can be treated with segmental or atypical resection.

### **HCC in patients with cirrhosis**

HCC in patients with cirrhosis is a challenge due to both pre-existing liver damage and possible tumor multifocality. Portal hypertension and reduced functional capacity of the cirrhotic liver significantly increase the peri-operative risk. These facts influence two significant decisions regarding surgery: patient selection and the choice of the surgical therapeutic method.

The resection margin of HCC in cirrhotic patients does not represent a significant predictive factor for recurrence, unless residual tumor directly invades the raw surface of the liver<sup>[1]</sup>. In most HCC patients, tumor recurrence results from disseminated tumor, and in the remaining patients, recurrence is caused by metachronous tumors that arise in the oncogenic cirrhotic liver, as is typical in the cirrhosis that develops after hepatitis C infection<sup>[1]</sup>. Because of the difficulty to prevent recurrence by resection with an adequate safety margin, resection (preferably segmentectomy or subsegmentectomy rather than wedge resection) should be as limited as possible. Because of the threat of insufficient liver function coupled with a greater risk of mortality, the decision to perform major resection should be considered with caution.

The reduced functional reserve capacity in patients with cirrhosis of the liver limits the choice of surgical therapy. Various tests have been developed to quantify liver function.

Refined selection criteria and technical advances, including a broader knowledge of segmental anatomy, vascular occlusion techniques, and the use of intra-operative ultrasonography, have facilitated resection and improved outcome. Operative mortality rates have decreased to less than 5%<sup>[2,9]</sup>. A considerable decrease in intra-operative blood loss has been achieved by means of numerous technical improvements such as the use of ultrasonographic dissectors and bipolar and argon beamer coagulation. In individual cases, hilar occlusion

(the Pringle manoeuvre) has become either unnecessary or the occlusion time can be shortened, both of which result in reduced ischemia-reperfusion damage. Despite a decrease in the operative mortality rate and improved results after resection, overall survival after the resection of HCC has changed little due to absence of effective adjuvant treatment to eliminate postoperative recurrence.

**Liver function assessment:** The clinical assessment of hepatic function by the use of “Child” system, developed to understand the significance of cirrhotic liver injury and portal hypertension as they related to patient survival after portal-systemic shunt surgery was used mainly in the past in its original version. In general, Child class A or Child class B patients may tolerate a resection of up to 50% and 25% of liver parenchyma, respectively. However, evaluating hepatic reserve by means of the CTP classification may lead to an inconsistent predictive value, because as Child class A patients may already have significant functional impairment and may demonstrate an increase in the bilirubin level as well as portal hypertension and fluid retention. These features indicate advanced liver disease and preclude resection. Limited discriminatory ability, subjective interpretation of parameters, and variability in the measurement of laboratory parameters are further limitations of CPT. Makuuchi *et al*<sup>[18]</sup> was first to described three parameters in patients with cirrhosis associated with morbidity after hepatectomy. Ascites, abnormal serum bilirubin and ICG clearance were defined as independent factors affected postoperative morbidity. They noted that a cut-off ICG clearance level below 20% is adequate for safe hepatectomy. Other groups use a cut-off level of 14% to discriminate high-risk candidates<sup>[19]</sup>. If that level is greater than 40% postoperative liver failure is likely, even with minimal resection. However, ICG clearance criteria are not absolute and every effort for further extension depends on the liver remnant size and severity of cirrhosis. In addition ICG retention measurement is cumbersome requiring accurate sampling. Furthermore, the dynamic tests (ICG, Galactose elimination capacity *etc*) cannot take into account all the complexities of liver function and, therefore, they have limitations. ICG clearance is not a true index of parenchymal function because there is also a substantial influence of hepatic blood flow. Clearance is considered to be impaired when 15% or more of the dye remains within the plasma 15 min following the injection of 0.5 mg/kg ICG. Thus, patients with CP scores of 5 or 6 (Child A) and ICG15 of greater than 14% are the “high risk” CP-A patients with limited functional reserve.

Nuclear imaging has been used recently to evaluate liver function. A functional imaging with great promise involves receptor targeting with radio-labelled synthetic asialoglycoproteins (99 m-Tc-GSA). GSA provides volumetric receptor data and kinetic distribution curves. However, GSA is still preliminary and although is correlated with CP and ICG clearance its potential role in evaluating resection must demonstrate that they are

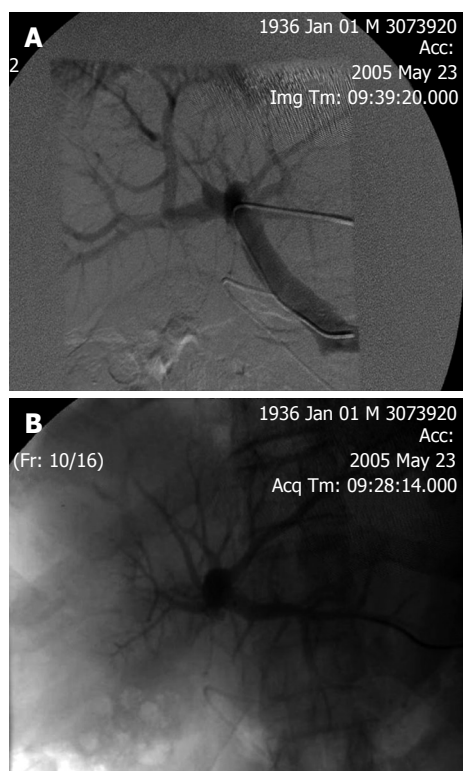
improvements over CP stratification.

In Europe and North America, the selection of optimal candidates for LR is usually based on the degree of portal hypertension and an elevated bilirubin level. Portocaval pressure gradient > 10 mmHg or the presence of oesophageal varices (grade 2, 3) are good indicators of portal hypertension (PH). A low platelet count < 100 000/mL and splenomegaly is also used as a surrogate marker of PH. A bilirubin concentration that is within normal limits and a hepatic vein pressure gradient of less than 10 mmHg (measured by hepatic vein catheterization) are the best predictors of excellent outcome after resection and are associated with almost no risk of postoperative liver failure<sup>[2]</sup>. In the setting of Child A cirrhosis with none of these factors presented, a 70% 5-year survival was documented despite the fact that portal hypertension and abnormal bilirubin decrease long-term survival by half. Measurement of the liver remnant volume is helpful in selecting patients for major hepatic resection, however, preoperative evaluation of the severity of cirrhosis may be mandatory by biopsy of non- tumorous liver for histological grading.

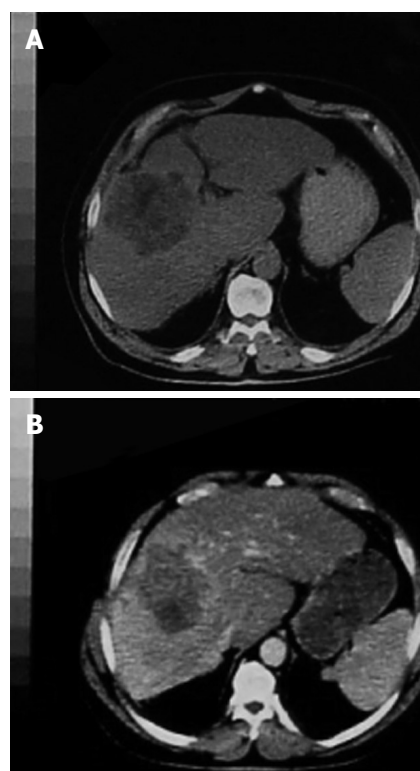
The Model for End-Stage Liver Disease (MELD) score has gained widespread acceptance to prioritize candidates for liver transplantation. Few studies<sup>[20-23]</sup> including the one performed in our department<sup>[24]</sup>, demonstrated a strong correlation of postoperative morbidity after hepatectomy and MELD value. More precisely a cut-off value of 8 was associated with higher morbidity and a cut-off value of 11 with high mortality rate. In this particular situation, our group suggests liver transplantation rather than resection.

Apart from the factors mentioned above the presence of co-morbid illnesses such as cardiovascular disease has been shown to increase the risk of hepatectomy. While the presence of severe co-morbid illnesses such as congestive heart failure and chronic renal failure should be considered a contraindication for hepatectomy, HCC patients with less severe co-morbid illnesses, such as diabetes, may still benefit from hepatic resection provided with meticulous peri-operative care. However, the importance of optimum peri-operative control of the blood glucose level and vigilant postoperative care in such cases needs to be emphasized. Finally, the Memorial Sloan Kettering data reported by Jarnagin<sup>[25-28]</sup> suggests that experience may play a critical, positive role in patient selection. The avoidance of greater than four segment resections in “bad-risk” Child-Pugh Class A patients is a clear-cut goal, unless the option of portal vein occlusion is to be pursued.

**Portal vein embolization:** Portal vein embolization (PVE) has been applied in the setting of inadequate liver remnant volume to induce hypertrophy. Although the concept of contra-lateral liver hypertrophy after PVE has been challenged by some due to the impaired cirrhotic liver regenerative capacity, a better selection of those patients not amenable to major resection is feasible. Proposed guidelines for PVE application include less than 40% remnant liver volume in the



**Figure 1** A: Before portal vein embolization (PVE); B: After portal vein embolization (PVE).



**Figure 2** CT indicated, A: Before PVE; B: Six weeks after PVE on the same patient. Hypertrophy of the left hepatic lobe is obvious.

non-cirrhotic group of patients underwent major hepatectomy or less than 60% in the group of cirrhotic individuals with ICG15 < 20%.

The volume of functional liver mass left after resection is an important factor in the development of postoperative complications and subsequent mortality<sup>[29,30]</sup>. Liver volume measurement for adult living donor liver transplantation is standardized to achieve a graft weight to recipient weight of at least 1% because of the clear link between adequate functional hepatic mass of the donor graft to recipient weight and postoperative complications.

In the non-transplant LR setting, the minimum acceptable liver volume remaining post-resection has not been well assessed but is generally thought to be about 25% of the normal liver volume. In cases where there is liver dysfunction such as cirrhosis or cholestasis, 40% of the normal volume is acceptable<sup>[31]</sup>. The advent of PVE allows optimization of the remnant liver volume in cases where it is projected to be less than ideal. However, no randomized trials examining the effectiveness of portal vein embolization on liver regeneration or its impact on LR exist. The use of portal vein embolization of the hepatic lobe that hosts the tumor to induce compensatory hypertrophy in the non-affected liver before major resection is controversial. Uncontrolled tumor progression because of the proliferation of malignant cells stimulated by this method and the risk of variceal bleeding resulting from acute portal hypertension are some of the concerns.

PVE blocks portal flow to the side of the liver ipsilateral to the lesion to be resected (Figure 1A and B)

and causes an increase in size of the future liver remnant (FLR) (Figure 2A and B). The increased size is due to both clonal expansion and cellular hypertrophy<sup>[32]</sup>. The assumption that increase in liver volume correlates with increased function post-PVE has been demonstrated in studies showing increase in asialoglycoprotein receptor binding sites in the FLR before resection<sup>[33-36]</sup>. Percutaneous transhepatic PVE can be performed by either of two standard approaches: the transhepatic ipsilateral<sup>[37]</sup> and transhepatic contralateral<sup>[38]</sup>.

Many commercially available embolic agents have been used for preoperative PVE without significant differences in degrees or rates of hypertrophy of the nonembolized segments<sup>[39]</sup>. The ideal agent is well tolerated by the patient and causes permanent embolization of the portal vein and its branches with minimal risk of recanalization<sup>[40]</sup>. In addition, it should be widely available and easily administered without causing inflammatory reaction and be associated with a low risk of post-embolization syndrome and hepatic necrosis. Although each of the embolic agents has advantages and disadvantages, no single agent has been proven to be consistently superior<sup>[41,42]</sup>.

All patients have a volumetric assessment of their liver volumes both before PVE and before surgery using CT imaging. Post-embolization CT is essential for assessment of liver volume change and planning of LR<sup>[39]</sup>. The average interval for the CT volumetric study from portal vein embolization to surgery is 4 to 6 wk. CT is used to make direct measurements of total liver volume, volume to be resected, and volume of the future liver remnant. The future liver remnant volume



is considered to be the volume of the liver segments expected to remain after hepatectomy. More precisely Computed Tomography scans of the liver are necessary. Serial transverse scans at 1-cm intervals from the dome of the liver to the most inferior part of the organ must be obtained, with enhancement by intravenous bolus injection of contrast and with the patient suspending respiration in expiration. Each slice of the liver is traced with a cursor, and computer calculates the corresponding area. The middle hepatic vein and gallbladder are used as landmarks to define the borders between the right and left livers. Segment IV volume is measured using the middle hepatic vein and the umbilical portion of the left portal vein as landmarks. The total volumes measured (whole liver volume, tumor volume, and remnant liver volume) are calculated by multiplying the area of each part by the interval thickness and by adding all the interval volumes of each part. The estimated rate of remnant functional liver parenchyma (ERRFLP) is calculated by the formula:  $\text{FLR volume} = (\text{remnant liver volume} \times 100) / (\text{total liver volume} - \text{tumor volume})^{[43,44]}$ .

The increase in FLR volume after portal vein embolization can be calculated with the following formula:  $(\text{volume of the FLR before surgery} - \text{volume of the FLR before PVE}) \times 100 / (\text{volume of the FLR before PVE})$ . The increase in the %FLR after portal vein embolization can also be calculated from:  $(\% \text{FLR after PVE} - \% \text{FLR before surgery})$ . The total liver volume can be estimated on the basis of the body surface area using the formula;  $\text{total liver volume} = 706.2 \times \text{body surface area} + 2.4$ . The calculated range of percentage increase of future liver remnant volume following portal vein embolization is 8% to 27% in different studies including our experience.

**Preoperative imaging:** Selection of candidates with HCC for LR requires adequate preoperative staging<sup>[1,2]</sup>. Sensitivity and diagnostic value of preoperative imaging (CT, MR) approximates 80% but is limited for satellite nodules or less than 1 cm lesions. MR angiography although is more sensitive for identification of 1-2 cm lesions has limitations for subcentimeter tumors. Intraoperative ultrasound is more accurate for very early less than 1 cm HCC. Positron emission tomography (PET) is also useful in the identification of extra-hepatic disease that considerably influence clinical decision-making. If there is any suspicion of lymph node metastasis or tumor dissemination, diagnostic laparoscopy with intraoperative ultra-sonography is useful to avoid an unnecessary laparotomy. There is widespread acceptance that liver biopsy is not necessary as a routine for lesions more than 2 cm with imaging compatible for HCC in a patient with history of underlying cirrhosis or high AFP levels.

## PROGNOSTIC FACTORS

The most significant predictive factors for early recurrence are the size and number of tumors, the presence of satellite nodules, the histologic grade, the severity of cirrhosis, and the serum AFP level<sup>[45-49]</sup>.

Tumor size and the number of nodules are important factors that predict vascular invasion. However, tumor size is not an absolute contraindication for LR<sup>[50]</sup>. Long-term survival varies from 66% in cases of less than 5 cm lesions to 37% in the group of large (> 5 cm) HCC<sup>[46-50]</sup>. Vascular invasion is an independent factor affect prognosis and is strongly associated with both size and histological grade. More precisely lesions less than 2 cm in diameter have a 20% rate of micro-vascular invasion although the rate increases progressively for lesions more than 5 cm in diameter (60%-90%). Even though vascular invasion is not easily identified preoperatively by imaging techniques, tumor size and grading are surrogate markers. According to the results of a study reported recently, a tumor size larger than 5 cm was an indicator of high histological grade in more than 40% of patients with HCC. Needle core biopsy (NCB) is notorious, unreliable to confirm preoperatively tumor grading due to heterogenous nature of HCC as reported by Pawlik and associates<sup>[51]</sup>.

The number of lesions is strongly related with incidence of recurrence<sup>[49]</sup>. Long-term survival is 57% for solitary lesions but only 26% for multifocal HCC. Other factors affect prognosis includes AFP levels, age and concomitant diseases<sup>[46,49]</sup>. In addition, genetic signature of the tumor is attributed to both disease free and overall survival<sup>[46,49,50]</sup>. The role of hepatic resection for bilobar HCCs is controversial. Bilobar HCCs may represent advanced disease with intrahepatic metastasis or may represent multifocal HCCs derived from multicentric hepatocarcinogenesis. Major hepatectomy in one lobe combined with wedge resection for a smaller lesion in the other lobe is possible in some cases. Alternatively, hepatic resection in one lobe can be combined with local ablation of a smaller lesion in the other lobe using ethanol injection or newer ablative modalities such as radiofrequency ablation. In a recent study by Poon and associates, they demonstrated that hepatic resection for patients with bilobar HCCs resulted in a better survival outcome than non-resectional therapies<sup>[52]</sup>. Hence, in accordance with others we recommend that hepatic resection should be considered in selected patients with bilobar HCCs, especially those with a small solitary lesion in the contra lateral lobe that are amenable to wedge resection or local ablative therapy.

Although hepatic resection with removal of tumor thrombus in the inferior vena cava or main portal vein has been advocated by some authors<sup>[53]</sup>, most liver surgeons consider the presence of tumor thrombus in the inferior vena cava or main portal vein a contraindication for hepatic resection because the prognosis is usually poor even with such an aggressive approach. However, hepatic resection for patients with tumor invasion of the hepatic veins or major intrahepatic branches of the portal vein is justified because favourable survival results may be expected compared with non-surgical treatment<sup>[54,55]</sup>.

A major drawback of LR in the setting of HCC is the high recurrence rate (70%) due to intrahepatic dissemination or *de novo* appearance of new lesions.

Molecular techniques differentiate widespread liver disease (60%-70%) from de novo HCC development (30%-40%). Intrahepatic metastases occurred early (less than 2 years) after LR and related to primary tumor biologic aggressiveness (low grade, vascular invasion, satellite nodules). De novo HCC occurrence is related mostly to the underlying liver disease and appears later.

## TREATMENT STRATEGY

Although surgery remains the gold standard for HCC in patients with or without cirrhosis, most individuals are ineligible for surgical intervention. In fact, only 20% to 30% of cases are amenable to resection using the previous mentioned selection criteria. Despite the difficulty of exposing patients to the risks and consequences of transplantation-associated immune suppression, liver transplantation is the ultimate treatment option in patients with HCC who fulfil Milan selection criteria. Transplantation restores liver function and decrease tumor recurrence due to removal of the oncogenic potential dysplastic lesions. However when compared with LR the results of liver transplantation in patients with HCC and without cirrhosis are less favorable. Shortage of available donors is an additional limitation associated with a high dropout rate especially in large tumors that do not fulfil Milan criteria. Recently, several groups compared resection and transplantation demonstrated similar survival rates when the different tumor invasiveness was taken into account<sup>[47]</sup>. The inclusion of patients with more advanced cancer in the waiting list for transplantation result in a higher dropout rate that leads in turn to poor survival rates in an intent-to-treat analysis. In such cases, LR if feasible is the only curative option. The relative benefits of transplantation and resection are, therefore, likely to depend upon local organ allocation policy and waiting times. Another approach to reduce both waiting-list dropout rate and the demand for donor organs is primary resection followed by salvage transplantation in case of recurrence. Two studies have compared this strategy of primary transplantation with conflicting conclusions. Opposite results applying salvage transplantation have been published by two groups reporting peri-operative mortality ranging from 5% to 30%. The BCLC group proposed a policy of listing patients for liver transplant without evident HCC based on pathological risk of recurrence after resection (vascular invasion or satellites). However, in current clinical practice the applicability of this policy is low, about 10% of cases especially if there is underlying hepatitis C.

The recent development of laparoscopic LR has added new possibilities for the limited removal of peripheral lesions<sup>[56]</sup>. The avoidance of long sub-costal incisions seems to be associated with reduced morbidity and earlier recovery. Re-operations are easy after laparoscopic resection, which allows it to be used as a neoadjuvant treatment in those awaiting liver transplantation without compromising subsequent surgery. An additional advantage of resecting small

HCCs is that it allows histopathological study of the whole tumour and identifies patients at high risk of intra-hepatic recurrence (those with poorly differentiated tumor, microvascular invasion, and satellite nodules). This raises the concept of pre-emptive transplantation when these criteria apply to the resected specimen.

## CONCLUSION

In conclusion, the selection of the treatment options in patients with HCC must be based on the patient's condition, the number and size of the hepatic tumors, the functional reserve capacity and the available resources. LR is strongly recommended in non-cirrhotic and selected cirrhotic individuals. Appropriate selection of candidates achieved by accurate estimation of hepatic liver reserve is of paramount importance. Technical advances in imaging and surgery facilitates resection and improves outcome.

## REFERENCES

- 1 Schwartz M, Roayaie S, Konstadoulakis M. Strategies for the management of hepatocellular carcinoma. *Nat Clin Pract Oncol* 2007; **4**: 424-432
- 2 Llovet JM, Schwartz M, Mazzaferro V. Resection and liver transplantation for hepatocellular carcinoma. *Semin Liver Dis* 2005; **25**: 181-200
- 3 Abulkhir A, Limongelli P, Healey AJ, Damrah O, Tait P, Jackson J, Habib N, Jiao LR. Preoperative portal vein embolization for major liver resection: a meta-analysis. *Ann Surg* 2008; **247**: 49-57
- 4 Delis SG, Bakoyiannis A, Tassopoulos N, Athanasiou K, Madariaga J, Dervenis C. Radiofrequency-assisted liver resection. *Surg Oncol* 2008; **17**: 81-86
- 5 Delis SG, Madariaga J, Bakoyiannis A, Dervenis Ch. Current role of bloodless liver resection. *World J Gastroenterol* 2007; **13**: 826-829
- 6 Llovet JM, Bru C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. *Semin Liver Dis* 1999; **19**: 329-338
- 7 Prospective validation of the CLIP score: a new prognostic system for patients with cirrhosis and hepatocellular carcinoma. The Cancer of the Liver Italian Program (CLIP) Investigators. *Hepatology* 2000; **31**: 840-845
- 8 Leung TW, Tang AM, Zee B, Lau WY, Lai PB, Leung KL, Lau JT, Yu SC, Johnson PJ. Construction of the Chinese University Prognostic Index for hepatocellular carcinoma and comparison with the TNM staging system, the Okuda staging system, and the Cancer of the Liver Italian Program staging system: a study based on 926 patients. *Cancer* 2002; **94**: 1760-1769
- 9 Llovet JM, Bruix J. Novel advancements in the management of hepatocellular carcinoma in 2008. *J Hepatol* 2008; **S20**-S37
- 10 Mazzaferro V, Regalia E, Doci R, Andreola S, Pulvirenti A, Bozzetti F, Montalto F, Ammatuna M, Morabito A, Gennari L. Liver transplantation for the treatment of small hepatocellular carcinomas in patients with cirrhosis. *N Engl J Med* 1996; **334**: 693-699
- 11 Mazzaferro V, Chun YS, Poon RT, Schwartz ME, Yao FY, Marsh JW, Bhoori S, Lee SG. Liver transplantation for hepatocellular carcinoma. *Ann Surg Oncol* 2008; **15**: 1001-1007
- 12 Roayaie S, Llovet JM. Liver transplantation for hepatocellular carcinoma: is expansion of criteria justified? *Clin Liver Dis* 2005; **9**: 315-328
- 13 Yao FY, Ferrell L, Bass NM, Watson JJ, Bacchetti P, Venook

- A, Ascher NL, Roberts JP. Liver transplantation for hepatocellular carcinoma: expansion of the tumor size limits does not adversely impact survival. *Hepatology* 2001; **33**: 1394-1403
- 14 **Majno PE**, Adam R, Bismuth H, Castaing D, Ariche A, Krissat J, Perrin H, Azoulay D. Influence of preoperative transarterial lipiodol chemoembolization on resection and transplantation for hepatocellular carcinoma in patients with cirrhosis. *Ann Surg* 1997; **226**: 688-701; discussion 701-703
  - 15 **Llovet JM**. Updated treatment approach to hepatocellular carcinoma. *J Gastroenterol* 2005; **40**: 225-235
  - 16 **Makuuchi M**, Kokudo N, Arii S, Futagawa S, Kaneko S, Kawasaki S, Matsuyama Y, Okazaki M, Okita K, Omata M, Saida Y, Takayama T, Yamaoka Y. Development of evidence-based clinical guidelines for the diagnosis and treatment of hepatocellular carcinoma in Japan. *Hepatol Res* 2008; **38**: 37-51
  - 17 **Shi M**, Guo RP, Lin XJ, Zhang YQ, Chen MS, Zhang CQ, Lau WY, Li JQ. Partial hepatectomy with wide versus narrow resection margin for solitary hepatocellular carcinoma: a prospective randomized trial. *Ann Surg* 2007; **245**: 36-43
  - 18 **Miyagawa S**, Makuuchi M, Kawasaki S, Kakazu T. Criteria for safe hepatic resection. *Am J Surg* 1995; **169**: 589-594
  - 19 **Poon RT**, Fan ST. Hepatectomy for hepatocellular carcinoma: patient selection and postoperative outcome. *Liver Transpl* 2004; **10**: S39-S45
  - 20 **Cucchetti A**, Ercolani G, Vivarelli M, Cescon M, Ravaioli M, La Barba G, Zanella M, Grazi GL, Pinna AD. Impact of model for end-stage liver disease (MELD) score on prognosis after hepatectomy for hepatocellular carcinoma on cirrhosis. *Liver Transpl* 2006; **12**: 966-971
  - 21 **Teh SH**, Christein J, Donohue J, Que F, Kendrick M, Farnell M, Cha S, Kamath P, Kim R, Nagorney DM. Hepatic resection of hepatocellular carcinoma in patients with cirrhosis: Model of End-Stage Liver Disease (MELD) score predicts perioperative mortality. *J Gastrointest Surg* 2005; **9**: 1207-1215; discussion 1215
  - 22 **Northup PG**, Wanamaker RC, Lee VD, Adams RB, Berg CL. Model for End-Stage Liver Disease (MELD) predicts nontransplant surgical mortality in patients with cirrhosis. *Ann Surg* 2005; **242**: 244-251
  - 23 **Befeler AS**, Palmer DE, Hoffman M, Longo W, Solomon H, Di Bisceglie AM. The safety of intra-abdominal surgery in patients with cirrhosis: model for end-stage liver disease score is superior to Child-Turcotte-Pugh classification in predicting outcome. *Arch Surg* 2005; **140**: 650-654; discussion 655
  - 24 **Delis S**, Biliatis I, Athanassiou K. Model for end-stage liver disease. MELD score as a prognostic factor for postoperative morbidity and mortality in cirrhotic patients undergoing hepatectomy for HCC. *Gut* 2006; **56**: A145
  - 25 **Kooby DA**, Jarnagin WR. Surgical management of hepatic malignancy. *Cancer Invest* 2004; **22**: 283-303
  - 26 **Fong Y**, Sun RL, Jarnagin W, Blumgart LH. An analysis of 412 cases of hepatocellular carcinoma at a Western center. *Ann Surg* 1999; **229**: 790-799; discussion 799-800
  - 27 **Cha CH**, Ruo L, Fong Y, Jarnagin WR, Shia J, Blumgart LH, DeMatteo RP. Resection of hepatocellular carcinoma in patients otherwise eligible for transplantation. *Ann Surg* 2003; **238**: 315-321; discussion 321-323
  - 28 **Liau KH**, Ruo L, Shia J, Padela A, Gonen M, Jarnagin WR, Fong Y, D'Angelica MI, Blumgart LH, DeMatteo RP. Outcome of partial hepatectomy for large (> 10 cm) hepatocellular carcinoma. *Cancer* 2005; **104**: 1948-1955
  - 29 **Melendez J**, Ferri E, Zwillman M, Fischer M, DeMatteo R, Leung D, Jarnagin W, Fong Y, Blumgart LH. Extended hepatic resection: a 6-year retrospective study of risk factors for perioperative mortality. *J Am Coll Surg* 2001; **192**: 47-53
  - 30 **Shirabe K**, Shimada M, Gion T, Hasegawa H, Takenaka K, Utsunomiya T, Sugimachi K. Postoperative liver failure after major hepatic resection for hepatocellular carcinoma in the modern era with special reference to remnant liver volume. *J Am Coll Surg* 1999; **188**: 304-309
  - 31 **Azoulay D**, Castaing D, Krissat J, Smail A, Hargreaves GM, Lemoine A, Emile JF, Bismuth H. Percutaneous portal vein embolization increases the feasibility and safety of major liver resection for hepatocellular carcinoma in injured liver. *Ann Surg* 2000; **232**: 665-672
  - 32 **Abdalla EK**, Hicks ME, Vauthey JN. Portal vein embolization: rationale, technique and future prospects. *Br J Surg* 2001; **88**: 165-175
  - 33 **Kubo S**, Shiomi S, Tanaka H, Shuto T, Takemura S, Mikami S, Uenishi T, Nishino Y, Hirohashi K, Kawamura E, Kinoshita H. Evaluation of the effect of portal vein embolization on liver function by (99m)tc-galactosyl human serum albumin scintigraphy. *J Surg Res* 2002; **107**: 113-118
  - 34 **Kudo M**, Todo A, Ikekubo K, Yamamoto K, Vera DR, Stadalnik RC. Quantitative assessment of hepatocellular function through in vivo radioreceptor imaging with technetium 99m galactosyl human serum albumin. *Hepatology* 1993; **17**: 814-819
  - 35 **Vera DR**, Topcu SJ, Stadalnik RC. In vitro quantification of asialoglycoprotein receptor density from human hepatic microsomes. *Methods Enzymol* 1994; **247**: 394-402
  - 36 **Yumoto Y**, Umeda M, Ohshima K, Ogawa H, Kurokawa T, Kajitani M, Yumoto E, Hanafusa T, Tsuboi H, Higashi T. Estimation of remnant liver function before hepatectomy by means of technetium-99m-diethylenetriamine-pentaacetic acid galactosyl human albumin. *Cancer Chemother Pharmacol* 1994; **33** Suppl: S1-S6
  - 37 **Madoff DC**, Abdalla EK, Gupta S, Wu TT, Morris JS, Denys A, Wallace MJ, Morello FA Jr, Ahrar K, Murthy R, Lunagomez S, Hicks ME, Vauthey JN. Transhepatic ipsilateral right portal vein embolization extended to segment IV: improving hypertrophy and resection outcomes with spherical particles and coils. *J Vasc Interv Radiol* 2005; **16**: 215-225
  - 38 **Kinoshita H**, Sakai K, Hirohashi K, Igawa S, Yamasaki O, Kubo S. Preoperative portal vein embolization for hepatocellular carcinoma. *World J Surg* 1986; **10**: 803-808
  - 39 **Abdalla EK**, Barnett CC, Doherty D, Curley SA, Vauthey JN. Extended hepatectomy in patients with hepatobiliary malignancies with and without preoperative portal vein embolization. *Arch Surg* 2002; **137**: 675-680; discussion 680-681
  - 40 **de Baere T**, Roche A, Vavasseur D, Therasse E, Indushekar S, Elias D, Bognel C. Portal vein embolization: utility for inducing left hepatic lobe hypertrophy before surgery. *Radiology* 1993; **188**: 73-77
  - 41 **de Baere T**, Roche A, Elias D, Lasser P, Lagrange C, Bousson V. Preoperative portal vein embolization for extension of hepatectomy indications. *Hepatology* 1996; **24**: 1386-1391
  - 42 **Imamura H**, Shimada R, Kubota M, Matsuyama Y, Nakayama A, Miyagawa S, Makuuchi M, Kawasaki S. Preoperative portal vein embolization: an audit of 84 patients. *Hepatology* 1999; **29**: 1099-1105
  - 43 **Kubota K**, Makuuchi M, Kusaka K, Kobayashi T, Miki K, Hasegawa K, Harihara Y, Takayama T. Measurement of liver volume and hepatic functional reserve as a guide to decision-making in resectional surgery for hepatic tumors. *Hepatology* 1997; **26**: 1176-1181
  - 44 **Vauthey JN**, Chaoui A, Do KA, Bilimoria MM, Fenstermacher MJ, Charnsangavej C, Hicks M, Alsfasser G, Lauwers G, Hawkins IF, Caridi J. Standardized measurement of the future liver remnant prior to extended liver resection: methodology and clinical associations. *Surgery* 2000; **127**: 512-519
  - 45 **Hasegawa K**, Kokudo N, Imamura H, Matsuyama Y, Aoki T, Minagawa M, Sano K, Sugawara Y, Takayama T, Makuuchi M. Prognostic impact of anatomic resection for hepatocellular carcinoma. *Ann Surg* 2005; **242**: 252-259
  - 46 **Portolani N**, Coniglio A, Ghidoni S, Giovanelli M, Benetti A, Tiberio GA, Giulini SM. Early and late recurrence after

- liver resection for hepatocellular carcinoma: prognostic and therapeutic implications. *Ann Surg* 2006; **243**: 229-235
- 47 **Poon RT**, Fan ST, Lo CM, Liu CL, Wong J. Difference in tumor invasiveness in cirrhotic patients with hepatocellular carcinoma fulfilling the Milan criteria treated by resection and transplantation: impact on long-term survival. *Ann Surg* 2007; **245**: 51-58
- 48 **Shah SA**, Cleary SP, Wei AC, Yang I, Taylor BR, Hemming AW, Langer B, Grant DR, Greig PD, Gallinger S. Recurrence after liver resection for hepatocellular carcinoma: risk factors, treatment, and outcomes. *Surgery* 2007; **141**: 330-339
- 49 **Ramacciato G**, Mercantini P, Nigri GR, Ravaioli M, Cautero N, Di Benedetto F, Masetti M, Grazi GL, Ziparo V, Ercolani G, Pinna AD. Univariate and multivariate analysis of prognostic factors in the surgical treatment of hepatocellular carcinoma in cirrhotic patients. *Hepatogastroenterology* 2006; **53**: 898-903
- 50 **Lee SG**, Hwang S, Jung JP, Lee YJ, Kim KH, Ahn CS. Outcome of patients with huge hepatocellular carcinoma after primary resection and treatment of recurrent lesions. *Br J Surg* 2007; **94**: 320-326
- 51 **Pawlik TM**, Gleisner AL, Anders RA, Assumpcao L, Maley W, Choti MA. Preoperative assessment of hepatocellular carcinoma tumor grade using needle biopsy: implications for transplant eligibility. *Ann Surg* 2007; **245**: 435-442
- 52 **Liu CL**, Fan ST, Lo CM, Ng IO, Poon RT, Wong J. Hepatic resection for bilobar hepatocellular carcinoma: is it justified? *Arch Surg* 2003; **138**: 100-104
- 53 **Konishi M**, Ryu M, Kinoshita T, Inoue K. Surgical treatment of hepatocellular carcinoma with direct removal of the tumor thrombus in the main portal vein. *Hepatogastroenterology* 2001; **48**: 1421-1424
- 54 **Minagawa M**, Makuuchi M, Takayama T, Ohtomo K. Selection criteria for hepatectomy in patients with hepatocellular carcinoma and portal vein tumor thrombus. *Ann Surg* 2001; **233**: 379-384
- 55 **Poon RT**, Fan ST, Ng IO, Wong J. Prognosis after hepatic resection for stage IVA hepatocellular carcinoma: a need for reclassification. *Ann Surg* 2003; **237**: 376-383
- 56 **Cherqui D**, Laurent A, Tayar C, Chang S, Van Nhieu JT, Loriau J, Karoui M, Duvoux C, Dhumeaux D, Fagniez PL. Laparoscopic liver resection for peripheral hepatocellular carcinoma in patients with chronic liver disease: midterm results and perspectives. *Ann Surg* 2006; **243**: 499-506

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Hugh James Freeman, MD, FRCPC, FACP, Series Editor

## Heterogeneity of colorectal adenomas, the serrated adenoma, and implications for screening and surveillance

Hugh James Freeman

Hugh James Freeman, Department of Medicine (Gastroenterology), University of British Columbia, Vancouver V6T 1W5, Canada

Author contribution: Freeman HJ contributed all to this paper.  
Correspondence to: Dr. Hugh James Freeman, MD, FRCPC, FACP, Department of Medicine (Gastroenterology), University of British Columbia Hospital, 2211 Wesbrook Mall, Vancouver V6T 1W5, Canada. [hugfree@shaw.ca](mailto:hugfree@shaw.ca)

Telephone: +1-604-8227216 Fax: +1-604-8227236

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Atlanta, GA 30322, United States; Alessandro Fichera, MD, FACS, FASCRS, Assistant Professor, Department of Surgery -University of Chicago, 5841 S. Maryland Ave, MC 5031, Chicago IL 60637, United States

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### Abstract

Current algorithms for screening and surveillance for colon cancer are valuable, but may be limited by the underlying nature of the targeted neoplastic lesions. Although part of the success of adenoma removal relates to interruption of so-called "adenoma-carcinoma sequence", an alternate serrated pathway to colon cancer may pose difficulties with the ultimate results achieved by traditional colonoscopic methods. The endpoint carcinoma in this unique pathway may be derived from a dysplastic serrated adenoma. These tend to be located primarily in the right colon, especially in females, and are frequently associated with co-existent colon cancer. Unfortunately, however, there are few, if any, other identifiable risk factors, including age or family history of colon polyps or colon cancer. Moreover, this alternate serrated pathway may itself also be quite biologically heterogeneous as reflected in sessile serrated adenomas (SSA) with virtually exclusive molecular signatures defined by the presence of either BRAF or KRAS mutations. Screening algorithms in the future may need to be modified and individualized, depending on new information that likely will emerge on the natural history of these biologically heterogeneous lesions that differs from traditional adenomatous polyps.

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**Key words:** Colorectal adenomas; Serrated polyps; Polyp heterogeneity; Colonoscopy screening; Colorectal cancer

**Peer reviewers:** Vincent W Yang, Professor and Director, 201 Whitehead Research Building, 615 Michael Street,

### INTRODUCTION

Clinician endoscopists have witnessed an evolution in the application of the colonoscope. Initially, it was used primarily as an investigative modality to explore patient symptoms (abdominal pain, diarrhea, bleeding). Later, it also became widely applied as a therapeutic tool, particularly for removal of colorectal neoplasms. Now, colonoscopy has increasingly been used to screen for colon polyps and cancer in those with few or no symptoms demanding peace of mind. As a result, various agencies have developed (and promoted) guidelines for use that might aid in this endeavour, largely based on various risk factors, including familial colon polyps and cancers. For some, these are described as "evidence-based" with the goal of being "cost-effective" for an increasingly scarce service resource. These guidelines have also been based, at least in part, on the "Vogelstein" hypothesis that colon cancer results from a multistage and sequential series of mutational events that proceed from a benign adenomatous proliferation of altered epithelial cells to an increasingly larger and more complex invasive neoplastic (and even metastatic) lesion or lesions, the so-called "polyp-cancer sequence"<sup>[1]</sup>. Reasonably, interruption of this sequence could be accomplished by an intervening polypectomy and reduce the individual's risk for colon cancer. Recent information emphasizing the heterogeneous nature of these precursor colon epithelial polyps has suggested that this perspective may be an oversimplification of a more difficult problem.

### TRADITIONAL ADENOMAS AND SCREENING

Although different degrees of altered cytological

Table 1 Comparison of SSA and TSA to TA-TVA-VA polyps

	SSA	TSA	TA-TVA-VA
Location	Right colon	Throughout, 60% left	Throughout, 60% left
Shape	Flat	Pedunculated	Pedunculated
Cytodysplasia	Minimal	Present	Present
Growth	Bottom-up	Bottom-up	Top-down
Serration	Present	Present	Absent
Basal crypt	Dilation present	Dilation absent	Dilation may be present
Horizontal crypts	Present	Absent	May be present
Branched crypts	Present	Absent	May be present
Basal serration	Present	Absent	Absent
Nuclear shape	Round or oval	Tall columnar	Tall columnar
Cytoplasm	Eosinophilic	Eosinophilic	Basophilic

SSA: Sessile serrated adenoma; TSA: Traditional serrated adenoma; TA: Tubular adenoma; TVA: Tubulovillous adenoma; VA: Villous adenoma. Adapted from Li and Burgart<sup>[11]</sup>.

differentiation and atypia have often been described for individual polyps, recognition that colon polyp heterogeneity might have some prognostic clinical significance has traditionally been limited to discerning the degree of villous architectural change in the resected polyp (as opposed to tubular change), estimating polyp size (or more precisely, dimension) along with the number of colon polyps (albeit macroscopically visible). Any one (or more) of these has been considered by some to warrant alteration in the general guideline leading to shortened intervals of screening and/or surveillance, particularly if there were multiple polyps of large size that were histologically complex, primarily with villous architecture. Other factors may have also been entered into the equation in the mind of the practicing clinician including a background of chronic inflammatory bowel disease, certain ethnic or racial backgrounds, and, likely, individual financial means to facilitate performance of the actual screening procedure.

## ALTERNATE SERRATED PATHWAY

More recently, there has also been increased recognition that the serrated polyp (including the hyperplastic polyp (HP) with its serrated morphological features) may be more than a simple clinically innocuous bystander in the process of cancer development<sup>[2,3]</sup>.

These polyps appear quite distinct from traditional adenomatous polyps and may also exhibit morphological and molecular heterogeneity. Recent evidence suggests that some subtypes may pose a substantive potential risk for eventual malignant transformation. As such, it appears that this serrated pathway may represent an alternate road to development of colon cancer with potentially important implications for the “guideline approach” to screening and surveillance for colonic neoplastic lesions.

Some of the difficulty in this area relates to the pathological terminology along with evolution in methods of classification of serrated lesions that reflects the so-called “saw-tooth” architectural appearance of this polyp group<sup>[4]</sup>. Moreover, distinction between different forms of serrated polyps and interobserver

Table 2 Comparison of SSA polyps and HP polyps

	SSA	HP
Location	Right colon	Rectosigmoid
Shape	Flat	Pedunculated or flat
Size	> 5 mm	< 5 mm
Cytologic dysplasia	Minimal	Absent
Basal crypt dilation	Yes	No
Horizontal crypts	Yes	No
Branched crypts	Yes	No
Basal crypt serration	Yes	No
Nuclear shape	Round to oval	Flat or low columnar
Cytoplasmic eosinophilia	Prominent	Not prominent

SSA: Sessile serrated adenoma; HP: Hyperplastic polyp. Adapted from Li and Burgart<sup>[11]</sup>.

agreement among expert pathologists may be limited<sup>[5]</sup>. It appears that HPs are the most common type and these have been further sub-divided into microvesicular, goblet-cell rich and mucin-poor types. Other kinds of serrated polyps include sessile serrated adenomas (SSA), traditional serrated adenomas (TSA) and mixed polyps containing components of sessile serrated and tubular adenomas. Significant differences in the expression of specific genetic and molecular markers have also been shown between SSA and TSA<sup>[6]</sup>. While HPs seem to remain small and localized to the distal colorectum, other serrated polyps may progress to cancer through an apparently unique pathway. An early recognized form of this entity, initially labeled “hyperplastic polyposis” or “serrated adenomatous polyposis”, consisted of larger sessile polyps developing mainly in the right colon. These were associated with synchronous colon cancer in over 50%<sup>[7,8]</sup>. For TSA, dysplasia or intramucosal carcinoma were also noted in almost 50%<sup>[9]</sup>. Of note, about 10% of all colon polyps may be SSA type and about 15% have multiple lesions, based on detection with magnification chromoendoscopy<sup>[10]</sup>. Most were located in the right colon, particularly in females, however, there was no correlation with age or personal or family history of colon polyps or colon cancer<sup>[10]</sup>. Other characteristics of SSA are listed in Tables 1 and 2.

## MOLECULAR HETEROGENEITY OF SSA TYPE POLYPS

The molecular heterogeneity of lesions in this alternate serrated pathway has been nicely reviewed elsewhere by O'Brien<sup>[3]</sup>. The carcinomas that occur demonstrate microsatellite instability (MSI-high) due to hMLH1 inactivation and consequent DNA mismatch repair (MMR). In addition, heterogeneity is evident in that some carcinomas are also microsatellite stable (MSS or MSI-low). The pathway is believed to originate in a HP, or precursor aberrant crypt focus, and progresses through an intermediate disordered type of HP that eventually becomes dysplastic (dysplastic serrated polyp), and ultimately to a serrated adenocarcinoma<sup>[3]</sup>. Definition of the phenomenon of epigenetic mutagenesis by CpG-island methylation and its key role in sporadic MSI colorectal carcinomas have been stated to be at the molecular genetic core of this newly defined serrated

pathway<sup>[3]</sup>. CpG island methylation phenotype (CIMP) refers to nonrandom methylation of gene promoter regions that concordantly affects multiple susceptible suppressor, mutator, and other genes that have roles in carcinogenesis<sup>[12]</sup>. Epigenetic gene silencing of cancer-related genes has been shown in precursor polyps and endpoint carcinomas in the serrated polyp pathway<sup>[3]</sup>. This mechanism differs from the mutagenic process of the traditional “adenoma-carcinoma sequence”, where adenomas progress to carcinoma by deletions and homozygous loss of suppressor genes due to APC mutation-induced chromosomal instability<sup>[1]</sup>. Another interesting observation has been the discovery of an oncogene BRAF mutation in these neoplasms<sup>[10]</sup>. A specific activating mutation (V600E) of this phosphokinase appears to be present in most CIMP-high and MSI colon cancers, serrated polyps, including hyperplastic aberrant crypt foci, and dysplastic serrated adenomas. Although the BRAF serrated pathway is predominant (up to 80% of SSA), a second is associated with KRAS mutations. It contrasts with the BRAF pathway in that most of these are distal rather than proximal lesions with lower levels of CpG-island methylation and MSS or MSI-L rather than MSI endpoint carcinomas<sup>[3]</sup>.

## FUTURE SCREENING FOR COLON POLYPS

The high proportion of SSA is noteworthy as these are not exactly rare and most disconcerting is that routine colonoscopy may not be adequate for their detection<sup>[2,13]</sup>.

Moreover, there does not appear to be a definite profile of high risk for this SSA type that might actually lead to initiation of the screening process, particularly family history<sup>[2]</sup>. Thus, current screening algorithms may not be adequate for detection. Moreover, hyperplastic/serrated polyposis has also been observed in patients with chronic inflammatory bowel disease<sup>[14]</sup>. A prudent approach has been suggested to include complete resection and surveillance examinations as often as the intervals defined for the more traditional adenomatous polyps but this approach is not necessarily reflective of the natural biological history of these lesions. A prudent colonoscopist will also emphasize prior to embarking on a screening procedure with the patient that small lesions may be not be readily detectable. While still the gold standard for polyp detection, colonoscopic procedures have a definitive “miss rate” so that it can come close to, but does not appear able to reach perfection. Recent comparative and prospective studies using pan-colonic narrow-band imaging suggest that its use for surveillance of even small adenomas may be superior to conventional colonoscopy and equivalent to chromoendoscopy<sup>[15-18]</sup>. More widespread application of these evolving technologies in the future may also impact on the detection of serrated adenomas and current screening and surveillance guidelines.

## REFERENCES

- 1 Vogelstein B, Fearon ER, Hamilton SR, Kern SE, Preisinger AC, Leppert M, Nakamura Y, White R, Smits AM, Bos JL. Genetic alterations during colorectal-tumor development. *N Engl J Med* 1988; **319**: 525-532
- 2 Lauwers GY, Chung DC. The serrated polyp comes of age. *Gastroenterology* 2006; **131**: 1631-1634
- 3 O'Brien MJ. Hyperplastic and serrated polyps of the colorectum. *Gastroenterol Clin North Am* 2007; **36**: 947-968, viii
- 4 Snover DC, Jass JR, Fenoglio-Preiser C, Batts KP. Serrated polyps of the large intestine: a morphologic and molecular review of an evolving concept. *Am J Clin Pathol* 2005; **124**: 380-391
- 5 Farris AB, Misdraji J, Srivastava A, Muzikansky A, Deshpande V, Lauwers GY, Mino-Kenudson M. Sessile serrated adenoma: challenging discrimination from other serrated colonic polyps. *Am J Surg Pathol* 2008; **32**: 30-35
- 6 Torlakovic EE, Gomez JD, Driman DK, Parfitt JR, Wang C, Benerjee T, Snover DC. Sessile serrated adenoma (SSA) vs. traditional serrated adenoma (TSA). *Am J Surg Pathol* 2008; **32**: 21-29
- 7 Torlakovic E, Snover DC. Serrated adenomatous polyposis in humans. *Gastroenterology* 1996; **110**: 748-755
- 8 Leggett BA, Devereaux B, Biden K, Searle J, Young J, Jass J. Hyperplastic polyposis: association with colorectal cancer. *Am J Surg Pathol* 2001; **25**: 177-184
- 9 Longacre TA, Fenoglio-Preiser CM. Mixed hyperplastic adenomatous polyps/serrated adenomas. A distinct form of colorectal neoplasia. *Am J Surg Pathol* 1990; **14**: 524-537
- 10 Spring KJ, Zhao ZZ, Karamatic R, Walsh MD, Whitehall VL, Pike T, Simms LA, Young J, James M, Montgomery GW, Appleyard M, Hewett D, Togashi K, Jass JR, Leggett BA. High prevalence of sessile serrated adenomas with BRAF mutations: a prospective study of patients undergoing colonoscopy. *Gastroenterology* 2006; **131**: 1400-1407
- 11 Li SC, Burgart L. Histopathology of serrated adenoma, its variants, and differentiation from conventional adenomatous and hyperplastic polyps. *Arch Pathol Lab Med* 2007; **131**: 440-445
- 12 O'Brien MJ, Yang S, Mack C, Xu H, Huang CS, Mulcahy E, Amoroso M, Farraye FA. Comparison of microsatellite instability, CpG island methylation phenotype, BRAF and KRAS status in serrated polyps and traditional adenomas indicates separate pathways to distinct colorectal carcinoma end points. *Am J Surg Pathol* 2006; **30**: 1491-1501
- 13 Cappell MS. Reducing the incidence and mortality of colon cancer: mass screening and colonoscopic polypectomy. *Gastroenterol Clin North Am* 2008; **37**: 129-160, vii-viii
- 14 Srivastava A, Redston M, Farraye FA, Yantiss RK, Odze RD. Hyperplastic/serrated polyposis in inflammatory bowel disease: a case series of a previously undescribed entity. *Am J Surg Pathol* 2008; **32**: 296-303
- 15 Su MY, Hsu CM, Ho YP, Chen PC, Lin CJ, Chiu CT. Comparative study of conventional colonoscopy, chromoendoscopy, and narrow-band imaging systems in differential diagnosis of neoplastic and nonneoplastic colonic polyps. *Am J Gastroenterol* 2006; **101**: 2711-2716
- 16 Chiu HM, Chang CY, Chen CC, Lee YC, Wu MS, Lin JT, Shun CT, Wang HP. A prospective comparative study of narrow-band imaging, chromoendoscopy, and conventional colonoscopy in the diagnosis of colorectal neoplasia. *Gut* 2007; **56**: 373-379
- 17 Inoue T, Murano M, Murano N, Kuramoto T, Kawakami K, Abe Y, Morita E, Toshina K, Hoshiro H, Egashira Y, Umegaki E, Higuchi K. Comparative study of conventional colonoscopy and pan-colonic narrow-band imaging system in the detection of neoplastic colonic polyps: a randomized, controlled trial. *J Gastroenterol* 2008; **43**: 45-50
- 18 Adler A, Pohl H, Papanikolaou IS, Abou-Rebyeh H, Schachschal G, Veltzke-Schlieker W, Khalifa AC, Setka E, Koch M, Wiedenmann B, Rosch T. A prospective randomised study on narrow-band imaging versus conventional colonoscopy for adenoma detection: does narrow-band imaging induce a learning effect? *Gut* 2008; **57**: 59-64



REVIEW

## Liver cell transplantation for Crigler-Najjar syndrome type I : Update and perspectives

Philippe A Lysy, Mustapha Najimi, Xavier Stéphenne, Annick Bourgois, Françoise Smets, Etienne M Sokal

Philippe A Lysy, Mustapha Najimi, Xavier Stéphenne, Annick Bourgois, Françoise Smets, Etienne M Sokal, Université Catholique de Louvain, Cliniques Universitaires Saint Luc, HPED Department, PEDI Unit, Laboratory of Pediatric Hepatology and Cell Therapy, Brussels B-1200, Belgium

**Author contributions:** Sokal EM, Smets F, and Lysy PA designed research; Lysy PA, Bourgois A, Smets F and Sokal EM performed research; Lysy PA, Najimi M, Bourgois A, Smets F and Sokal EM analyzed data; and Lysy PA, Stéphenne X, Smets F, Sokal EM wrote the paper.

**Correspondence to:** Etienne M Sokal, Pediatric Hepatology and Cell Therapy, Université Catholique de Louvain, Cliniques Saint Luc, 10 av. Hippocrate, Brussels B-1200, Belgium. [sokal@pedi.ucl.ac.be](mailto:sokal@pedi.ucl.ac.be)

Telephone: +32-2-7641387 Fax: +32-2-7648909

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Transplant Research Program, University of California, Davis Medical Center, Sacramento, CA 95817, United States; Dr. J Michael Millis, Department of Surgery, University of Chicago, Chicago 60637, United States; Roger Williams, Professor, The Institute of Hepatology, 69-75 Chenies Mews, London, WC1E 6HX, United Kingdom

Lysy PA, Najimi M, Stéphenne X, Bourgois A, Smets F, Sokal EM. Liver cell transplantation for Crigler-Najjar syndrome type I : Update and perspectives. *World J Gastroenterol* 2008; 14(22): 3464-3470 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3464.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3464>

### Abstract

Liver cell transplantation is an attractive technique to treat liver-based inborn errors of metabolism. The feasibility and efficacy of the procedure has been demonstrated, leading to medium term partial metabolic control of various diseases. Crigler-Najjar is the paradigm of such diseases in that the host liver is lacking one function with an otherwise normal parenchyma. The patient is at permanent risk for irreversible brain damage. The goal of liver cell transplantation is to reduce serum bilirubin levels within safe limits and to alleviate phototherapy requirements to improve quality of life. Preliminary data on Gunn rats, the rodent model of the disease, were encouraging and have led to successful clinical trials. Herein we report on two additional patients and describe the current limits of the technique in terms of durability of the response as compared to alternative therapeutic procedures. We discuss the future developments of the technique and new emerging perspectives.

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**Key words:** Hepatocyte transplantation; Cell therapy; Inborn error of metabolism; Crigler-Najjar; Liver regeneration; Animal models

**Peer reviewers:** Jian Wu, MD, PhD, Internal Medicine/

### INTRODUCTION

Crigler-Najjar (CN) syndrome is the paradigm of an inborn error of liver metabolism affecting the function of one enzyme, the 1A1 isoform of the bilirubin-uridine diphosphate glucuronosyltransferase (UGT1A1)<sup>[1]</sup>. The parenchyma and thousands of other metabolic functions are normal, but the patient is at risk for severe neurological complications. Quality of life is deeply impaired, requiring phototherapy up to 12 h daily with efficacy lessening with ageing (probably due to unfavorable body surface/weight ratio and to increased skin thickness and pigmentation). Orthotopic liver transplantation (OLT) is a curative for the disorder<sup>[2,3]</sup>, but seems disproportionate to correct one single missing enzymatic function in an otherwise normal liver. Patients and physicians are often reluctant to undertake such an irreversible procedure and are seeking less invasive alternative options. Indeed, up to 15% of OLT patients require re-transplantation, and progressive fibrosis of the graft is a subject of concern at long term<sup>[4]</sup>.

Auxiliary liver transplantation (ALT) is another curative approach that has the advantage of being reversible. However, ALT remains associated with major pitfalls. In addition to being an invasive surgical procedure, the technique is difficult mainly because of perilous anastomosis that can hamper the venous in- or outflow and can lead to graft atrophy/ischemia or vascular thrombosis. Another complication is the small-for-size liver syndrome, defined as liver impairment, following inadequate liver mass replacement<sup>[5]</sup>. The diagnosis of rejection is difficult because of minimal enzyme elevation.



**Table 1** Representative liver cell transplantation experiments in the Gunn rat model

Donor cells	Injection site	Hepatic injury	Outcome	Cell tracking	References
50 × 10 <sup>6</sup> free or encapsulated congenic Hc	Peritoneum	None	34.8% serum bilirubin reduction with encapsulated Hc vs 13.5% with free Hc at 1 mo	Light and electron microscopy	22
10 × 10 <sup>6</sup> syngeneic Hc	Liver	Hepatectomy	Significant reduction of serum bilirubin up to 4 wk Apparition of conjugates in bile	ND	24
10 × 10 <sup>6</sup> congenic Hc	Spleen	None	Significant reduction of serum bilirubin up to 12 mo Apparition of bile conjugates at 4 mo	ND	27
2-20 × 10 <sup>6</sup> congenic Hc	Portal vein	Right portal vein ligation	Significant reduction of serum bilirubin when injury with 2 × 10 <sup>6</sup> Hc or with 20 × 10 <sup>6</sup> without injury up to 30 d Conjugates in bile after 10 d	UGT1A1 activity, WB, PCR for <i>ugt1</i> gene	28
5 × 10 <sup>6</sup> congenic Hc	Spleen	Hepatic irradiation ± Hepatectomy	Normalization of serum bilirubin only with combined injury Conjugates in bile detected up to 5 mo	UGT1A1 activity, WB, IHC	29
10 × 10 <sup>6</sup> congenic Hc	Spleen	Hepatic irradiation ± FasL-induced apoptosis	Normalization of serum bilirubin up to 160 d Conjugates in bile at 150 d Estimation of repopulation at 52 ± 15% when combined injury	UGT1A1 activity, WB, IHC	31
40 × 10 <sup>6</sup> fetal or adult syngeneic Hc	Spleen	Retrorsine + Triiodothyronine	Significant reduction of serum bilirubin (+ conjugates in bile) up to 90 d (no difference between fetal and adult cells)	PCNA	32

Hc: Hepatocyte; IHC: Immunohistochemistry; PCNA: Proliferating cell nuclear antigen; WB: Western blot.

Successful long-term results were recently obtained with gene therapy in Gunn rats<sup>[6,7]</sup>. This technique was described to depend on vector serotypes and allowed a reduction of serum bilirubin up to 64% after one year<sup>[8]</sup>. Globally, this technique is still facing with anti-UGT1A1 antibody production in the host organism, impeding the perpetuation of the metabolic effect<sup>[9]</sup>. Although encouraging, *ex vivo* gene transfer and cell injection is closely related to the quality of cell preparation<sup>[10,11]</sup> and has not been documented in CN patients.

Other experimental protocols have been described, such as tin-mesoporphyrin treatment, for which feasibility has been demonstrated in two 17 year old patients<sup>[12]</sup>, or treatment with chimeric oligonucleotides that allowed a significant reduction of serum bilirubin in Gunn rats for up to 11 mo<sup>[13]</sup>.

Since the princeps report by Fox *et al*<sup>[14]</sup>, liver cell therapy (LCT) appeared as a new alternative treatment, which is intermediate between whole organ transplantation and gene therapy. Cells can be infused safely in the diseased liver, and are expected to bring sufficient enzyme activity to restore bilirubin metabolism, setting the patients within safer metabolic limits and improving quality of life. LCT has been shown to be able to restore metabolic function not only in CN patients<sup>[15]</sup>, but also in disorders of ammonium metabolism<sup>[16,17]</sup>, glucose metabolism<sup>[18]</sup>, clotting factor deficiencies<sup>[19]</sup>, and even complex enzyme systems such as Refsum disease<sup>[20]</sup>.

However, the technique remains insufficient; metabolic control is partial and durability of the result is limited to less than one year in most cases. Our aim is to review the current knowledge on the role of LCT to treat CN patients, report two additional patients, and

review animal experiments performed as preclinical studies.

## LCT FOR CN DISEASE TYPE I

### Lessons from the animal model

The Gunn rat model represents the rodent equivalent of CN disease and is characterized by a single mutation in the *ugt1A1* gene. In this model, many experimental protocols using free or encapsulated liver cells have been designed with syngeneic/congenic or allogeneic transplantation procedures<sup>[21-32]</sup>. Table 1 summarizes representative experiments. The best results were obtained when a hepatic injury was caused before LCT to create a niche and a regenerative stimulus for engrafting cells. The explanation for why the injury was beneficial is Gunn rats global liver function is normal, except for bilirubin conjugation, and the lack of host hepatocyte impairment fails to provide to donor cells a proliferative advantage. The repopulation rate necessary to observe a metabolic efficacy ranges from 5% to 10%<sup>[33]</sup>. Significant lowering of serum bilirubin could be observed up to 12 mo while using congenic procedures<sup>[27]</sup>.

### On the clinical side

Reports of human LCT for CN disease have shown encouraging results. The first demonstration of the efficacy of the technique was provided by Fox *et al*<sup>[14]</sup>. In this case, 7.5 × 10<sup>9</sup> viable liver cells were infused in a 10-year-old patient and the effect was a significant decrease of bilirubin levels for up to 11 mo (Table 2). UGT1A1 enzyme activity was detected in the host liver and glucuronoconjugates were found in bile confirming

Table 2 Summary of clinical liver cell transplantation procedures for liver-based inborn errors of metabolism

Indication	<i>n</i>	Patient age	Cell amount (% liver cell mass)	Follow-up	References
Familial hypercholesterolemia	5	7-41 yr		Partial reduction of LDL (3/5 patients)	69
				Donor hepatocytes detected by ISH at 4 mo	
				Decrease of bilirubin levels up to 11 mo	14
CN disease type I	1	10 yr	$7.5 \times 10^9$ (5%)	Detection of UGT1A1 enzyme activity and of glucurono-conjugates in bile	
				50%-65% reduction of bilirubin up to 3 mo	34
				Donor hepatocytes not detected by short tandem repeat analysis at 40 d	
	2	18 mo/3 yr	ND	50%/30% reduction of serum bilirubin over 7 mo/ND follow-up	33
				Donor hepatocytes detected in one case by short tandem repeat analysis at 8 mo	
				Donor Y-chromosomes detected by PCR at 7 d	20
Infantile refsum disease	1	4 yr	$2 \times 10^9$		
Inherited coagulation factor VII deficiency	2	3 mo/2 yr	$1.1 \times 10^9$ / $2.2 \times 10^9$ (4%/3%)	Decrease in the factor VII requirements	19
PFIC 2	2	ND	$0.3 \times 10^9$	No improvement	33
				Fasting tolerance: up to 7 h	18
Glycogen storage disease type I a	1	47 yr	$2 \times 10^9$ (1%)	Increase of glycemia	
				Improvement of diet	
				G6Pase activity detected	
Urea cycle disease	1 (OTC)	5 yr	$1 \times 10^9$	Improvement of ammonia levels	70
				Detection of enzyme activity	
	1 (OTC)	0 d	$10.5 \times 10^9$	Transient metabolic improvement between 20 and 31 d of life	71
	1 (OTC)	1 d	$1.9 \times 10^9$	Improvement of ammonia levels	72
				Increased urea synthesis	
	1 (OTC)	14 mo	$2.4 \times 10^9$ (6%)	Improvement of psychomotor development and of ammonia levels	16
				Urea neo-synthesis	
				Improvement of psychomotor development and of ammonia levels	17
	1 (ASL)	3.5 yr	$4.7 \times 10^9$ (9%)	Donor hepatocytes detected by FISH at 12 mo and by enzyme activity at 8 mo	

ASL: Arginino-succinate lyase; (F)ISH: (Fluorescent) in situ hybridization; LDL: Low density lipoproteins; ND: Not documented; OTC: Ornithine transcarbamylase; PCR: Polymerase chain reaction; PFIC: Progressive familial intrahepatic cholestasis.

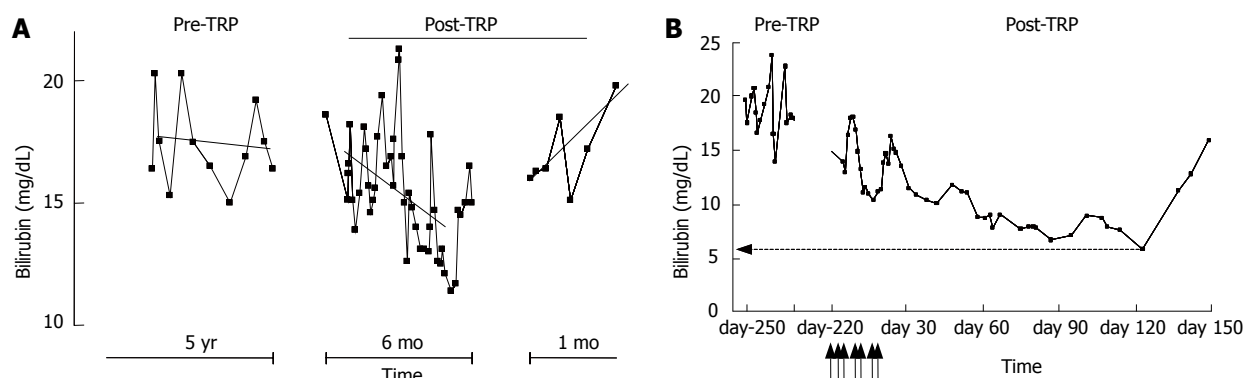
Table 3 Presentation of LCT procedures in two Crigler-Najjar disease type I patients

	Patient 1	Patient 2
Age/Gender	9 yr/Female	1 yr/Female
Infusion procedure	Porth-a-cath in jejunal vein	Broviac in portal vein
Timing of infusions	18 infusions/5 mo	14 infusions/15 d
Donor cells	Fresh and cryopreserved from 3 donors	Fresh and cryopreserved from 1 donor
Cell amount	6.1 billion	2.6 billion
	0.16 billion/kg	0.35 billion/kg
% Liver cell mass	4%	8%
Mean viability	80%	83%

the integration of functional, healthy hepatocytes. Dhawan *et al* reported two additional patients ages 18 mo and 3 years, in which the reduction of serum bilirubin reached up to 50% and 30%, respectively over a follow-up period up of 7 mo (Table 2). Donor hepatocyte engraftment was illustrated by short tandem repeat analysis at 8 mo follow-up. Ambrosino *et al* also described a decrease of bilirubin levels up to 3 mo post-LCT, whereas they did not detect donor cells by using a short tandem repeat assay at 40 d follow-up<sup>[34]</sup>.

We performed LCT in two CN pediatric cases (Table 3, Figure 1). The first patient was a 9 year old girl in whom a port-a-cath was placed in the jejunal

vein. She received 18 cell infusions from three different donors over a period of 5 mo for a total of 4% of her estimated liver cell mass. Mean cell viability was high (80%) and no adverse events were noticed during the procedure. Pre-transplant serum bilirubin values attained  $17.5 \pm 0.49$  mg/dL (mean  $\pm$  SD) and dropped after LCT to the lowest value of 11.4 mg/dL (mean  $\pm$  SD:  $13.6 \pm 0.42$  mg/dL,  $P < 0.001$ ). After a period of 6 mo, bilirubin values increased suddenly without a concomitant event and the patient was scheduled for OLT. For the second patient, the protocol was revised in order to provide a higher amount of cells within a shorter infusion period. She was 1 year old at the time



**Figure 1** Evolution of serum bilirubin before and after LCT in two CN patients performed in our center. **A:** After fluctuating over a period of 5 yr, serum bilirubin of patient 1 decreased significantly to the lesser value of 11.4 mg/dL in 6 mo. Subsequently, increasing values were observed and the patient was listed for OLT. **B:** For patient 2, after cell infusions, the serum bilirubin dramatically decreased to the value of 6 mg/dL in 4 mo. At this time, concomitantly to an EBV infection, higher values were observed and the patient underwent OLT. Arrows indicate the timing of cell infusions. TRP: Transplantation.

of the procedure and received 14 infusions from one single donor over 15 d to reach a total of 8.6% of her estimated liver cell mass. Cells were infused *via* a broviac catheter surgically inserted *via* a colonic vein to the spleno-mesaraic confluent. Cell viability (mean 83%) and clinical tolerance were optimal. With pre-LCT levels of  $17.6 \pm 3.5$  mg/dL (mean  $\pm$  SD), the serum bilirubin dramatically decreased to values of  $13.3 \pm 2.4$  mg/dL (mean  $\pm$  SD) with the lowest value at 6 mg/dL. Skin jaundice reduced rapidly and the daily phototherapy schedule was alleviated from 10 to 8 h without any influence on the bilirubin levels. After 4 mo of progressive decrease of serum bilirubin, the values increased suddenly following an intercurrent Epstein-Barr virus (EBV) infection. The child underwent OLT without complications related to the previous LCT. Both patients received a methylprednisolone bolus and tacrolimus the day before and for 12 d after LCT. Subsequently they were given tacrolimus as long-term monotherapy.

## PERSPECTIVES

At present, LCT remains limited by incomplete and time-limited metabolic control, mainly due to unfavorable immunological cell interactions, impaired donor cell quality and poor repopulation rates. Whereas the immunogenicity of liver cells is quite different compared to whole liver<sup>[35]</sup>, the same immunosuppression protocols are applied for LCT and OLT. Additional fundamental *in vivo* studies are necessary for the development of the optimal immunosuppression protocol. In that way, Wu *et al* recently compared the effects of tacrolimus, rapamycin and mycophenolate mofetil on the engraftment and proliferation of engrafted liver cells in a allogeneic setting<sup>[36]</sup>. They observed a deleterious effect of rapamycin on the proliferation of the transplanted cells. Serrano *et al* reported the lack of toxicity of tacrolimus and methylprednisolone on human hepatocytes *in vitro*<sup>[37]</sup>. Other experimental protocols were designed to reduce the immunological pressure occurring in LCT procedures. For example, Mashalova

*et al* obtained similar engraftment levels with syngeneic or allogeneic hepatocytes after their transduction with adenoviral early region 3 genes, suggesting a protective effect against rejection<sup>[38]</sup>. This was related to the down-expression of Fas receptor at the cell surface leading to inhibition of Fas-mediated apoptosis. Protocols combining LCT with bone marrow transplantation with<sup>[39]</sup> or without<sup>[40]</sup> elimination of natural killer cells are being investigated. Liver cell encapsulation aiming to protect cells from the immune system has demonstrated promising results in Gunn rats<sup>[41-43]</sup>. The technique is reversible and allows delivery of the cells to extrahepatic sites that are easy to access for sampling. However, major remaining hurdles are the creation of an adequate 'intracapsular' microenvironment allowing long-term cell functionality and the restriction of this technique to an enzyme-delivery role. Host immunity can be modulated by co-transplantation of immunomodulatory cells, as developed by Le Blanc *et al*, using mesenchymal stem cells to control graft *versus* host disease in the bone marrow transplant setting<sup>[44,45]</sup>. These cells and others, as non-parenchymal cells<sup>[46]</sup> or liver-derived mesenchymal lineages<sup>[47,48]</sup>, could provide permissive factors or a microenvironment allowing more favorable immunological cell interactions, although this has not been tested so far in LCT protocols. Study of inner mechanisms of cell rejection may also lead to improved clinical efficiency of LCT. For example, it has been shown recently that human hepatocytes exert a procoagulant activity depending on tissue factor expression<sup>[49]</sup>, as previously demonstrated with pancreatic islet cells<sup>[50,51]</sup>. In this work, St  phenne *et al* demonstrated the improvement of the procoagulant activity by incubating the cells with N-acetylcysteine, making this drug valuable for additional *in vivo* studies.

Enhancement of liver cell engraftment capacity is another challenge. Engraftment depends on liver cell quality and host liver environment. While LCT is highly dependent on banking of cryopreserved cells, this procedure has been demonstrated to deteriorate cell quality. Indeed, although cryopreserved/thawed hepatocytes have been shown to possess *in vivo* clonal

replicative potential identical to freshly isolated cells<sup>[52]</sup>, their *in vivo* potential seems to be restricted in time<sup>[53-55]</sup> and their *in vitro* functionality remains lower than that of freshly isolated hepatocytes<sup>[56]</sup>. Furthermore, we recently demonstrated that, with the current protocols, cryopreservation/thawing of hepatocytes induces cell alteration and especially mitochondrial defects (complex 1 impairment)<sup>[57]</sup>. Intracellular ice formation remains the major factor affecting the quality of cells. Protection delivered by non-permeating cryoprotectants must be further analyzed in terms of cell death and mitochondrial functions. New perspectives, such as vitrification, to avoid the crystalline state, coupled or not with encapsulation, must be validated in the future while considering the problem of hepatocyte de-differentiation at long term that could occur in this type of configuration.

Actions on the liver microenvironment have been evaluated in a recent report using monocrotaline, which is an alkaloid showing toxicity against liver endothelial and Kupffer cells<sup>[58]</sup>. Authors reported an enhanced liver cell engraftment in a syngeneic background mainly related to endothelial cell damage. Comparable studies were performed on dipeptidyl peptidase IV<sup>-/-</sup>F344 rats using doxorubicin, irinotecan, or vincristine<sup>[59]</sup>. In this study, Kim *et al* showed improved cell engraftment after doxorubicin treatment attributed to endothelial cell disruption. While interesting, these approaches will not be applicable in a clinical setting. Physical alteration of the liver architecture was studied by Dagher *et al* on nonhuman primates using partial portal vein ligation or embolization in an autologous LCT procedure<sup>[60]</sup>. The authors reported hepatic regeneration rates up to 10% obtained at short term (15 d) after embolization of the portal vein. Others have successfully used chemicals as vascular endothelial growth factor delivered *in situ*<sup>[61]</sup> or by peripheral route<sup>[62]</sup> to promote cell engraftment.

As stem cells were recently described to have a hepatocyte differentiation potential<sup>[63,64]</sup>, these are currently considered with growing interest for liver cell therapy. The most potent candidates are mesenchymal stem cells isolated from various tissues, with predilection for bone marrow<sup>[65]</sup> and umbilical cord<sup>[66]</sup>. Liver progenitor cells<sup>[67]</sup> or mesenchymal-like cells<sup>[47,48]</sup> also deserve detailed attention. However, stem cells only display partial hepatocyte-like functionality<sup>[64,68]</sup> and further advance is necessary to consider such cell types for therapy.

## CONCLUSION

While LCT seems currently efficient and safe to improve the quality of life of CN diseased patients for a medium period of time, the technique still requires development to be considered for longer term or curative purposes. Advances must be focused on the quality of cell preparations together with the management of immunological barriers hampering reliable cell engraftment. Furthermore, other research areas, such as gene or stem cell therapy, are currently encountering

exciting expansion, and combined therapeutic approaches would be justified in the near future.

## REFERENCES

- 1 **Bosma PJ**, Seppen J, Goldhoorn B, Bakker C, Oude Elferink RP, Chowdhury JR, Chowdhury NR, Jansen PL. Bilirubin UDP-glucuronosyltransferase 1 is the only relevant bilirubin glucuronidating isoform in man. *J Biol Chem* 1994; **269**: 17960-17964
- 2 **Kaufman SS**, Wood RP, Shaw BW Jr, Markin RS, Rosenthal P, Gridelli B, Vanderhoof JA. Orthotopic liver transplantation for type I Crigler-Najjar syndrome. *Hepatology* 1986; **6**: 1259-1262
- 3 **Sokal EM**, Silva ES, Hermans D, Reding R, de Ville de Goyet J, Buts JP, Otte JB. Orthotopic liver transplantation for Crigler-Najjar type I disease in six children. *Transplantation* 1995; **60**: 1095-1098
- 4 **Evans HM**, Kelly DA, McKiernan PJ, Hubscher S. Progressive histological damage in liver allografts following pediatric liver transplantation. *Hepatology* 2006; **43**: 1109-1117
- 5 **Heaton N**. Small-for-size liver syndrome after auxiliary and split liver transplantation: donor selection. *Liver Transpl* 2003; **9**: S26-S28
- 6 **Bellodi-Privato M**, Aubert D, Pichard V, Myara A, Trivin F, Ferry N. Successful gene therapy of the Gunn rat by *in vivo* neonatal hepatic gene transfer using murine oncoretroviral vectors. *Hepatology* 2005; **42**: 431-438
- 7 **van der Wegen P**, Louwen R, Imam AM, Buijs-Offerman RM, Sinaasappel M, Grosveld F, Scholte BJ. Successful treatment of UGT1A1 deficiency in a rat model of Crigler-Najjar disease by intravenous administration of a liver-specific lentiviral vector. *Mol Ther* 2006; **13**: 374-381
- 8 **Seppen J**, Bakker C, de Jong B, Kunne C, van den Oever K, Vandenbergh K, de Waart R, Twisk J, Bosma P. Adeno-associated virus vector serotypes mediate sustained correction of bilirubin UDP glucuronosyltransferase deficiency in rats. *Mol Ther* 2006; **13**: 1085-1092
- 9 **Seppen J**, van Til NP, van der Rijt R, Hiralall JK, Kunne C, Elferink RP. Immune response to lentiviral bilirubin UDP-glucuronosyltransferase gene transfer in fetal and neonatal rats. *Gene Ther* 2006; **13**: 672-677
- 10 **Seppen J**, Tada K, Ottenhoff R, Sengupta K, Chowdhury NR, Chowdhury JR, Bosma PJ, Oude Elferink RP. Transplantation of Gunn rats with autologous fibroblasts expressing bilirubin UDP-glucuronosyltransferase: correction of genetic deficiency and tumor formation. *Hum Gene Ther* 1997; **8**: 27-36
- 11 **Nguyen TH**, Birraux J, Wildhaber B, Myara A, Trivin F, Le Coultre C, Trono D, Chardot C. Ex vivo lentivirus transduction and immediate transplantation of uncultured hepatocytes for treating hyperbilirubinemic Gunn rat. *Transplantation* 2006; **82**: 794-803
- 12 **Galbraith RA**, Drummond GS, Kappas A. Suppression of bilirubin production in the Crigler-Najjar type I syndrome: studies with the heme oxygenase inhibitor tinmesoporphyrin. *Pediatrics* 1992; **89**: 175-182
- 13 **Kren BT**, Parashar B, Bandyopadhyay P, Chowdhury NR, Chowdhury JR, Steer CJ. Correction of the UDP-glucuronosyltransferase gene defect in the gunn rat model of crigler-najjar syndrome type I with a chimeric oligonucleotide. *Proc Natl Acad Sci USA* 1999; **96**: 10349-10354
- 14 **Fox IJ**, Chowdhury JR, Kaufman SS, Goertzen TC, Chowdhury NR, Warkentin PI, Dorko K, Sauter BV, Strom SC. Treatment of the Crigler-Najjar syndrome type I with hepatocyte transplantation. *N Engl J Med* 1998; **338**: 1422-1426
- 15 **Najimi M**, Sokal E. Liver cell transplantation. *Minerva Pediatr* 2005; **57**: 243-257



- 16 **Stephenne X**, Najimi M, Smets F, Reding R, de Ville de Goyet J, Sokal EM. Cryopreserved liver cell transplantation controls ornithine transcarbamylase deficient patient while awaiting liver transplantation. *Am J Transplant* 2005; **5**: 2058-2061
- 17 **Stephenne X**, Najimi M, Sibille C, Nassogne MC, Smets F, Sokal EM. Sustained engraftment and tissue enzyme activity after liver cell transplantation for argininosuccinate lyase deficiency. *Gastroenterology* 2006; **130**: 1317-1323
- 18 **Muraca M**, Gerunda G, Neri D, Vilei MT, Granato A, Feltracco P, Meroni M, Giron G, Burlina AB. Hepatocyte transplantation as a treatment for glycogen storage disease type 1a. *Lancet* 2002; **359**: 317-318
- 19 **Dhawan A**, Mitry RR, Hughes RD, Lehec S, Terry C, Bansal S, Arya R, Wade JJ, Verma A, Heaton ND, Rela M, Mieli-Vergani G. Hepatocyte transplantation for inherited factor VII deficiency. *Transplantation* 2004; **78**: 1812-1814
- 20 **Sokal EM**, Smets F, Bourgois A, Van Maldergem L, Buts JP, Reding R, Bernard Otte J, Evrard V, Latinne D, Vincent MF, Moser A, Soriano HE. Hepatocyte transplantation in a 4-year-old girl with peroxisomal biogenesis disease: technique, safety, and metabolic follow-up. *Transplantation* 2003; **76**: 735-738
- 21 **Cobourn CS**, Makowka L, Falk JA, Falk RE. Allogeneic intrasplenic hepatocyte transplantation in the Gunn rat using cyclosporine A immunosuppression. *Transplant Proc* 1987; **19**: 1002-1003
- 22 **Dixit V**, Darvasi R, Arthur M, Brezina M, Lewin K, Gitnick G. Restoration of liver function in Gunn rats without immunosuppression using transplanted microencapsulated hepatocytes. *Hepatology* 1990; **12**: 1342-1349
- 23 **te Velde AA**, Bosman DK, Oldenburg J, Sala M, Maas MA, Chamuleau RA. Three different hepatocyte transplantation techniques for enzyme deficiency disease and acute hepatic failure. *Artif Organs* 1992; **16**: 522-526
- 24 **Zhang H**, Miescher-Clemens E, Drugas G, Lee SM, Colombani P. Intrahepatic hepatocyte transplantation following subtotal hepatectomy in the recipient: a possible model in the treatment of hepatic enzyme deficiency. *J Pediatr Surg* 1992; **27**: 312-315; discussion 315-316
- 25 **Holzman MD**, Rozga J, Neuzil DF, Griffin D, Moscioni AD, Demetriou AA. Selective intraportal hepatocyte transplantation in albuminemic and Gunn rats. *Transplantation* 1993; **55**: 1213-1219
- 26 **Albani AP**, Campanati L, Arosio E, Gatti S, Gridelli B, Orsenigo R, Grizzi F, Doglia M, Fassati LR, Galmarini D. Hepatocyte injection in Gunn rats' thymus and spleen. *Transplant Proc* 1994; **26**: 3443-3445
- 27 **Kokudo N**, Otsu I, Okazaki T, Takahashi S, Sanjo K, Adachi Y, Makino S, Nozawa M. Long-term effects of intrasplenically transplanted adult hepatocytes and fetal liver in hyperbilirubinemic Gunn rats. *Transpl Int* 1995; **8**: 262-267
- 28 **Ilan Y**, Roy-Chowdhury N, Prakash R, Jona V, Attavar P, Guha C, Tada K, Roy-Chowdhury J. Massive repopulation of rat liver by transplantation of hepatocytes into specific lobes of the liver and ligation of portal vein branches to other lobes. *Transplantation* 1997; **64**: 8-13
- 29 **Guha C**, Parashar B, Deb NJ, Garg M, Gorla GR, Singh A, Roy-Chowdhury N, Vikram B, Roy-Chowdhury J. Normal hepatocytes correct serum bilirubin after repopulation of Gunn rat liver subjected to irradiation/partial resection. *Hepatology* 2002; **36**: 354-362
- 30 **Kim BH**, Han YS, Dong SH, Kim HJ, Chang YW, Lee JI, Chang R. Temporary amelioration of bilirubin conjugation defect in Gunn rats by transplanting conditionally immortalized hepatocytes. *J Gastroenterol Hepatol* 2002; **17**: 690-696
- 31 **Takahashi M**, Deb NJ, Kawashita Y, Lee SW, Furgueil J, Okuyama T, Roy-Chowdhury N, Vikram B, Roy-Chowdhury J, Guha C. A novel strategy for in vivo expansion of transplanted hepatocytes using preparative hepatic irradiation and FasL-induced hepatocellular apoptosis. *Gene Ther* 2003; **10**: 304-313
- 32 **Cubero FJ**, Maganto P, Mula N, Ortiz A, Barrutia MG, Codesal FJ, Arahuetes RM. Hepatic proliferation in Gunn rats transplanted with hepatocytes: effect of retrorsine and tri-iodothyronine. *Cell Prolif* 2005; **38**: 137-146
- 33 **Dhawan A**, Mitry RR, Hughes RD. Hepatocyte transplantation for liver-based metabolic disorders. *J Inherit Metab Dis* 2006; **29**: 431-435
- 34 **Ambrosino G**, Varotto S, Strom SC, Guariso G, Franchin E, Miotto D, Caenazzo L, Basso S, Carraro P, Valente ML, D'Amico D, Zancan L, D'Antiga L. Isolated hepatocyte transplantation for Crigler-Najjar syndrome type 1. *Cell Transplant* 2005; **14**: 151-157
- 35 **Crispe IN**, Giannandrea M, Klein I, John B, Sampson B, Wuensch S. Cellular and molecular mechanisms of liver tolerance. *Immunol Rev* 2006; **213**: 101-118
- 36 **Wu YM**, Joseph B, Gupta S. Immunosuppression using the mTOR inhibition mechanism affects replacement of rat liver with transplanted cells. *Hepatology* 2006; **44**: 410-419
- 37 **Serrano T**, Mitry RR, Terry C, Lehec SC, Dhawan A, Hughes RD. The effects of immunosuppressive agents on the function of human hepatocytes in vitro. *Cell Transplant* 2006; **15**: 777-783
- 38 **Mashalova EV**, Guha C, Roy-Chowdhury N, Liu L, Fox IJ, Roy-Chowdhury J, Horwitz MS. Prevention of hepatocyte allograft rejection in rats by transferring adenoviral early region 3 genes into donor cells. *Hepatology* 2007; **45**: 755-766
- 39 **Wesolowska A**, Olszewski WL, Durlik M. Transplantation of hepatocytes: elimination of recipient natural killer cells with irradiation and bone marrow reconstitution prevent early graft dysfunction. *Transplant Proc* 2003; **35**: 2358-2360
- 40 **Yoshida N**, Kawahara T, Futagawa S. Induction of donor-specific tolerance to allogeneic hepatocytes by allogeneic bone marrow transplantation. *Hepatol Res* 2003; **26**: 148-153
- 41 **Dixit V**, Darvasi R, Arthur M, Lewin K, Gitnick G. Cryopreserved microencapsulated hepatocytes--transplantation studies in Gunn rats. *Transplantation* 1993; **55**: 616-622
- 42 **Gomez N**, Balladur P, Calmus Y, Baudrimont M, Honiger J, Delelo R, Myara A, Crema E, Trivin F, Capeau J, Nordlinger B. Evidence for survival and metabolic activity of encapsulated xenogeneic hepatocytes transplanted without immunosuppression in Gunn rats. *Transplantation* 1997; **63**: 1718-1723
- 43 **Liu ZC**, Chang TM. Coencapsulation of hepatocytes and bone marrow stem cells: in vitro conversion of ammonia and in vivo lowering of bilirubin in hyperbilirubemia Gunn rats. *Int J Artif Organs* 2003; **26**: 491-497
- 44 **Le Blanc K**. Immunomodulatory effects of fetal and adult mesenchymal stem cells. *Cytotherapy* 2003; **5**: 485-489
- 45 **Le Blanc K**, Rasmusson I, Sundberg B, Gotherstrom C, Hassan M, Uzunel M, Ringden O. Treatment of severe acute graft-versus-host disease with third party haploidentical mesenchymal stem cells. *Lancet* 2004; **363**: 1439-1441
- 46 **Ding XM**, Xue WJ, Ji ZZ, Tian PX. Infusion of donor hepatic non-parenchymal cells prolongs survival of skin allografts in mice: role of microchimerism and IL-4. *Hepatobiliary Pancreat Dis Int* 2007; **6**: 34-37
- 47 **Herrera MB**, Bruno S, Buttiglieri S, Tetta C, Gatti S, Deregibus MC, Bussolati B, Camussi G. Isolation and characterization of a stem cell population from adult human liver. *Stem Cells* 2006; **24**: 2840-2850
- 48 **Najimi M**, Khuu DN, Lysy PA, Jazouli N, Abarca J, Sempoux C, Sokal EM. Adult-derived human liver mesenchymal-like cells as a potential progenitor reservoir of hepatocytes? *Cell Transplant* 2007; **16**: 717-728
- 49 **Stephenne X**, Vosters O, Najimi M, Beuneu C, Dung KN, Wijns W, Goldman M, Sokal EM. Tissue factor-dependent procoagulant activity of isolated human hepatocytes: relevance to liver cell transplantation. *Liver Transpl* 2007; **13**: 599-606
- 50 **Beuneu C**, Vosters O, Movahedi B, Rimmelink M, Salmon

- I, Pipeleers D, Pradier O, Goldman M, Verhasselt V. Human pancreatic duct cells exert tissue factor-dependent procoagulant activity: relevance to islet transplantation. *Diabetes* 2004; **53**: 1407-1411
- 51 **Beuneu C**, Vosters O, Ling Z, Pipeleers D, Pradier O, Goldman M, Verhasselt V. N-Acetylcysteine derivative inhibits procoagulant activity of human islet cells. *Diabetologia* 2007; **50**: 343-347
  - 52 **Jamal HZ**, Weglarz TC, Sandgren EP. Cryopreserved mouse hepatocytes retain regenerative capacity in vivo. *Gastroenterology* 2000; **118**: 390-394
  - 53 **David P**, Alexandre E, Chenard-Neu MP, Audet M, Wolf P, Jaeck D, Azimzadeh A, Richert L. Engraftment and function of freshly isolated and cryopreserved Sprague Dawley rat hepatocytes after intrasplenic transplantation in analbuminemic rats. *Transplant Proc* 2000; **32**: 2796-2797
  - 54 **David P**, Alexandre E, Audet M, Chenard-Neu MP, Wolf P, Jaeck D, Azimzadeh A, Richert L. Engraftment and albumin production of intrasplenically transplanted rat hepatocytes (Sprague-Dawley), freshly isolated versus cryopreserved, into Nagase analbuminemic rats (NAR). *Cell Transplant* 2001; **10**: 67-80
  - 55 **Fuller BJ**, Lewin J, Sage L. Ultrastructural assessment of cryopreserved hepatocytes after prolonged ectopic transplantation. *Transplantation* 1983; **35**: 15-18
  - 56 **Loven AD**, Olsen AK, Friis C, Andersen B. Phase I and II metabolism and carbohydrate metabolism in cultured cryopreserved porcine hepatocytes. *Chem Biol Interact* 2005; **155**: 21-30
  - 57 **Stephenn X**, Najimi M, Ngoc DK, Smets F, Hue L, Guigas B, Sokal EM. Cryopreservation of human hepatocytes alters the mitochondrial respiratory chain complex 1. *Cell Transplant* 2007; **16**: 409-419
  - 58 **Joseph B**, Kumaran V, Berishvili E, Bhargava KK, Palestro CJ, Gupta S. Monocrotaline promotes transplanted cell engraftment and advances liver repopulation in rats via liver conditioning. *Hepatology* 2006; **44**: 1411-1420
  - 59 **Kim KS**, Joseph B, Inada M, Gupta S. Regulation of hepatocyte engraftment and proliferation after cytotoxic drug-induced perturbation of the rat liver. *Transplantation* 2005; **80**: 653-659
  - 60 **Dagher I**, Boudechiche L, Branger J, Coulomb-Lhermine A, Parouchev A, Sentilhes L, Lin T, Groyer-Picard MT, Vons C, Hadchouel M, Pariente D, Andreoletti M, Franco D, Weber A. Efficient hepatocyte engraftment in a nonhuman primate model after partial portal vein embolization. *Transplantation* 2006; **82**: 1067-1073
  - 61 **Kedem A**, Perets A, Gamlieli-Bonshtein I, Dvir-Ginzberg M, Mizrahi S, Cohen S. Vascular endothelial growth factor-releasing scaffolds enhance vascularization and engraftment of hepatocytes transplanted on liver lobes. *Tissue Eng* 2005; **11**: 715-722
  - 62 **Shani-Peretz H**, Tsiperson V, Shoshani G, Veitzman E, Neufeld G, Baruch Y. HVEGF165 increases survival of transplanted hepatocytes within portal radicles: suggested mechanism for early cell engraftment. *Cell Transplant* 2005; **14**: 49-57
  - 63 **Nussler A**, Konig S, Ott M, Sokal E, Christ B, Thasler W, Brulport M, Gabelein G, Schormann W, Schulze M, Ellis E, Kraemer M, Nocken F, Fleig W, Manns M, Strom SC, Hengstler JG. Present status and perspectives of cell-based therapies for liver diseases. *J Hepatol* 2006; **45**: 144-159
  - 64 **Lysy PA**, Campard D, Smets F, Najimi M, Sokal EM. Stem cells for liver tissue repair: current knowledge and perspectives. *World J Gastroenterol* 2008; **14**: 864-875
  - 65 **Lysy PA**, Campard D, Smets F, Malaise J, Mourad M, Najimi M, Sokal EM. Persistence of a chimerical phenotype after hepatocyte differentiation of human bone marrow mesenchymal stem cells. *Cell Prolif* 2008; **41**: 36-58
  - 66 **Campard D**, Lysy PA, Najimi M, Sokal EM. Native umbilical cord matrix stem cells express hepatic markers and differentiate into hepatocyte-like cells. *Gastroenterology* 2008; **134**: 833-848
  - 67 **Fiegel HC**, Lange C, Kneser U, Lambrecht W, Zander AR, Rogiers X, Kluth D. Fetal and adult liver stem cells for liver regeneration and tissue engineering. *J Cell Mol Med* 2006; **10**: 577-587
  - 68 **Hengstler JG**, Brulport M, Schormann W, Bauer A, Hermes M, Nussler AK, Fandrich F, Ruhnke M, Ungefroren H, Griffin L, Bockamp E, Oesch F, von Mach MA. Generation of human hepatocytes by stem cell technology: definition of the hepatocyte. *Expert Opin Drug Metab Toxicol* 2005; **1**: 61-74
  - 69 **Grossman M**, Rader DJ, Muller DW, Kolansky DM, Kozarsky K, Clark BJ 3rd, Stein EA, Lupien PJ, Brewer HB Jr, Raper SE. A pilot study of ex vivo gene therapy for homozygous familial hypercholesterolaemia. *Nat Med* 1995; **1**: 1148-1154
  - 70 **Strom SC**, Fisher RA, Rubinstein WS, Barranger JA, Towbin RB, Charron M, Miele L, Pisarov LA, Dorko K, Thompson MT, Reyes J. Transplantation of human hepatocytes. *Transplant Proc* 1997; **29**: 2103-2106
  - 71 **Horslen SP**, McCowan TC, Goertzen TC, Warkentin PI, Cai HB, Strom SC, Fox IJ. Isolated hepatocyte transplantation in an infant with a severe urea cycle disorder. *Pediatrics* 2003; **111**: 1262-1267
  - 72 **Mitry RR**, Dhawan A, Hughes RD, Bansal S, Lehec S, Terry C, Heaton ND-, Karani JB, Mieli-Vergani G, Rela M. One liver, three recipients: segment IV from split-liver procedures as a source of hepatocytes for cell transplantation. *Transplantation* 2004; **77**: 1614-1616

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# Food-borne parasitic zoonosis: Distribution of trichinosis in Thailand

Natthawut Kaewpitoon, Soraya Jatesadapattaya Kaewpitoon, Prasit Pengsaa

Natthawut Kaewpitoon, Soraya Jatesadapattaya Kaewpitoon, Research and Diagnostic Center for Parasitic Infectious Diseases, Northeastern Region, College of Medicine and Public Health, Ubon Rajathane University, 85 Warinchamrap District, Ubonratchathani 34190, Thailand  
Prasit Pengsaa, College of Medicine and Public Health, Ubon Rajathane University, 85 Warinchamrap District, Ubonratchathani 34190, Thailand

**Author contributions:** Kaewpitoon N designed, reviewed, analyzed and wrote the paper; Kaewpitoon SJ reviewed and wrote the paper; Pengsaa P collected proof.

**Correspondence to:** Natthawut Kaewpitoon, Research and Diagnostic Center for Parasitic Infectious Diseases, Northeastern Region, College of Medicine and Public Health, Ubon Rajathane University, 85 Warinchamrap District, Ubonratchathani 34190, Thailand. [natthawut.k@ubu.ac.th](mailto:natthawut.k@ubu.ac.th)

Telephone: +66-4535-3909 Fax: +66-4535-3901

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## Abstract

Trichinosis is among the most common food-borne parasitic zoonoses in Thailand and many outbreaks are reported each year. This paper investigates the distribution of the disease in regions of north, north-east, central and south Thailand. Between the earliest recorded outbreak of trichinosis in Mae Hong Son Province in 1962 and 2006, there have been 135 outbreaks involving 7340 patients and 97 deaths in Thailand. The highest number of cases, 557, was recorded in 1983. Most infected patients were in the 35-44 year age group, and the disease occurred more frequently in men than women during 1962-2003, with no significant sex difference during 2004-2006. Outbreaks were most common in the northern areas, especially in rural areas where raw and under-cooked pork and/or wild animals are eaten. Human infections occur annually in northern Thailand during communal feasts celebrating the Thai New Year. Trichinosis causes have been reported every year, supporting the need for planning education programs.

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**Key words:** Food-born parasitic; Zoonosis; Trichinosis; Thailand

**Peer reviewer:** Yasuji Arase, MD, Department of Gastroen-

terology, Toranomon Hospital, 2-2-2 Toranomonminato-ku, Tokyo 105-8470, Japan

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## INTRODUCTION

Trichinosis is a parasitic disease of mammals caused by the nematode parasite *Trichinella spp.* It is an important zoonotic infection caused by humans eating raw or inadequately cooked meat of infected animals. Infection is most common in omnivores (horses, humans, pigs and rats) and carnivores (cats, dogs, and seals), and pigs and rodents play an important role in the epidemiology of the disease. The main source of infection in Thailand has been pigs, but wild boar, jackal and black bear have also been identified as sources of trichinosis<sup>[1]</sup>. Unlike other parasitic infections, trichinosis has been a major public health problem in Asia, including Korea and Thailand<sup>[2-4]</sup>.

Since 1962, more than 7300 infections and 97 deaths have occurred in Thailand in 135 outbreaks (morbidity rate 0.04 per 100 000 people); however, this figure may underestimate the actual number of cases<sup>[4]</sup>. Until recently, *T. spiralis*, *T. pseudospiralis* and *T. papuae* were the only human-infecting species in Thailand. *T. spiralis* was the causative agent of most early outbreaks of trichinosis<sup>[5]</sup>. More recently, *T. pseudospiralis* and *T. papuae* infections in some areas of Thailand have been reported<sup>[6,7]</sup>. The first outbreak of trichinosis in Thailand in 1962 involved 56 patients and resulted in 11 deaths in the Mae Sariang District, Mae Hong Son Province<sup>[5]</sup>. The highest annual number of hospital recorded trichinosis cases recorded in Thailand was 557 in 1983. Historically, most infections result from consumption of raw pork in the form of “lahb” and “nahm,” favorite dishes of north, Thailand<sup>[8]</sup>. The incidence of *T. spiralis* larvae in dog meat in the areas favoring dog meat consumption is a major public health problem in the future, the popular food of people in the northeast area. Srikitjakarn *et al* reported *T. spiralis* was found in 1.67% of 421 samples of dog meat in Tarae District, Sakonnakon Province<sup>[9]</sup>. Raw dog meat was a source of infection in Kaeng Khlo District, Chaiyaphum Province in December 1984<sup>[10]</sup>.

From the past to present, trichinosis is still reported every year in Thailand; therefore, this article reviews the literature on the distribution, prevalence and cause of trichinosis in Thailand, including the annual epidemiological surveillance, a report by the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand, 1971-2006. Morbidity is described by year, month of the year, age group, region and province.

## REPORT CASES OF TRICHINOSIS PER 100 000 PER CAPITA BY YEAR, THAILAND

The first outbreak of trichinosis in Thailand, in 1962, involved 56 patients resulting in 11 deaths<sup>[5]</sup>. In an April 1973 outbreak, 31 persons were involved, ranging from 9 to 72 years, and one adult female died<sup>[8]</sup>. In 1980, trichinosis was reported, the infection being caused by the consumption of wild squirrel<sup>[11]</sup>. An epidemic of trichinosis involving 177 patients and 13 deaths occurred in 1981<sup>[12]</sup>. The highest annual number of hospital recorded trichinosis cases was 557 in 1983<sup>[13]</sup>. This figure is considered an underestimation of the actual number of cases involved in the outbreaks. Khambooruang reported 118 discrete outbreaks of the disease involving 5400 patients and 95 deaths<sup>[14]</sup>. In 8 cases of childhood trichinosis reported<sup>[15]</sup>. An outbreak of trichinosis affecting 59 individuals resulting in one death occurred in during 1994-1995. This was the first report of an epidemic of human infection caused by *T. pseudospiralis*<sup>[6]</sup>. Takahashi *et al* (2000) reported 120 outbreaks from 1962 to 2000 involving nearly 6700 patients and 97 deaths<sup>[16]</sup>. Chotmongkol *et al* presented the progressive generalized muscle hypertrophy weakness for 3 mo with the 49-year-old man who infected with *T. spiralis*<sup>[17]</sup>. More than 7300 infections with 97 deaths occurred in about 130 outbreaks, since 1962<sup>[18]</sup>. Recently, *T. papuae* was identified as the etiological agent of trichinosis involving 19 people after eating raw wild pig<sup>[13]</sup>. More than 5 outbreaks were reported by the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand. Reported morbidity rate of trichinosis cases increased from 0.04 in 1997 to 0.57 in 1998. In 1999 and 2000, the number of reported morbidity rate decreased to 0.03 and 0.21 respectively. No cases were recorded in 2001, but then 0.46, 0.20 and 0.34 occurred in 2002, 2003 and 2004, respectively. In 2005 and 2006, 0.12 and 0.03 of morbidity rates were reported by the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health (Figure 1A). Morbidity rate of trichinosis cases during 1997, 1998, 1999, 2000, 2001, 2002, 2003, 2004, 2005 and 2006 were 61, 351, 16, 128, 0, 289, 126, 154, 73 and 19, respectively. More than 135 outbreaks have been reported totaling 7340 patients and 97 deaths. Most of cases were classified by occupation and place of treatment were agriculture and community hospital<sup>[18]</sup>.

## REPORT CASES OF TRICHINOSIS BY MONTH, THAILAND

The distribution of trichinosis was reported by month

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from 1962 and 2006, more frequently during October to January. Cases were reported most frequently in months during communal feasts celebrating the Thai New Year, though few of them have been documented. During 2002 to 2006, the number of reported trichinosis cases differed from previously recorded reports. In 2002, the highest of number of trichinosis cases was reported in January. The number of cases was decreased in 2003 overall, and February had the highest of reported trichinosis cases. In 2004, May and June were the months with the highest trichinosis cases, and in 2005, October was the highest trichinosis report month. The most recent data from 2006 shows that June is the month with the highest number of trichinosis cases (Figure 1B)<sup>[18]</sup>.

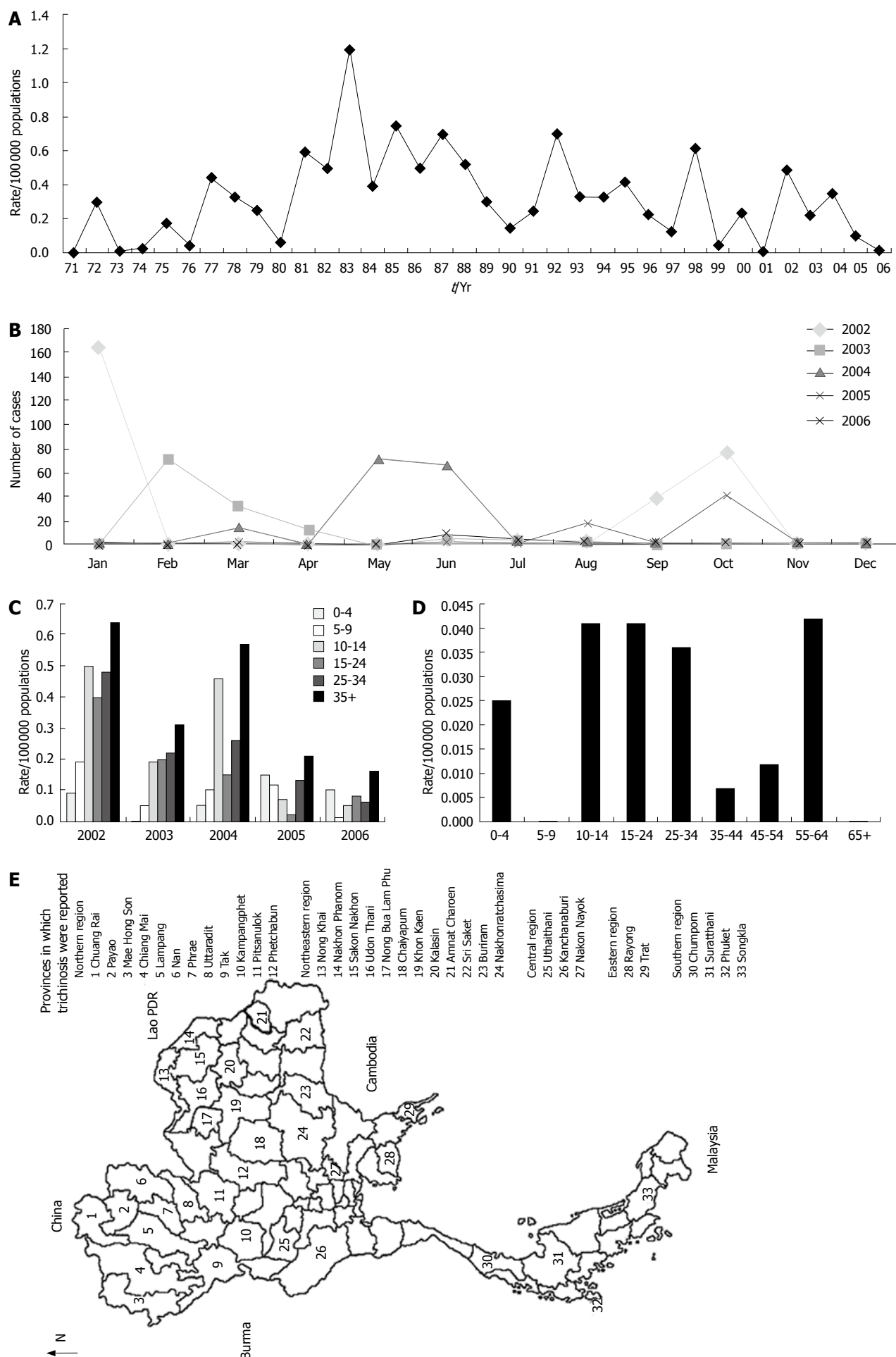
## REPORT CASES OF TRICHINOSIS PER 100 000 PER CAPITA BY AGE GROUP

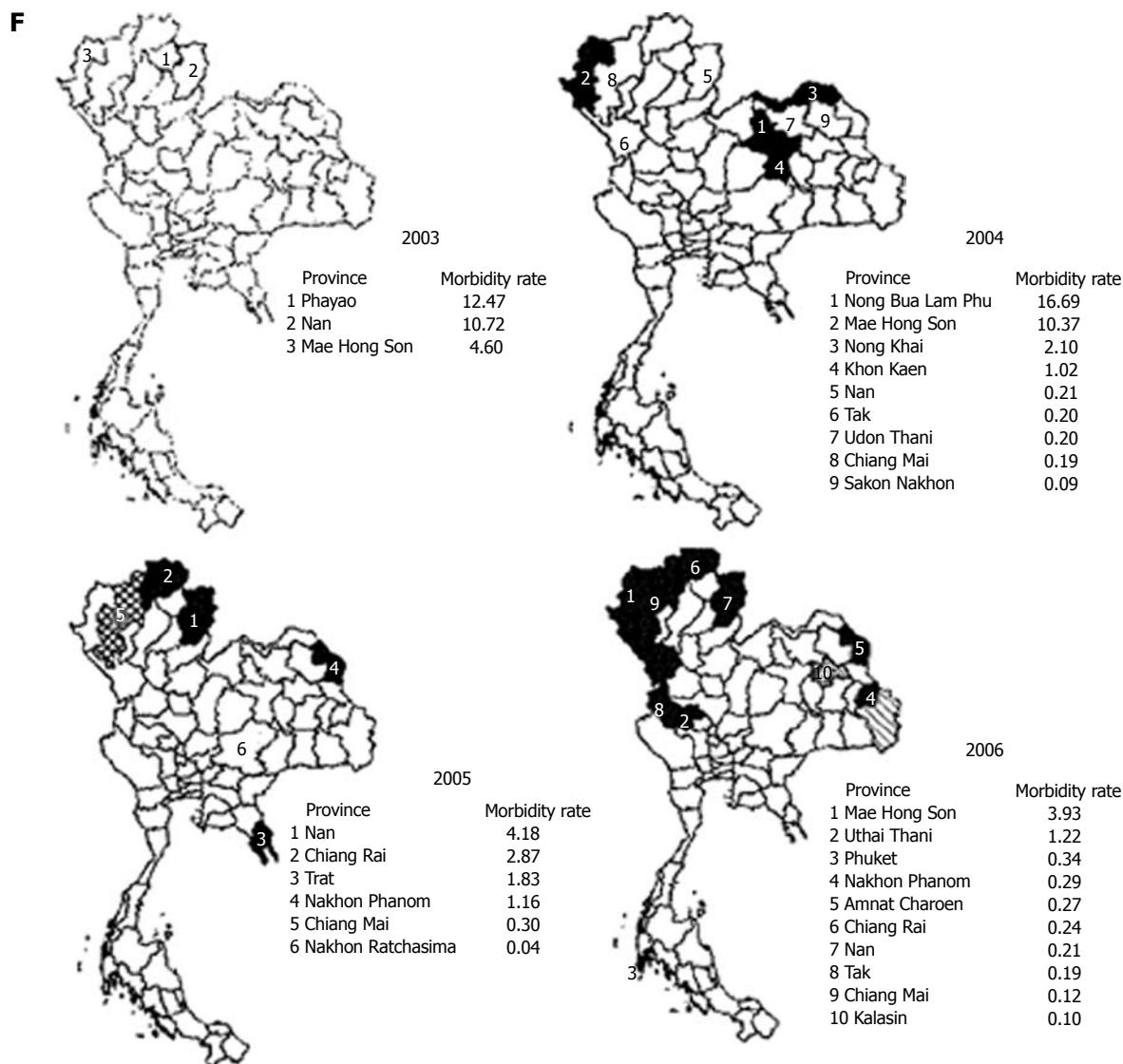
Previous research demonstrates variability in reports of trichinosis by age-group. Of 31 persons in an outbreak of trichinosis occurred in Chiang Rai Province, ranging from 9 to 72 years, one adult female died<sup>[8]</sup>. In Thailand, 8 cases of childhood trichinosis were reported in several studies<sup>[15]</sup>. Charkrit reported the youngest patient was about 1 year old<sup>[19]</sup>. Chotmongkol *et al* presented the progressive generalized muscle hypertrophy and weakness over a three-month period in a 49-year-old man infected with *T. spiralis*<sup>[17]</sup>. Kaewpitoon *et al* have reported that trichinosis cases are common in the 35-44 groups<sup>[4]</sup>. Since 2002, the distribution of human trichinosis cases by age groups has been collected by the annual epidemiological surveillance reports. Most patients are in the 35-44 groups, and many are over the age of 65. In 2002 and 2005, trichinosis cases were most frequently reported in the 35-44 groups, and this figure is very similar to previously reported research<sup>[4,17]</sup>. In 2006, the cases reported by the annual epidemiological surveillance varied considerably from reports of the past. Most, 66.67% patients were 15 to 44 years old. The youngest and oldest patients were 4 and 62 years old (Figure 1C). Reported cases of trichinosis per 100 000 people by age group in 2006, were similarly (0.036-0.042) across five age-groups as, that is, 10-14, 15-24, 25-34, 35-44 and 55-64 years old (Figure 1D). Infection occurs in men and women at the ratio of 1.7:1, 1:1.03, 1:1 and 1:1 in 2003, 2004, 2005 and 2006, respectively<sup>[18]</sup>.

## REPORT CASES OF TRICHINOSIS BY REGION, THAILAND

Trichinosis is more common in temperate than in tropical regions. The epidemiological surveillance reports of trichinosis have been conducted almost every year and data investigations reveal that outbreaks have occurred predominantly in rural areas. The annual epidemiological surveillance reports from 1962 to 2006 found consistently high numbers of cases in the north region. The north part of Thailand is responsible for 77.94% of all cases reported from 2002 to 2006. The northeast region was responsible for 21.61% of cases







**Figure 1** Reported cases of trichinosis in Thailand. **A:** Morbidity rate (per 100 000 populations) of trichinosis cases was reported by the Bureau of Epidemiology, Department of Disease Control, Ministry of Public Health, 1971-2006; **B:** Reported by month, 2002-2006; **C:** Reported per 100 000 populations by age-group, 2000-2006; **D:** Reported per 100 000 populations by age-group, 2006; **E:** Distribution by region during 1962-2006; **F:** Reported per 100 000 populations by Province, 2004-2006.

in 2004. In 2004, there were 124 reported cases in the northeast, representing the first time that a region other than the north had the highest number of cases. These results showed that trichinosis is a serious problem, particularly in the north and northeast regions of Thailand. Only small numbers of trichinosis cases were recorded in the central and south regions in 2005. The numbers of cases in other parts of Thailand were very few. In the central region, the infection rate was 0.3% of the total number of cases; the south region accounted for only 0.15% of cases. In 2006, most reported cases occurred in the north region (Figure 1E)<sup>[18]</sup>.

## REPORT CASES OF TRICHINOSIS PER 100 000 PEOPLE, BY PROVINCE

Most outbreaks that occurred during 1962 to 2006 were located in the north region. The most severely affected

areas in the north region were the highland provinces of Chiang Rai, Nan, Chiang Mai, Mae Hong, Son Payao, Lampang, Phrae, Uttaradit, Pitsanuloke, Phetchabun, Tak and Kamphangphet<sup>[8,12,19-21]</sup>. The first outbreak of trichinosis occurred in the Mae Sariang District, Mae Hong Son Province<sup>[5]</sup>. The second outbreak was in 1963 at Prao District, Chiang Mai Province. In April 1973, an outbreak of trichinosis occurred in Mae Sruay District, Chiang Rai Province. 31 persons were involved, and 1 adult female died. All trichinosis cases included a history of having consumed raw pork in the form of “lahb” and “nahm,” favorite dishes of north Thailand<sup>[8]</sup>. An epidemic of trichinosis involving 177 patients resulted in 13 deaths in Kok-Ta-Back Village, Nong-Pai District, Petchabun Province<sup>[12]</sup>. Takahashi *et al* (2000) reported 120 outbreaks from 1962 to 2000 involving nearly 6700 patients and 97 deaths. The highest numbers of cases were in Chiang Mai, Chiang Rai and Nan provinces, 1776, 1739 and 894 respectively. The numbers of cases

in other parts of Thailand were very few. The northeast region was responsible for the highest number of cases in some years mainly in 2004. Provinces involved were Non Bua Lam Phu, Nong Khai, Udon Thani, Sakon Nakhon, Nakhon Phanom, Amnat Charoen, Kalasin, Khon kaen, Chaiyapum, Nakhonratchasima, Buriram and Sri Saket. In the central region, Uthaithani, Kanchanaburi, Nakhon Nayok, Rayong and Trat provinces have been reported the number of trichinosis cases. In 1994, in the south of Thailand, an outbreak of trichinosis affecting 59 individuals resulted in 1 death in Chumphon Province. This was the first report of an epidemic of human infection caused by *T. pseudospiralis*<sup>[6]</sup>. At this time, Chumphon, Suratthani, Songkla and Phuket were the only provinces of the south region in which cases of trichinosis were observed. This is interesting because Phuket province is a popular destination for Thai and foreigner travelers.

The reported cases of trichinosis per 100 000 populations by province during 2003 to 2006 were classified by the bureau of the Epidemiology, Department of Disease Control, Ministry of Public Health, Thailand. In 2003, most trichinosis cases were reported from Payao, Nan and Mae Hong Son province, morbidity rate were 12.47, 10.72 and 4.60, respectively. The provinces with the highest rates of trichinosis cases in 2004 were Nong Bua Lam Phu, Mae Hong Son, Nong Khai, Khon Kaen, Nan, Tak, Udon Thani, Chiang Mai and Sakon Nakhon, 16.69, 10.37, 2.10, 1.02, 0.21, 0.20, 0.20, 0.19 and 0.09, respectively. Meanwhile, 2005 was comprised 6 provinces have been shown that the highest morbidity rate were Nan, Chiang Rai, Trat, Nakhon Phanom, Chiang Mai and Nakhon Ratchasima as 4.18 2.87 1.83 1.16 0.30 and 0.04, respectively. Recent, Mae Hong Son, Uthai Thani, Phuket, Nakhon Phanom, Amnat Charoen, Chiang Rai, Nan, Tak, Chiang Mai and Kalasin as 3.93, 1.22, 0.34, 0.29, 0.27, 0.24, 0.21, 0.19, 0.12 and 0.10, were the top ten leading rate of trichinosis cases in 2006 (Figure 1F)<sup>[18]</sup>.

## CONCLUSION

Studies of trichinosis in Thailand, since the first recorded outbreak in 1962 until 2006, show that most outbreaks occurred in the north of the country, an area in which popular, traditional dishes involve meat from pigs and wild boars, often eaten raw or under-cooked. Overall, more than 135 outbreaks have been reported during 1962-2006 involving 7340 patients and 97 deaths. Trichinosis is still a serious problem food-borne parasitic zoonoses in Thailand. No vaccines have been developed. Treatment exists for Trichinosis in humans if diagnosed promptly. Better prevention and control of trichinosis requires health education programs to improve the knowledge, attitude and behavior of people in the high-risk areas.

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## REFERENCES

- 1 **Suriyanon V**, Klunklin K. Human trichinosis: analysis of cases during the tenth outbreak in North Thailand. *Southeast Asian J Trop Med Public Health* 1972; **3**: 390-396
- 2 **Yamaguchi T**. Present status of trichinellosis in Japan. *Southeast Asian J Trop Med Public Health* 1991; **22** Suppl: 295-301
- 3 **Sohn WM**, Kim HM, Chung DI, Yee ST. The first human case of *Trichinella spiralis* infection in Korea. *Korean J Parasitol* 2000; **38**: 111-115
- 4 **Kaewpitoon N**, Kaewpitoon SJ, Philasri C, Leksomboon R, Maneenin C, Sirilaph S, Pengsaa P. Trichinosis: epidemiology in Thailand. *World J Gastroenterol* 2006; **12**: 6440-6445
- 5 **Boonthanom P**, Nawarat A. The outbreaks of trichinosis at Amphur Mae Sarialng. *Bull Pub Health* 1963; **33**: 301-308
- 6 **Jongwutiwes S**, Chantachum N, Kraivichian P, Siriyasatien P, Putaporntip C, Tamburrini A, La Rosa G, Sreesunpasirikul C, Yingyoud P, Pozio E. First outbreak of human trichinellosis caused by *Trichinella pseudospiralis*. *Clin Infect Dis* 1998; **26**: 111-115
- 7 **Kusolsuk T**, Khumjui C, Dekumyoy P, Thaengkun U, Sanguankait S, Maipanich W, Nuamtanong S, Pubampen S, Yoonuan T, Thonghong A, Waikagul J. *Trichinella papuae* in Ban-Rai District, Uthaithani Province, Thailand. In: Proceedings of the 5th Food and Waterborne Parasitic Zoonosis, Bangkok, Thailand, 2006: 28-30
- 8 **Khamboonruang C**, Nateewatana N. Trichinosis: A recent outbreak in Northern Thailand. *Southeast Asian J Trop Med Public Health* 1975; **6**: 74-78
- 9 **Srikitjakarn L**, Korakovit M, Toboran P, Sutaravong V, Bettermann G, Lingelback W. *Trichinella spiralis* in dog meat for human consumption in Sakon Nakon province. *Thai Vet Med Assoc* 1981; **32**: 271-277
- 10 **Chitchang S**, Vongmek V, Leelasupasri S. Trichinosis for dog meat at Changwat Chaiyaphum. *Royal Thai Army Med J* 1985; **38**: 305-309
- 11 **Wiwatanaworapant T**. Trichinosis: a case report. *Siriraj Hosp Gaz* 1980; **32**: 220-222
- 12 **Chalermchaikit T**, Nawarat A, Muangyai M, Brahmasa R, Chumkasian P. Epidemiological surveillance of trichinosis outbreak in Petchaboon province. *Thai J Vet Med* 1982; **12**: 1-23
- 13 **Morakote N**, Khamboonruang C, Siriprasert V, Suphawitayanukul S, Marcanantachoti S, Thamasonthi W. The value of enzyme-linked immunosorbent assay (ELISA) for diagnosis of human trichinosis. *Trop Med Parasitol* 1991; **42**: 172-174
- 14 **Khamboonruang C**. The present status of trichinellosis in Thailand. *Southeast Asian J Trop Med Public Health* 1991; **22** Suppl: 312-315
- 15 **Limsuwan S**, Thisyakorn U. Trichinosis in children, report of 8 cases. *Thai J Pediatrics* 1993; **32**: 265-270
- 16 **Takahashi Y**, Mingyuan L, Waikagul J. Epidemiology of trichinellosis in Asia and the Pacific Rim. *Vet Parasitol* 2000; **93**: 227-239
- 17 **Chotmongkol V**, Intapan PM, Koonmee S, Kularbkaew C, Aungaree T. Case report: acquired progressive muscular hypertrophy and trichinosis. *Am J Trop Med Hyg* 2005; **72**: 649-650
- 18 **Bureau of Epidemiology**. Trichinosis in Thailand. Annual Epidemiological Surveillance Report. Department of Disease Control, Ministry of Public Health, Thailand 1971-2006
- 19 **Charkrit S**. Study on clinical manifestations of trichinosis in Payao province. *Com Dis J* 1998; **24**: 242-247
- 20 **Limsuwan S**, Siriprasert V. A clinical study on trichinosis in Changwat Phayao, Thailand. *Southeast Asian J Trop Med Public Health* 1994; **25**: 305-308
- 21 **Pinanong M**, Tongma S, Hongphinyo V. Human trichinosis: an outbreak at Petchaboon province. *Royal Thai Air Force Med Gaz* 1985; **31**: 87-94



## TOPIC HIGHLIGHT

Simon D Taylor-Robinson, MD, Series Editor

# Non-invasive means of measuring hepatic fat content

Sanjeev R Mehta, E Louise Thomas, Jimmy D Bell, Desmond G Johnston, Simon D Taylor-Robinson

Sanjeev R Mehta, Desmond G Johnston, Simon D Taylor-Robinson, Division of Medicine, Imperial College London, St Mary's Campus, Praed Street, London W2 1NY, United Kingdom

E Louise Thomas, Jimmy D Bell, Imaging Sciences Department, Division of Clinical Sciences, Imperial College London, Hammersmith Campus, Du Cane Road, London W12 0HS, United Kingdom

Supported by Grants from the Novo Nordisk UK Research Foundation (supporting S.R.M), Pfizer Global Research and Development (Sandwich, UK), the British Medical Research Council and the United Kingdom Department of Health Research and Development Initiative

Correspondence to: Dr. Sanjeev R Mehta, Department of Metabolic Medicine, Division of Medicine, Imperial College London, St Mary's Campus, 2nd Floor, Mint Wing, St Mary's Hospital, Praed Street, London W2 1NY, United Kingdom. [s.mehta@imperial.ac.uk](mailto:s.mehta@imperial.ac.uk)

Telephone: +44-207-8866120 Fax: +44-207-8861790

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the quantification of intrahepatocellular lipid (IHCL) levels. Both techniques will be useful tools in future longitudinal clinical studies, either in examining the natural history of conditions causing hepatic steatosis (e.g. non-alcoholic fatty liver disease), or in testing new treatments for these conditions.

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**Key words:** Adipose tissue; Ectopic fat; Hepatic fat; Insulin resistance; Ultrasound; Computerized tomography; Magnetic resonance imaging; Magnetic resonance spectroscopy

**Peer reviewers:** Dr. Markus Reiser, Professor, Gastroenterology-Hepatology, Ruhr-University Bochum, Bürkle-de-la-Camp-Platz 1, Bochum 44789, Germany; Stefan Georg Hübscher, MD, Professor, Department of Pathology, University of Birmingham, Birmingham B15 2TT, United Kingdom

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## Abstract

Hepatic steatosis affects 20% to 30% of the general adult population in the western world. Currently, the technique of choice for determining hepatic fat deposition and the stage of fibrosis is liver biopsy. However, it is an invasive procedure and its use is limited, particularly in children. It may also be subject to sampling error. Non-invasive techniques such as ultrasound, Computerized tomography (CT), magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy ( $^1\text{H}$  MRS) can detect hepatic steatosis, but currently cannot distinguish between simple steatosis and steatohepatitis, or stage the degree of fibrosis accurately. Ultrasound is widely used to detect hepatic steatosis, but its sensitivity is reduced in the morbidly obese and also in those with small amounts of fatty infiltration. It has been used to grade hepatic fat content, but this is subjective. CT can detect hepatic steatosis, but exposes subjects to ionizing radiation, thus limiting its use in longitudinal studies and in children. Recently, magnetic resonance (MR) techniques using chemical shift imaging have provided a quantitative assessment of the degree of hepatic fatty infiltration, which correlates well with liver biopsy results in the same patients. Similarly, *in vivo*  $^1\text{H}$  MRS is a fast, safe, non-invasive method for

## INTRODUCTION

In humans, adipose tissue is an important “energy bank” in which excess energy is stored and then released to meet the energy needs of the body. In the fed state and during periods of excess calorie intake, the excess energy is stored within adipose tissue as triglycerides. In the fasting state and during starvation, triglycerides within adipose tissue can be rapidly broken down by hormone-sensitive lipase to generate fatty acids. Oxidation of fatty acids releases more energy than that of carbohydrate, protein or triglycerides. Fatty acids are thus the most efficient “fuel” to meet the body's energy needs<sup>[1]</sup>.

In addition to playing an important role in energy homeostasis, adipose tissue is now well recognised as an endocrine organ<sup>[2,3]</sup>. It is known to produce and secrete a wide variety of bioactive peptides, known as adipokines. These include proteins, such as leptin, adiponectin, resistin, as well as the recently described visfatin and retinol binding protein 4<sup>[4-8]</sup>. In addition, proinflammatory cytokines, such as tumour necrosis factor alpha and interleukin-6, and acute phase reactants,



such as C-reactive protein are also secreted by the adipocyte. All of these adipokines may act at both a local (paracrine) and systemic (endocrine) level and contribute to the development of obesity-related disorders, such as insulin resistance, Type 2 diabetes and cardiovascular disease<sup>[2,3]</sup>.

Adipose tissue stores may become saturated, either due to failure to develop adequate adipose tissue mass (lipodystrophy), or to expand these stores sufficiently to accommodate increased energy intake. When this occurs, lipid starts to accumulate in non-adipose cells. Ectopic storage of lipids in organs, such as the liver, skeletal muscle and pancreas (the “lean body mass”) is thought to play a critical role in the development of insulin resistance and Type 2 diabetes<sup>[9]</sup>. The accumulation of ectopic fat has been shown to affect both renal and cardiovascular function and may contribute to the development of cardiovascular disease<sup>[10]</sup>.

Evidence to support the hypothesis that ectopic fat accumulation plays a crucial role in insulin resistance comes from the study of subjects with lipodystrophies. These subjects have insufficient adipose tissue mass and hence store excess energy as triglycerides within organs, such as liver and skeletal muscle. As a result, individuals may develop insulin resistance and are at increased risk of developing Type 2 diabetes<sup>[11-14]</sup>.

## RELEVANCE OF HEPATIC FAT ACCUMULATION

Fatty infiltration of the liver is not a new phenomenon. However, until the last few decades, fatty liver, particularly that associated with insulin resistance rather than alcohol excess—“non-alcoholic fatty liver disease”, was considered to be a relatively benign condition. This notion was challenged when several reports documented the development of liver failure in some patients following jejunal bypass operations for morbid obesity<sup>[15-17]</sup>. The liver histology in such patients was indistinguishable from that seen in alcoholic steatohepatitis<sup>[18]</sup>. Similar hepatic lesions were subsequently described in obese patients who had neither abused alcohol nor undergone bariatric surgery<sup>[19-21]</sup> and in patients with diabetes mellitus<sup>[22-24]</sup>. In 1980, Ludwig and colleagues introduced the term “non-alcoholic steatohepatitis” (NASH) to describe these histological findings in patients who did not consume alcohol<sup>[25]</sup>. A variety of other terms have been used to describe this entity with non-alcoholic fatty liver disease now being the preferred term.

Accumulation of fat within the liver is of particular importance in that, over time, it may lead to the development of steatohepatitis and ultimately cirrhosis, end-stage liver failure and hepatocellular carcinoma<sup>[26-29]</sup>.

## MECHANISMS OF HEPATIC FAT ACCUMULATION

The mechanisms leading to hepatic fat accumulation

remain poorly understood. The liver synthesizes triglycerides from free fatty acids. Free fatty acids are derived from lipolysis of triglycerides within adipose tissue, diet or *de novo* lipogenesis<sup>[1]</sup>. Once taken up by the liver, free fatty acids can either be oxidised in the mitochondria to form adenosine triphosphate (ATP), or esterified to produce triglycerides for storage, or incorporated into very low density lipoprotein (VLDL) particles. Triglycerides accumulate in the liver when their synthesis exceeds their export *via* VLDL<sup>[1,30,31]</sup>.

The development of non-invasive techniques for assessing hepatic fat content *in vivo* in humans has renewed interest in studying the mechanisms leading to hepatic fat accumulation. Ultrasound, computerized tomography (CT), magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy (<sup>1</sup>H MRS) have all played a prominent role in this.

## METHODS TO STUDY HEPATIC FAT

Currently, the gold standard for determining hepatic fat severity and morphology is a liver biopsy<sup>[31,32]</sup>. However, this procedure has several drawbacks, including discomfort, owing to its invasive nature, risk of infection, haematoma formation, or more significant internal bleeding, and biliary leakage. Furthermore, biopsies are subject to sampling error, because less than 1/50000th of the liver is available for histological analysis.

Non-invasive imaging techniques, such as ultrasound, Computerized tomography (CT), magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy can detect fatty infiltration of the liver, but unlike liver biopsy, they are limited in their ability to detect coexisting inflammation or fibrosis.

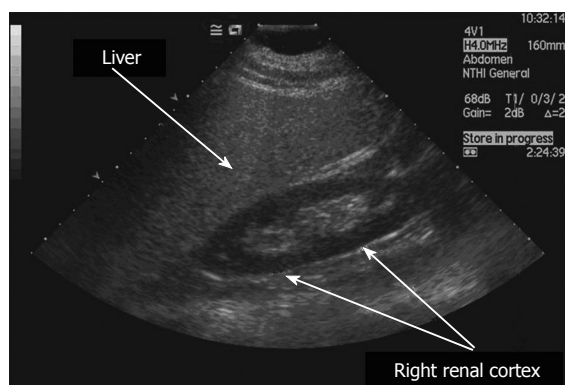
## NON-INVASIVE TECHNIQUES FOR ASSESSING HEPATIC FAT CONTENT

### Ultrasound

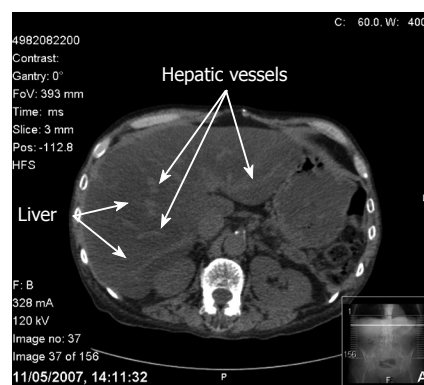
Hepatic ultrasound is a simple, non-invasive technique, which is widely used in clinical practice to detect fatty infiltration of the liver. As shown in Figure 1, hepatic steatosis causes increased echogenicity on ultrasound, so that liver appears brighter than the cortex of the ipsilateral kidney<sup>[33,34]</sup>.

Diffuse hepatic steatosis and diffuse fibrosis can have similar sonographic appearances and therefore it can sometimes be difficult to distinguish between them. Some groups have used the term “fatty fibrotic pattern” to describe this pattern of increased echogenicity, although the echo shadows tend to be coarser in the presence of pure fibrosis<sup>[35,36]</sup>.

In some cases, fatty infiltration of the liver may be patchy, rather than diffuse, in distribution. The non-uniformity may be so marked that the fat is deposited in one well-circumscribed region (focal fatty infiltration) or, alternatively, discrete areas of liver parenchyma remain uninvolved when the remainder of the liver is diffusely infiltrated with fat (focal fatty sparing). Both



**Figure 1** Ultrasound findings in hepatic steatosis. The steatotic liver is hyper-echoic, compared to the cortex of the right kidney. In addition, there is posterior attenuation of the ultrasound beam and reduced definition of the portal vein walls.



**Figure 2** CT findings in hepatic steatosis. In this contrast unenhanced scan, the liver appears darker than the spleen and the hepatic vessels appear bright. The increased brightness of the vessels relative to the liver parenchyma may erroneously suggest the use of contrast.

conditions may create diagnostic problems for the radiologist. For example, with focal fatty sparing, if the increased echogenicity of the majority of the liver is not appreciated, then the area of normal hepatic parenchyma may be misinterpreted as a pathological hypoechoic lesion<sup>[37]</sup>. If, however, the increased echogenicity of the remainder of the liver is appreciated, then the area of normal hepatic parenchyma may be helpful in making the diagnosis of fatty change<sup>[38]</sup>.

Several studies have assessed the sensitivity and specificity of ultrasound for detecting hepatic steatosis. In these, the sensitivity ranged from 60% to 94% and the specificity from 84% to 95%, respectively<sup>[35,39-41]</sup>. Another study combined fatty change with fibrosis and obtained a sensitivity of 98.7% and specificity of 94% for the “fatty fibrotic pattern”<sup>[42]</sup>. The sensitivity of ultrasound increases with increasing degrees of fatty infiltration. For example, in the presence of hepatic fat content of 10% to 19%, it has a sensitivity of 55%, which rises to 80% in the presence of > 30% fatty infiltration<sup>[43]</sup>. However, in the presence of morbid obesity (defined by a body mass index > 40 kg/m<sup>2</sup>), the sensitivity and specificity of ultrasound fall to 49% and 75%, respectively, possibly due to technical problems in performing ultrasound in such patients<sup>[44]</sup>.

Whereas ultrasound is a useful technique for detecting hepatic steatosis, particularly in severe cases, it is unable to provide a precise determination of hepatic fat content. Grading of hepatic fat content into broad categories (mild, moderate and severe steatosis) has been reported, using diagnostic criteria, based upon the visual assessment of hepatic echogenicity<sup>[35,41,42,45,46]</sup>. However, all of the above studies found the grading of hepatic fat content using ultrasound to be somewhat subjective. In addition, the most recent ones showed that ultrasound is very poor at discriminating small changes in hepatic fat content. For example, Fishbein and colleagues suggested from their study that an individual with hepatic steatosis undergoing a reduction of MRI hepatic fat fraction from 40% to 20% through successful intervention, would be unlikely to have a corresponding alteration in ultrasound appearance<sup>[45]</sup>.

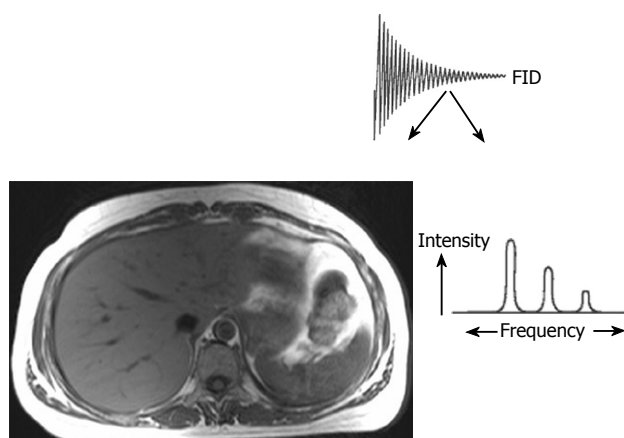
The operator dependency of ultrasound, its inability to precisely quantify hepatic fat content, and its inability to detect small changes in liver fat with time, all potentially limit its use in longitudinal clinical studies.

### Computerized tomography

Contrast-unenhanced Computerized tomography (CT) is the most accurate CT technique used to detect and characterise hepatic steatosis<sup>[47]</sup>. The CT diagnosis of hepatic steatosis is made by measuring the difference in liver and spleen attenuation values in Hounsfield units<sup>[48]</sup>. In individuals without hepatic steatosis, the mean attenuation value for the liver is at least 4 Hounsfield units greater than for the spleen<sup>[48]</sup>. However, in subjects with hepatic steatosis, the mean attenuation value for the liver is lower than that for the spleen, so the liver appears darker than the spleen, rather than brighter. This lower attenuation of the liver in hepatic steatosis is thought to be secondary to the accumulation of lipids (triglycerides and cholesterol) within the hepatocytes<sup>[49]</sup>. With severe hepatic steatosis, there is more marked contrast between the liver and intrahepatic vessels. The increased brightness of the vessels relative to the liver parenchyma may erroneously suggest the use of contrast (Figure 2)<sup>[36]</sup>.

Great care should be taken in diagnosing fatty infiltration of the liver on contrast-enhanced CT scans because contrast injection rate and timing of measurements can significantly influence the optimal liver-minus-spleen attenuation difference for diagnosing fatty liver<sup>[50,51]</sup>. It has been suggested that muscle, rather than spleen, may be a better qualitative standard of reference for diagnosing fatty liver on contrast-enhanced CT, and that fatty liver can be diagnosed if the liver has a lower attenuation value than muscle<sup>[52]</sup>. However, such a comparison works only if the degree of fatty infiltration is severe.

Although non-contrast-enhanced CT is very good for the qualitative diagnosis of macrovesicular steatosis of 30% or greater, there is conflicting evidence as to whether or not it can accurately quantify hepatic fat content. Some studies have demonstrated that

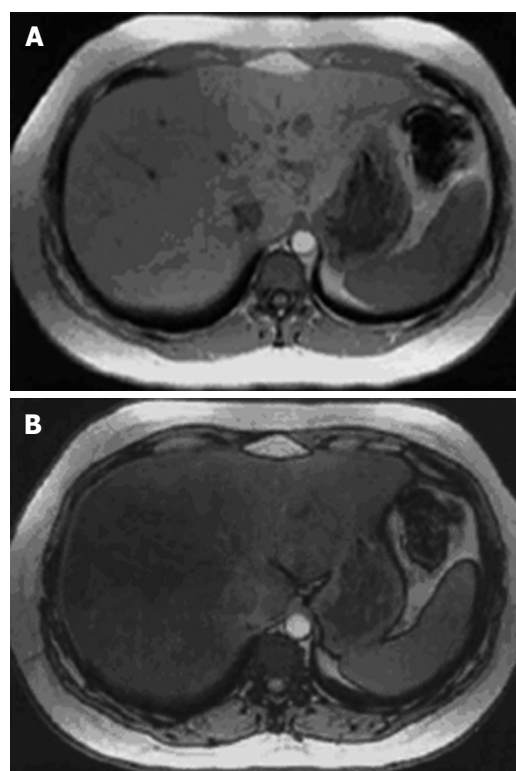


**Figure 3** Principles of magnetic resonance. The magnetic resonance (MR) signal or free induction decay (FID) may be converted by the mathematical process of Fourier transformation to form anatomical information (MR imaging) or localised biochemical information (MR spectroscopy). Modified from Taylor-Robinson SD Applications of magnetic resonance spectroscopy to chronic liver disease. *Clin Med* 2001; 1: 54-60 Copyright © 2001 Royal College of Physicians. Adapted by permission.

both the CT numbers for the liver and the ratio of CT numbers for the liver and the spleen show a good inverse correlation with the degree of steatosis seen on liver biopsy<sup>[47,53,54]</sup>. Nevertheless, it should be noted that a more recent study by Park and colleagues concluded that the diagnostic performance of unenhanced CT for quantitative assessment of macrovesicular steatosis is not clinically acceptable<sup>[55]</sup>. In addition, CT scanning has the drawback of exposing subjects to ionizing radiation. The above two factors limit its potential use in longitudinal studies and in children.

### **Magnetic resonance imaging and proton magnetic resonance spectroscopy**

**Principles of magnetic resonance imaging and spectroscopy:** The nuclear magnetic resonance (NMR) phenomenon was first reported by Bloch *et al* in 1946<sup>[56]</sup>. NMR techniques exploit the behaviour of certain atomic nuclei in an externally applied magnetic field. Magnetic resonance sensitive nuclei, such as hydrogen-1 ( $^1\text{H}$ ), carbon-13 ( $^{13}\text{C}$ ), nitrogen-15 ( $^{15}\text{N}$ ) and phosphorous-31 ( $^{31}\text{P}$ ) possess the quantum mechanical property of “spin”, a source of angular momentum intrinsic to nuclei with an odd mass number. When placed in a magnetic field they behave like magnetic dipoles, aligning parallel to or against the axis of the applied static magnetic field. When excited by irradiation with non-ionizing radiofrequency (rf) energy, this alignment of the nuclei is disturbed. During relaxation following excitation, the nuclei return to their original orientation, giving off a radiofrequency signal, which may then be detected by a receiver coil. This signal, known as the free induction decay (FID), can be resolved by a computer-based mathematical process known as Fourier transformation into either an image, providing anatomical information (MRI) or a frequency spectrum, providing biochemical information (MRS)<sup>[57,58]</sup>, as shown in Figure 3.



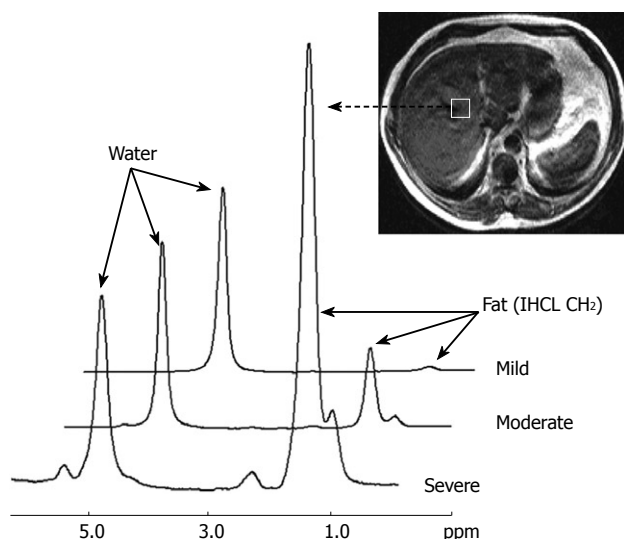
**Figure 4** In and opposed phase MR images of a liver illustrating the signal drop-off from an in-phase (A) to an opposed-phase image (B) in a patient with marked steatosis. Reproduced from Rinella *et al.* *Liver Transplantation* 2003; 9: 851-856. Copyright (2003) American Association for the Study of Liver Diseases. Reprinted with permission of Wiley-Liss, Inc., a subsidiary of John Wiley & Sons, Inc.

## **MAGNETIC RESONANCE IMAGING**

Nuclei from individual metabolites resonate at a given, but unique frequency, depending on the molecular structure of each compound. This is known as chemical shift and occurs because nuclei in different chemical environments experience slightly different magnetic field strengths. This variation in magnetic field strength results from the intrinsic “shielding” offered by the nearby electrons, which partially counteract the force of the main magnetic field. For example, the hydrogen nuclei in water (O-H bond) have less surrounding electrons than the hydrogen nuclei in lipid (C-H bond), so experience a slightly stronger magnetic field and, as a result, rotate at a slightly higher resonance frequency. The differences are extremely small and are typically measured using the dimensionless unit, parts per million (ppm)<sup>[59]</sup>.

Chemical shift MR imaging utilises this difference in resonance frequency of water and lipid to differentiate tissues containing only water from those containing both water and lipid<sup>[60]</sup>, as illustrated in Figure 4.

Applying this principle, Dixon developed a modified spin echo technique, now widely known as the Dixon method<sup>[61]</sup>. Using this technique, Lee and co-workers scanned the livers of five humans, two healthy volunteers with no evidence of liver disease and three patients with CT evidence of fatty infiltration of the liver<sup>[62]</sup>. In the subjects with CT evidence of hepatic fatty infiltration, there was a notable loss of signal



**Figure 5** Proton magnetic resonance spectra from three volunteers showing progressive degrees of hepatic fatty infiltration. Resonances from water and lipid (IHCL CH<sub>2</sub>) can be clearly seen. For each individual hepatic fatty infiltration was quantified using the equation: -Percentage fat = IHCL CH<sub>2</sub> peak area/Water peak area × 100. Shown are spectra from a liver with very mild fatty infiltration (1.0%), a liver with moderate fatty infiltration (10.2%), and a liver with severe fatty infiltration (74.9%). Adapted from Thomas *et al.* *Gut* 2005; 54: 122-127, with permission from the BMJ Publishing Group.

intensity on the out-of-phase images (because opposing signals from water and lipid tended to cancel each other out), and a less appreciable increase in signal intensity on the in-phase images. In contrast, in the healthy volunteers without hepatic steatosis, the signal intensity of the liver parenchyma was not altered between in-phase and out-of-phase images. They concluded that the Dixon method was capable of distinguishing fatty liver from normal liver, and that it might also be useful for the quantification of liver fat. Using the same technique, Heiken and colleagues scanned the livers of 35 subjects (12 healthy volunteers and 23 patients with CT evidence of fatty infiltration of the liver)<sup>[63]</sup>. They demonstrated the Dixon method to be a clinically useful technique for detecting and quantifying fatty infiltration of the liver and for differentiating non-uniform fatty infiltration from liver metastases<sup>[63]</sup>. However, this methodology has its limitations. It was time-consuming, and the quality of the images obtained was affected by respiratory and other motion artefacts, as well as by magnetic field inhomogeneities<sup>[61,64]</sup>.

Since these early studies, many researchers have developed modifications of the Dixon method with the aim to reduce these limitations. Levenson and colleagues used the technique to quantify hepatic fat in 16 subjects with a variety of liver abnormalities, and compared the imaging results with those from liver biopsies<sup>[65]</sup>. The results were reproducible and there was reasonably good correlation between the imaging and biopsy findings<sup>[65]</sup>. In pursuit of a quick, accurate, non-invasive evaluation of hepatic steatosis, Fishbein and co-workers developed a fast gradient echo technique, which allowed them to obtain images of the liver in both adults and children, using breath holding manoeuvres<sup>[66]</sup>. This technique not

only reduced the time taken for the scan, but had the advantage of reducing motion artefact due to respiration. Using the same scanning sequence, these authors compared hepatic MRI with ultrasound and liver biopsy in quantifying hepatic fat in 38 patients with a variety of liver diseases<sup>[45]</sup>. Both MRI and ultrasound assessment of steatosis severity correlated well with liver histology, but MRI was superior to ultrasound in detecting and quantifying minor degrees of hepatic steatosis. Several other groups have also demonstrated a good correlation between the severity of hepatic steatosis on MRI and liver biopsy<sup>[67-69]</sup>. Recently, Qayyum and colleagues studied 27 patients, 16 with cirrhosis, and compared two different MRI techniques in quantifying hepatic fat. Their preliminary results suggested that liver fat may be more accurately quantified with fat-saturated fast spin-echo MR imaging than with out-of-phase gradient echo MR imaging, especially in patients with cirrhosis<sup>[70]</sup>.

Further modifications continue to be made to the Dixon method for water and fat separation, such as the recently reported fast spin-echo triple-echo Dixon (FTED) technique, which enables both uniform water/fat separation and fast scanning with uncompromised scan parameters<sup>[71]</sup>. Thus, in the future, chemical shift MRI is likely to provide an accurate, safe and fast method of detecting and quantifying hepatic steatosis in both adults and children.

## PROTON MAGNETIC RESONANCE SPECTROSCOPY

Chemical shift magnetic resonance imaging enables the identification of tissues that contain a significant proportion of intracellular lipid. In contrast, proton magnetic resonance spectroscopy (<sup>1</sup>H MRS) facilitates the examination of the resonance frequencies of all hydrogen nuclei (protons) within a region of interest. Although the absolute differences in resonance frequencies in MRS are quite small, they can be separated out to form a spectrum. Frequency separation, and hence spectral resolution, is determined by the strength of the main magnetic field. The MR spectra are plotted on an axis of chemical shift. With MR spectroscopy, the concentration of any given molecule in a sample is represented by the area under the specific resonance peak within the spectrum. Quantification of hepatic fat using proton MR spectroscopy requires evaluation of the two dominant peaks within the unsuppressed MR spectrum, water at 4.7 ppm and lipid at 1.0-1.5 ppm<sup>[59]</sup>. Livers with fatty infiltration demonstrate an increase in the intensity of the lipid resonance peak as shown in Figure 5.

Since proton magnetic resonance spectroscopy allows direct measurement of the area under the lipid resonance, it can be used to provide a quantitative assessment of fatty infiltration of the liver. Allowance must be made for both T<sub>1</sub> and T<sub>2</sub> relaxation effects, which differ for lipid and water. A correction must also be made for unsaturated lipids, as a portion of the MR



signal from these molecules overlaps with the water resonance at 4.7 ppm<sup>[59]</sup>.

Allowing for these corrections, Longo and colleagues studied a population of subjects with non-alcoholic fatty liver disease and found the percentage hepatic lipid measurements using <sup>1</sup>H MRS correlated well with those obtained by CT and liver biopsy<sup>[54,72]</sup>. Thomsen and co-workers demonstrated similar results in patients with alcohol-induced fatty liver disease<sup>[73]</sup>. Since then, several other studies have shown <sup>1</sup>H MRS to be a fast, safe, non-invasive method for the quantification of hepatic fat content<sup>[74-78]</sup>. Recently, <sup>1</sup>H MRS has been used in a large United States population study to determine the prevalence of non-alcoholic fatty liver disease in the general adult population<sup>[79]</sup> and also in longitudinal clinical studies<sup>[80-84]</sup>. However, in most centres <sup>1</sup>H MRS remains largely a research tool, despite the fact that all commercially available MR scanners have MRS capabilities.

## CONCLUSION

Non-alcoholic fatty liver disease (NAFLD) is now recognized as the most common cause of chronic liver disease in the Western World<sup>[31]</sup>. Its prevalence will likely increase in the future in parallel with the predicted increase in obesity and Type 2 diabetes. Currently, the only proven treatments for this condition are lifestyle measures, such as dietary modification and exercise<sup>[82,85-87]</sup>.

Liver biopsy remains the gold standard investigation for determining hepatic fat deposition, but it is an invasive procedure and may be prone to sampling error.

Of the non-invasive imaging techniques used to detect fatty infiltration of the liver, ultrasound is the most widely used in clinical practice today. It will remain popular in the future due to its widespread availability and excellent tolerability. Magnetic resonance techniques using chemical shift imaging and *in vivo* <sup>1</sup>H MRS have an advantage over ultrasound in that they are able to detect small changes in liver fat content. They can be performed as an adjunct to whole body MRI, as part of the same examination, allowing a comparison to be made between hepatic fat content and whole body adipose tissue distribution in the same subject. Thus, in the future, as more technological advances are made and scanning times shorten further, it is anticipated that both techniques will be used much more widely, both in longitudinal clinical research studies, but also in clinical practice.

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## REFERENCES

- 1 **Sanyal AJ.** Mechanisms of Disease: pathogenesis of nonalcoholic fatty liver disease. *Nat Clin Pract Gastroenterol Hepatol* 2005; **2**: 46-53
- 2 **Kershaw EE, Flier JS.** Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab* 2004; **89**: 2548-2556
- 3 **Scherer PE.** Adipose tissue: from lipid storage compartment to endocrine organ. *Diabetes* 2006; **55**: 1537-1545
- 4 **Yang Q, Graham TE, Mody N, Preitner F, Peroni OD, Zabolotny JM, Kotani K, Quadro L, Kahn BB.** Serum retinol binding protein 4 contributes to insulin resistance in obesity and type 2 diabetes. *Nature* 2005; **436**: 356-362
- 5 **Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM.** Positional cloning of the mouse obese gene and its human homologue. *Nature* 1994; **372**: 425-432
- 6 **Scherer PE, Williams S, Fogliano M, Baldini G, Lodish HF.** A novel serum protein similar to C1q, produced exclusively in adipocytes. *J Biol Chem* 1995; **270**: 26746-26749
- 7 **Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA.** The hormone resistin links obesity to diabetes. *Nature* 2001; **409**: 307-312
- 8 **Fukuhara A, Matsuda M, Nishizawa M, Segawa K, Tanaka M, Kishimoto K, Matsuki Y, Murakami M, Ichisaka T, Murakami H, Watanabe E, Takagi T, Akiyoshi M, Ohtsubo T, Kihara S, Yamashita S, Makishima M, Funahashi T, Yamanaka S, Hiramatsu R, Matsuzawa Y, Shimomura I.** Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. *Science* 2005; **307**: 426-430
- 9 **Unger RH.** Minireview: weapons of lean body mass destruction: the role of ectopic lipids in the metabolic syndrome. *Endocrinology* 2003; **144**: 5159-5165
- 10 **Montani JP, Carroll JF, Dwyer TM, Antic V, Yang Z, Dulloo AG.** Ectopic fat storage in heart, blood vessels and kidneys in the pathogenesis of cardiovascular diseases. *Int J Obes Relat Metab Disord* 2004; **28** Suppl 4: S58-S65
- 11 **Capeau J, Magre J, Lascols O, Caron M, Bereziat V, Vigouroux C, Bastard JP.** Diseases of adipose tissue: genetic and acquired lipodystrophies. *Biochem Soc Trans* 2005; **33**: 1073-1077
- 12 **Monajemi H, Stroes E, Hegele RA, Fliers E.** Inherited lipodystrophies and the metabolic syndrome. *Clin Endocrinol (Oxf)* 2007; **67**: 479-484
- 13 **Simha V, Garg A.** Lipodystrophy: lessons in lipid and energy metabolism. *Curr Opin Lipidol* 2006; **17**: 162-169
- 14 **Bloomgarden ZT.** Gut hormones, obesity, polycystic ovarian syndrome, malignancy, and lipodystrophy syndromes. *Diabetes Care* 2007; **30**: 1934-1939
- 15 **Payne JH, Dewind LT, Commons RR.** Metabolic Observations in Patients with Jejunoileal Shunts. *Am J Surg* 1963; **106**: 273-289
- 16 **McGill DB, Humpherys SR, Baggenstoss AH, Dickson ER.** Cirrhosis and death after jejunoileal shunt. *Gastroenterology* 1972; **63**: 872-877
- 17 **Catlin R.** Liver dysfunction after intestinal bypass. *JAMA* 1976; **236**: 1693-1694
- 18 **Peters RL, Gay T, Reynolds TB.** Post-jejunoileal-bypass hepatic disease. Its similarity to alcoholic hepatic disease. *Am J Clin Pathol* 1975; **63**: 318-331
- 19 **Thaler H.** Relation of steatosis to cirrhosis. *Clin Gastroenterol* 1975; **4**: 273-280
- 20 **Adler M, Schaffner F.** Fatty liver hepatitis and cirrhosis in obese patients. *Am J Med* 1979; **67**: 811-816
- 21 **Nasrallah SM, Wills CE Jr, Galambos JT.** Hepatic morphology in obesity. *Dig Dis Sci* 1981; **26**: 325-327
- 22 **Itoh S, Tsukada Y, Motomura Y, Ichinoe A.** Five patients with nonalcoholic diabetic cirrhosis. *Acta Hepatogastroenterol*

- (Stuttg) 1979; **26**: 90-97
- 23 **Falchuk KR**, Fiske SC, Haggitt RC, Federman M, Trey C. Pericentral hepatic fibrosis and intracellular hyalin in diabetes mellitus. *Gastroenterology* 1980; **78**: 535-541
  - 24 **Hornboll P**, Olsen TS. Fatty changes in the liver: the relation to age, overweight and diabetes mellitus. *Acta Pathol Microbiol Immunol Scand [A]* 1982; **90**: 199-205
  - 25 **Ludwig J**, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; **55**: 434-438
  - 26 **Powell EE**, Cooksley WG, Hanson R, Searle J, Halliday JW, Powell LW. The natural history of nonalcoholic steatohepatitis: a follow-up study of forty-two patients for up to 21 years. *Hepatology* 1990; **11**: 74-80
  - 27 **Matteoni CA**, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999; **116**: 1413-1419
  - 28 **Dam-Larsen S**, Franzmann M, Andersen IB, Christoffersen P, Jensen LB, Sorensen TI, Becker U, Bendtsen F. Long term prognosis of fatty liver: risk of chronic liver disease and death. *Gut* 2004; **53**: 750-755
  - 29 **Teli MR**, James OF, Burt AD, Bennett MK, Day CP. The natural history of nonalcoholic fatty liver: a follow-up study. *Hepatology* 1995; **22**: 1714-1719
  - 30 **Angulo P**. Nonalcoholic fatty liver disease. *N Engl J Med* 2002; **346**: 1221-1231
  - 31 **Adams LA**, Angulo P, Lindor KD. Nonalcoholic fatty liver disease. *CMAJ* 2005; **172**: 899-905
  - 32 **Crowley H**, Lewis WD, Gordon F, Jenkins R, Khettry U. Steatosis in donor and transplant liver biopsies. *Hum Pathol* 2000; **31**: 1209-1213
  - 33 **Joseph AE**, Dewbury KC, McGuire PG. Ultrasound in the detection of chronic liver disease (the "bright liver"). *Br J Radiol* 1979; **52**: 184-188
  - 34 **Quinn SF**, Gosink BB. Characteristic sonographic signs of hepatic fatty infiltration. *AJR Am J Roentgenol* 1985; **145**: 753-755
  - 35 **Joseph AE**, Saverymutter SH, al-Sam S, Cook MG, Maxwell JD. Comparison of liver histology with ultrasonography in assessing diffuse parenchymal liver disease. *Clin Radiol* 1991; **43**: 26-31
  - 36 **Joy D**, Thava VR, Scott BB. Diagnosis of fatty liver disease: is biopsy necessary? *Eur J Gastroenterol Hepatol* 2003; **15**: 539-543
  - 37 **Kissin CM**, Bellamy EA, Cosgrove DO, Slack N, Husband JE. Focal sparing in fatty infiltration of the liver. *Br J Radiol* 1986; **59**: 25-28
  - 38 **Caturelli E**, Squillante MM, Andriulli A, Cedrone A, Cellerino C, Pompili M, Manoja ER, Rapaccini GL. Hypoechoic lesions in the 'bright liver': a reliable indicator of fatty change. A prospective study. *J Gastroenterol Hepatol* 1992; **7**: 469-472
  - 39 **Foster KJ**, Dewbury KC, Griffith AH, Wright R. The accuracy of ultrasound in the detection of fatty infiltration of the liver. *Br J Radiol* 1980; **53**: 440-442
  - 40 **Debonnie JC**, Pauls C, Fievez M, Wibin E. Prospective evaluation of the diagnostic accuracy of liver ultrasonography. *Gut* 1981; **22**: 130-135
  - 41 **Saverymutter SH**, Joseph AE, Maxwell JD. Ultrasound scanning in the detection of hepatic fibrosis and steatosis. *Br Med J (Clin Res Ed)* 1986; **292**: 13-15
  - 42 **Needleman L**, Kurtz AB, Rifkin MD, Cooper HS, Pasto ME, Goldberg BB. Sonography of diffuse benign liver disease: accuracy of pattern recognition and grading. *AJR Am J Roentgenol* 1986; **146**: 1011-1015
  - 43 **Ryan CK**, Johnson LA, Germin BI, Marcos A. One hundred consecutive hepatic biopsies in the workup of living donors for right lobe liver transplantation. *Liver Transpl* 2002; **8**: 1114-1122
  - 44 **Mottin CC**, Moretto M, Padoin AV, Swarowsky AM, Toneto MG, Glock L, Repetto G. The role of ultrasound in the diagnosis of hepatic steatosis in morbidly obese patients. *Obes Surg* 2004; **14**: 635-637
  - 45 **Fishbein M**, Castro F, Cheruku S, Jain S, Webb B, Gleason T, Stevens WR. Hepatic MRI for fat quantitation: its relationship to fat morphology, diagnosis, and ultrasound. *J Clin Gastroenterol* 2005; **39**: 619-625
  - 46 **Pacifico L**, Celestre M, Anania C, Paolantonio P, Chiesa C, Laghi A. MRI and ultrasound for hepatic fat quantification: relationships to clinical and metabolic characteristics of pediatric nonalcoholic fatty liver disease. *Acta Paediatr* 2007; **96**: 542-547
  - 47 **Bydder GM**, Kreel L, Chapman RW, Harry D, Sherlock S, Bassan L. Accuracy of computed tomography in diagnosis of fatty liver. *Br Med J* 1980; **281**: 1042
  - 48 **Piekarski J**, Goldberg HI, Royal SA, Axel L, Moss AA. Difference between liver and spleen CT numbers in the normal adult: its usefulness in predicting the presence of diffuse liver disease. *Radiology* 1980; **137**: 727-729
  - 49 **Kawata R**, Sakata K, Kunieda T, Saji S, Doi H, Nozawa Y. Quantitative evaluation of fatty liver by computed tomography in rabbits. *AJR Am J Roentgenol* 1984; **142**: 741-746
  - 50 **Johnston RJ**, Stamm ER, Lewin JM, Hendrick RE, Archer PG. Diagnosis of fatty infiltration of the liver on contrast enhanced CT: limitations of liver-minus-spleen attenuation difference measurements. *Abdom Imaging* 1998; **23**: 409-415
  - 51 **Jacobs JE**, Birnbaum BA, Shapiro MA, Langlotz CP, Slosman F, Rubesin SE, Horii SC. Diagnostic criteria for fatty infiltration of the liver on contrast-enhanced helical CT. *AJR Am J Roentgenol* 1998; **171**: 659-664
  - 52 **Panicek DM**, Giess CS, Schwartz LH. Qualitative assessment of liver for fatty infiltration on contrast-enhanced CT: is muscle a better standard of reference than spleen? *J Comput Assist Tomogr* 1997; **21**: 699-705
  - 53 **Pamilo M**, Sotaniemi EA, Suramo I, Lahde S, Arranto AJ. Evaluation of liver steatotic and fibrous content by computerized tomography and ultrasound. *Scand J Gastroenterol* 1983; **18**: 743-747
  - 54 **Longo R**, Ricci C, Masutti F, Vidimari R, Croce LS, Bercich L, Tiribelli C, Dalla Palma L. Fatty infiltration of the liver. Quantification by <sup>1</sup>H localized magnetic resonance spectroscopy and comparison with computed tomography. *Invest Radiol* 1993; **28**: 297-302
  - 55 **Park SH**, Kim PN, Kim KW, Lee SW, Yoon SE, Park SW, Ha HK, Lee MG, Hwang S, Lee SG, Yu ES, Cho EY. Macrovesicular hepatic steatosis in living liver donors: use of CT for quantitative and qualitative assessment. *Radiology* 2006; **239**: 105-112
  - 56 **Bloch F**, Hansen WW, Packard ME. Nuclear induction. *Phys Rev* 1946; **69**: 127
  - 57 **Cox JJ**. Development and applications of in vivo clinical magnetic resonance spectroscopy. *Prog Biophys Mol Biol* 1996; **65**: 45-81
  - 58 **Taylor-Robinson SD**. Applications of magnetic resonance spectroscopy to chronic liver disease. *Clin Med* 2001; **1**: 54-60
  - 59 **Siegelman ES**, Rosen MA. Imaging of hepatic steatosis. *Semin Liver Dis* 2001; **21**: 71-80
  - 60 **Venkataraman S**, Braga L, Semelka RC. Imaging the fatty liver. *Magn Reson Imaging Clin N Am* 2002; **10**: 93-103
  - 61 **Dixon WT**. Simple proton spectroscopic imaging. *Radiology* 1984; **153**: 189-194
  - 62 **Lee JK**, Dixon WT, Ling D, Levitt RG, Murphy WA Jr. Fatty infiltration of the liver: demonstration by proton spectroscopic imaging. Preliminary observations. *Radiology* 1984; **153**: 195-201
  - 63 **Heiken JP**, Lee JK, Dixon WT. Fatty infiltration of the liver: evaluation by proton spectroscopic imaging. *Radiology* 1985; **157**: 707-710
  - 64 **Outwater EK**, Blasbalg R, Siegelman ES, Vala M. Detection of lipid in abdominal tissues with opposed-phase gradient-echo images at 1.5 T: techniques and diagnostic importance. *Radiographics* 1998; **18**: 1465-1480

- 65 **Levenson H**, Greensite F, Hoefs J, Friloux L, Applegate G, Silva E, Kanel G, Buxton R. Fatty infiltration of the liver: quantification with phase-contrast MR imaging at 1.5 T vs biopsy. *AJR Am J Roentgenol* 1991; **156**: 307-312
- 66 **Fishbein MH**, Gardner KG, Potter CJ, Schmalbrock P, Smith MA. Introduction of fast MR imaging in the assessment of hepatic steatosis. *Magn Reson Imaging* 1997; **15**: 287-293
- 67 **Rinella ME**, McCarthy R, Thakrar K, Finn JP, Rao SM, Koffron AJ, Abecassis M, Blei AT. Dual-echo, chemical shift gradient-echo magnetic resonance imaging to quantify hepatic steatosis: Implications for living liver donation. *Liver Transpl* 2003; **9**: 851-856
- 68 **Hussain HK**, Chenevert TL, Londy FJ, Gulani V, Swanson SD, McKenna BJ, Appelman HD, Adusumilli S, Greenon JK, Conjeevaram HS. Hepatic fat fraction: MR imaging for quantitative measurement and display-early experience. *Radiology* 2005; **237**: 1048-1055
- 69 **Pilleul F**, Chave G, Dumortier J, Scoazec JY, Valette PJ. Fatty infiltration of the liver. Detection and grading using dual T1 gradient echo sequences on clinical MR system. *Gastroenterol Clin Biol* 2005; **29**: 1143-1147
- 70 **Qayyum A**, Goh JS, Kakar S, Yeh BM, Merriman RB, Coakley FV. Accuracy of liver fat quantification at MR imaging: comparison of out-of-phase gradient-echo and fat-saturated fast spin-echo techniques-initial experience. *Radiology* 2005; **237**: 507-511
- 71 **Ma J**, Son JB, Zhou Y, Le-Petross H, Choi H. Fast spin-echo triple-echo dixon (fTED) technique for efficient T2-weighted water and fat imaging. *Magn Reson Med* 2007; **58**: 103-109
- 72 **Longo R**, Pollesello P, Ricci C, Masutti F, Kvam BJ, Bercich L, Croce LS, Grigolato P, Paoletti S, de Bernard B. Proton MR spectroscopy in quantitative in vivo determination of fat content in human liver steatosis. *J Magn Reson Imaging* 1995; **5**: 281-285
- 73 **Thomsen C**, Becker U, Winkler K, Christoffersen P, Jensen M, Henriksen O. Quantification of liver fat using magnetic resonance spectroscopy. *Magn Reson Imaging* 1994; **12**: 487-495
- 74 **Thomas EL**, Hamilton G, Patel N, O'Dwyer R, Dore CJ, Goldin RD, Bell JD, Taylor-Robinson SD. Hepatic triglyceride content and its relation to body adiposity: a magnetic resonance imaging and proton magnetic resonance spectroscopy study. *Gut* 2005; **54**: 122-127
- 75 **Szczepaniak LS**, Babcock EE, Schick F, Dobbins RL, Garg A, Burns DK, McGarry JD, Stein DT. Measurement of intracellular triglyceride stores by H spectroscopy: validation in vivo. *Am J Physiol* 1999; **276**: E977-E989
- 76 **Kotronen A**, Westerbacka J, Bergholm R, Pietiläinen KH, Yki-Jarvinen H. Liver fat in the metabolic syndrome. *J Clin Endocrinol Metab* 2007; **92**: 3490-3497
- 77 **Seppala-Lindroos A**, Vehkavaara S, Hakkinen AM, Goto T, Westerbacka J, Sovijarvi A, Halavaara J, Yki-Jarvinen H. Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. *J Clin Endocrinol Metab* 2002; **87**: 3023-3028
- 78 **Ostberg JE**, Thomas EL, Hamilton G, Attar MJ, Bell JD, Conway GS. Excess visceral and hepatic adipose tissue in Turner syndrome determined by magnetic resonance imaging: estrogen deficiency associated with hepatic adipose content. *J Clin Endocrinol Metab* 2005; **90**: 2631-2635
- 79 **Browning JD**, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, Grundy SM, Hobbs HH. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. *Hepatology* 2004; **40**: 1387-1395
- 80 **Westerbacka J**, Lammi K, Hakkinen AM, Rissanen A, Salminen I, Aro A, Yki-Jarvinen H. Dietary fat content modifies liver fat in overweight nondiabetic subjects. *J Clin Endocrinol Metab* 2005; **90**: 2804-2809
- 81 **Tiikkainen M**, Hakkinen AM, Korshennikova E, Nyman T, Makimattila S, Yki-Jarvinen H. Effects of rosiglitazone and metformin on liver fat content, hepatic insulin resistance, insulin clearance, and gene expression in adipose tissue in patients with type 2 diabetes. *Diabetes* 2004; **53**: 2169-2176
- 82 **Thomas EL**, Brynes AE, Hamilton G, Patel N, Spong A, Goldin RD, Frost G, Bell JD, Taylor-Robinson SD. Effect of nutritional counselling on hepatic, muscle and adipose tissue fat content and distribution in non-alcoholic fatty liver disease. *World J Gastroenterol* 2006; **12**: 5813-5819
- 83 **Belfort R**, Harrison SA, Brown K, Darland C, Finch J, Hardies J, Balas B, Gastaldelli A, Tio F, Pulcini J, Berria R, Ma JZ, Dwivedi S, Havranek R, Fincke C, DeFronzo R, Bannayan GA, Schenker S, Cusi K. A placebo-controlled trial of pioglitazone in subjects with nonalcoholic steatohepatitis. *N Engl J Med* 2006; **355**: 2297-2307
- 84 **Thomas EL**, Potter E, Tosi I, Fitzpatrick J, Hamilton G, Amber V, Hughes R, North C, Holvoet P, Seed M, Betteridge DJ, Bell JD, Naoumova RP. Pioglitazone added to conventional lipid-lowering treatment in familial combined hyperlipidaemia improves parameters of metabolic control: relation to liver, muscle and regional body fat content. *Atherosclerosis* 2007; **195**: e181-e190
- 85 **Park HS**, Kim MW, Shin ES. Effect of weight control on hepatic abnormalities in obese patients with fatty liver. *J Korean Med Sci* 1995; **10**: 414-421
- 86 **Ueno T**, Sugawara H, Sujaku K, Hashimoto O, Tsuji R, Tamaki S, Torimura T, Inuzuka S, Sata M, Tanikawa K. Therapeutic effects of restricted diet and exercise in obese patients with fatty liver. *J Hepatol* 1997; **27**: 103-107
- 87 **Zelber-Sagi S**, Kessler A, Brazowsky E, Webb M, Lurie Y, Santo M, Leshno M, Blendis L, Halpern Z, Oren R. A double-blind randomized placebo-controlled trial of orlistat for the treatment of nonalcoholic fatty liver disease. *Clin Gastroenterol Hepatol* 2006; **4**: 639-644

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## COLORECTAL CANCER

# Risk of colorectal neoplasm in patients with acromegaly: A meta-analysis

Theodoros Rokkas, Dimitrios Pistiolas, Panos Sechopoulos, Georgios Margantinis, Georgios Koukoulis

Theodoros Rokkas, Dimitrios Pistiolas, Panos Sechopoulos, Georgios Margantinis, Georgios Koukoulis, Gastroenterology Clinic, Henry Dunant Hospital, 107 Messogion Ave., Athens 11526, Greece

**Author contributions:** Rokkas T conceived and designed the study, analysed data and wrote the paper; Pistiolas T analysed data and contributed to the writing of the paper; Sechopoulos P performed research and analysed data; Margantinis G and Koukoulis G performed research.

**Correspondence to:** Theodoros Rokkas, MD, PhD, FACP, AGAF, FEBG, Gastroenterology Clinic, Henry Dunant Hospital, Athens, Greece. [sakkor@otenet.gr](mailto:sakkor@otenet.gr)

Telephone: +30-210-6431334 Fax: +30-210-6431334

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**Peer reviewers:** Robert Flisiak, PhD, Department of Infectious Diseases, Medical University of Bialystok, 15-540 Bialystok, Zurawia str., 14, Poland; Dr. Mark S Pearce, Paediatric and Lifecourse Epidemiology Research Group School of Clinical Medical Sciences, University of Newcastle, Sir James Spence Institute, Royal Victoria Infirmary, Newcastle upon Tyne, NE1 4LP, United Kingdom; Yoshiharu Motoo, MD, PhD, FACP, FACP, Professor and Chairman, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

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## Abstract

**AIM:** To examine the risk of colorectal neoplasm in acromegalic patients by meta-analyzing all relevant controlled studies.

**METHODS:** Extensive English language medical literature searches for human studies, up to December 2007, were performed using suitable keywords. Pooled estimates [odds ratio (OR) with 95% confidence intervals (CI)] were obtained using either the fixed or random-effects model as appropriate. Heterogeneity between studies was evaluated with the Cochran *Q* test whereas the likelihood of publication bias was assessed by constructing funnel plots. Their symmetry was estimated by the adjusted rank correlation test.

**RESULTS:** For hyperplastic polyps the pooled ORs with 95% CI were 3.557 (2.587-4.891) by fixed effects model and 3.703 (2.565-5.347) by random effects model. The *Z* test values for overall effect were 7.81 and 6.984, respectively ( $P < 0.0001$ ). For colon adenomas the pooled ORs with 95% CI were 2.486 (1.908-3.238) (fixed effects model) and 2.537 (1.914-3.364) (random effects model). The *Z* test values were 6.747 and 6.472, respectively ( $P < 0.0001$ ). For colon cancer the pooled OR with 95% CI was identical for both fixed and random effects model (OR, 4.351; 95% CI, 1.533-12.354;  $Z = 2.762$ ,  $P = 0.006$ ). There was no significant heterogeneity and no publication bias in all the above meta-analyses.

**CONCLUSION:** Acromegaly is associated with an increased risk of colorectal neoplasm.

## INTRODUCTION

Acromegaly is a disease caused by excess secretion of growth hormone (GH), which is characterized by enlarged acral parts, coarse facial features, and visceromegaly. Acromegalic patients have a reduced life expectancy primarily due to cardiovascular, respiratory or cerebrovascular disease<sup>[1-3]</sup>. Acromegalics may also be at an increased risk for malignancies in several systems including the digestive tract, brain, kidney, breast and prostate<sup>[6-10]</sup>. Colon cancer incidence<sup>[6,7,10]</sup> and mortality rates<sup>[9]</sup> have been reported to be higher in acromegalics than expected. However, reported relative risks of colorectal cancer vary significantly depending on the study population and the study design. Moreover, the reported higher indices of colorectal neoplasia in acromegalics have not been a universal finding<sup>[11-13]</sup>. The main aim of this meta-analysis, therefore, was to examine the pooled risk of colorectal neoplasia (polyps and cancer) in acromegalic patients by meta-analyzing all relevant controlled studies. Secondary aims were to explore the possibility of heterogeneity between studies and to look for the existence of publication bias. This study is justified by the fact that, so far, no meta-analysis has been published examining the relationship between acromegaly and colorectal neoplasia.



## MATERIALS AND METHODS

### Data identification and extraction

We searched the MEDLINE/PUBMED and EMBASE databases up to December 2007 to identify all relevant English language medical literature for human studies under the search text terms; acromegaly AND (colon cancer OR colon polyps OR colorectal cancer OR colorectal polyps). We also performed a full manual search of all review articles, published editorials and of retrieved original studies. Data from each study were extracted independently by two authors (T.R and D.P) by using a predefined form, and disagreements were resolved by discussion and consensus.

### Selection criteria

Inclusion and exclusion criteria were delineated before the commencement of the literature search. Thus, eligible studies published as full articles were included in this meta-analysis if they met all of the following criteria: (1) written in the English language, (2) published as full articles and (3) a simultaneous control group was included.

Studies not meeting the aforementioned criteria, and in addition studies without data for retrieval, studies using historical control patients or autopsy data and duplicate publications were excluded. When two papers reported the same study the publication that was more informative was selected.

### Statistical analysis

Agreement in the selection of studies between the 2 reviewers was evaluated by the  $\kappa$  coefficient. We calculated the pooled odds ratios (ORs) and 95% confidence intervals (CI) and compared outcomes of individual studies by using the fixed<sup>[14]</sup> or the random effects model<sup>[15]</sup> as appropriate. Forest plots were constructed for visual display of OR (95% CI) of individual studies and pooled data. Heterogeneity between studies was evaluated with the Cochran  $Q$  test<sup>[16]</sup> and it was considered to be present if the  $Q$  test provided a  $P$  value of less than 0.10<sup>[16,17]</sup>. In the presence of significant statistical heterogeneity, sensitivity analyses were performed to search for the possible sources, such as sample size of each study, *etc.* These analyses were achieved by repeating the meta-analyses with exclusion of each individual study one at a time, in order to assess the overall effect of each study on the pooled ORs<sup>[18]</sup>. This indicates which particular studies are most influential and might help in the evaluation of the possibility that the conclusions result from the influence of a particular study. The likelihood of publication bias was assessed by constructing funnel plots<sup>[18]</sup> and their symmetry was estimated by the Begg and Mazumdar adjusted rank correlation test<sup>[19]</sup>.

All meta-analyses, sensitivity and meta-regression analyses in this study were performed by using suitable meta-analysis software (Comprehensive Meta Analysis-Version 2, Biostat Inc, Englewood, NJ, USA).

### Role of funding source

This was an investigator-initiated unfunded study. All

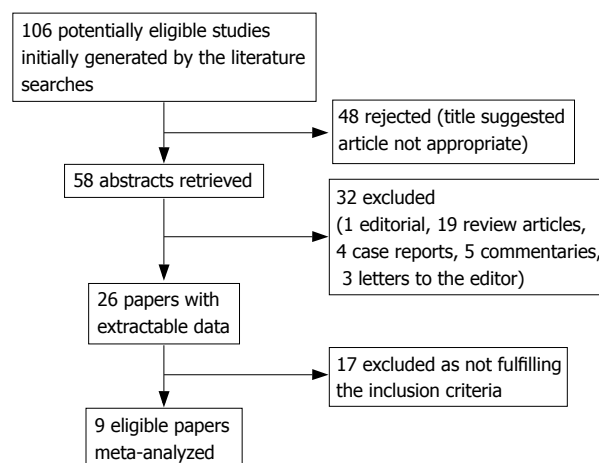


Figure 1 Flow diagram of the studies identified in this meta-analysis.

authors had access to the data and the statistical analysis report. Each author approved the final article and attests to the validity of the results.

## RESULTS

### Descriptive assessment and study characteristics

A flow chart describing the process of study selection is shown in Figure 1. Out of 106 titles initially generated by the literature searches, 48 were rejected as the title suggested that the articles were not appropriate. Of the remaining 58 abstracts, 32 were excluded for not having extractable data (editorials, review articles, case reports, commentaries and letters to the editor). Therefore, 26 papers remained candidates for eligibility. Of these, 17<sup>[6,8,9,11-13,20-30]</sup> were rejected on the basis of not fulfilling the inclusion criteria. Therefore 9 controlled studies remained eligible for meta-analysis<sup>[31-39]</sup>. Initial agreement between the reviewers for the selection of relevant articles was high ( $\kappa = 0.924$ , 95% CI, 0.851-0.997).

The main characteristics of the papers eligible for meta-analysis are shown in Table 1. The studies were conducted in different parts of the world; most were single centre studies and included 701 acromegaly patients and 1573 controls.

### Colon adenomas

Eight of the nine meta-analyzed studies<sup>[31-35,37-39]</sup> provided data concerning the frequency of colon adenomas in acromegaly patients and controls [149/641 (23.2%) *vs* 176/1,413 (12.45%)]. The pooled ORs (95%CI), by both the fixed and random effects model, were 2.486 (1.908-3.238) and 2.537 (1.914-3.364), respectively with  $Z$  test values for overall effect 6.747 and 6.472, respectively and  $P < 0.0001$  for both models (Figure 2). There was no significant heterogeneity ( $P = 0.371$ ) among these trials (Table 2). In addition there was no publication bias ( $P$  two tailed value 0.711, Table 2) as shown in the respective funnel plot (Figure 3).

### Colon hyperplastic polyps

Seven studies<sup>[31,33-38]</sup> provided data concerning the

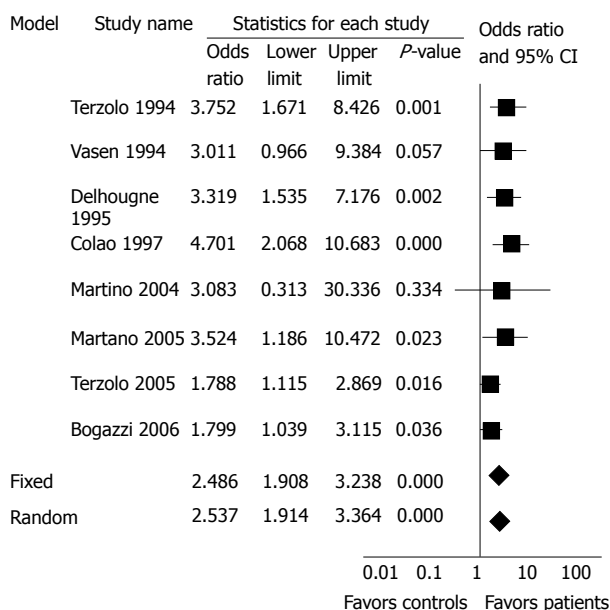
Table 1 The main characteristics of studies selected for meta-analysis

Ref.	1st author, year, country	Acromegaly patients		Control subjects		Type of the study	Control group composition
		n (M/F)	Mean age (range or SD yr)	n (M/F)	Mean age (range or SD, yr)		
31	Terzolo, 1994, Italy	31 (11/20)	52.2 (27-85)	236 (127/109)	50.1 (23-84)	Hospital based control study	Patients with rectal bright-red bleeding from hemorrhoids
32	Vasen, 1994, Germany and Holland	49 (30/19)	54 (30-75)	57 (28/29)	54 (34-72)	Hospital based control study	Patients with irritable bowel syndrome
33	Delhougne, 1995, Belgium and France	103 (49/54)	5 (12, SD)	138 (55/83)	53 (15, SD)	Hospital based control study	Patients with irritable bowel syndrome
34	Colao, 1997, Italy	50 (25/25)	20-70	318 (Sex matched) <sup>1</sup>	Age matched <sup>1</sup>	Hospital based control study	Patients with irritable bowel syndrome
35	Martino, 2004, Italy	75 (33/42)	54 (11, SD)	75 (33/42)	55 (10, SD)	Hospital based control study	Patients with irritable bowel syndrome
36	Bhansali, 2004, India	60 (35/25)	37.4 (13.2, SD)	160 (88/72)	38.2 (14, SD)	Hospital based control study	Patients with irritable bowel syndrome
37	Matano, 2005, Japan	19 (11/8)	46.7 (16.3, SD)	76 (44/32)	47.3 (16.5, SD)	Hospital based control study	Randomly selected from subjects referred for colonoscopy
38	Terzolo, 2005, Italy	235 (115/120)	49.1 (12.6, SD)	233 (156/77)	50.8 (12, SD)	Hospital based control study	Consecutive patients with nonspecific abdominal symptoms
39	Bogazzi, 2006, Italy	79 (33/46)	55.0 (11.1, SD)	280 (166/114)	50.9 (10.8, SD)	Hospital based control study	Consecutive donors for kidney or liver transplantation
	Total	701		1573			

<sup>1</sup>Paper did not provide detailed data on patients mean age, control subjects, sex distribution and control subjects age.

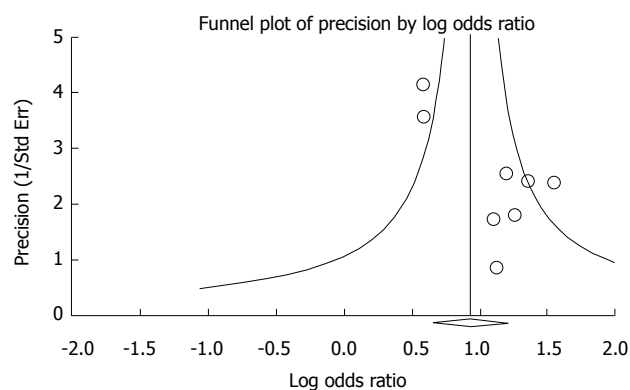
Table 2 Heterogeneity and publication bias results of meta-analyzed studies, concerning the three types of colonic lesion examined, i.e. colon adenomas, hyperplastic polyps and colon cancer

Type of colonic lesion	Heterogeneity					Publication bias	
	Number of studies	Q-value	df (Q)	I <sup>2</sup>	P value	Kendall's tau	P value (two-tailed)
Adenomatous polyps	8	7.584	7	7.700	0.371	0.143	0.711
Hyperplastic polyps	7	7.447	6	19.433	0.281	0.143	0.764
Cancer	3	1.068	2	0.000	0.586	-0.333	1.000



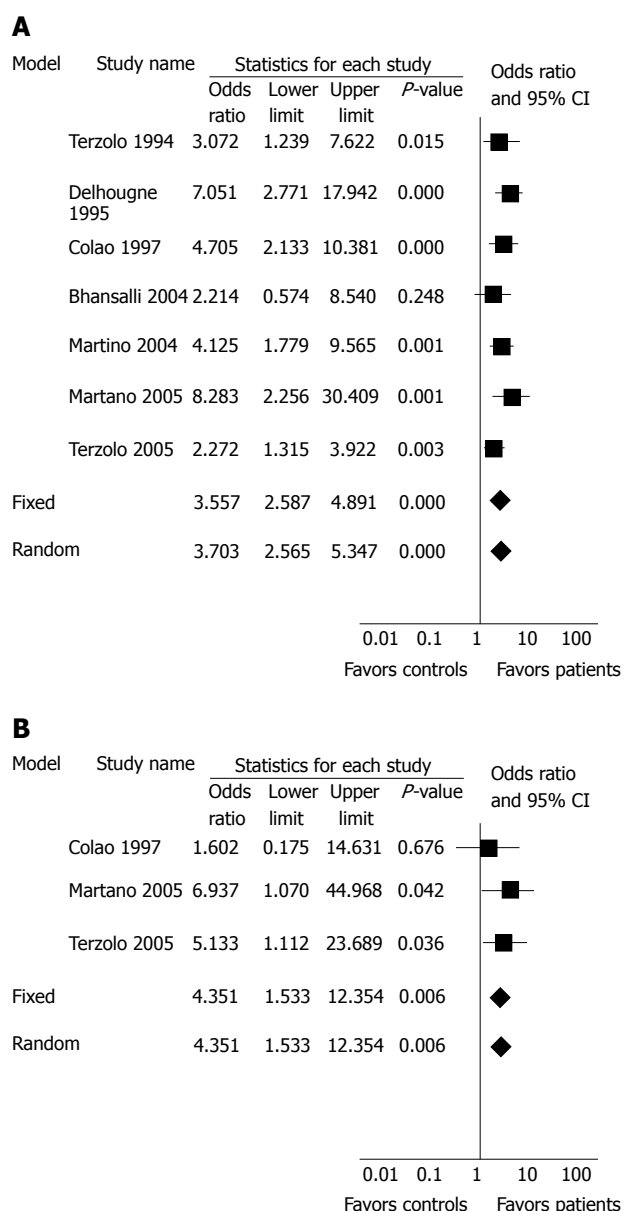
**Figure 2** Forest plot showing individual and pooled ORs (95% CIs) and P values in studies comparing the colon adenoma prevalence in acromegaly patients and controls.

frequency of colon hyperplastic polyps in acromegaly



**Figure 3** Funnel plot of selected studies examining colon adenoma prevalence in acromegaly patients and controls. No evidence of publication bias found ( $P = 0.711$ , by adjusted rank correlation test). Similarly no evidence of publication bias was found for the other colon neoplasias studied, i.e. colon hyperplastic polyps and colon cancer (see results, Table 2).

patients and controls [128/573 (22.3%) *vs* 91/1236 (7.36%)]. The pooled OR (95% CI), by both the fixed and random effects model, were 3.557 (2.587-4.891) and 3.703 (2.565-5.347), respectively, with Z test values for overall effect 7.81 and 6.984, respectively and  $P < 0.0001$  for both models (Figure 4A). There was no significant



**Figure 4** Forest plot showing individual and pooled ORs (95% CIs) and *P* values in studies comparing the colon hyperplastic polyp prevalence (A) and the colon cancer prevalence (B) in acromegaly patients and controls.

heterogeneity among these trials ( $P = 0.281$ ) and no publication bias ( $P = 0.764$ , Table 2).

### Colon cancer

Three studies<sup>[34,37,38]</sup> provided data concerning the frequency of colon cancer in acromegaly patients and controls [14/304 (4.6%) *vs* 8/627 (1.2%), respectively]. The pooled ORs (95%CI), by both the fixed and random effects model, were identical [4.351 (1.533-12.354) for both] with *Z* test for overall effect = 2.762 and  $P = 0.006$  (Figure 4B). There was no significant heterogeneity among these trials ( $P = 0.586$ ) and no publication bias ( $P = 1$ ).

## DISCUSSION

The study on the association between acromegaly

and cancer risk has focused primarily on colorectal cancer, but the findings of previous studies are far from conclusive and the matter of colorectal cancer in acromegaly has been debated in the literature<sup>[40-44]</sup>.

The discrepant results in the literature prompted us to undertake a meta-analysis on control trials in order to assess whether patients with acromegaly are at an increased risk of developing colorectal neoplasia, because if this is the case, then in these patients an aggressive approach to endoscopic management should be applied. In addition, our study is justified by the fact that, so far, no meta-analysis has been published examining the relationship between acromegaly and colorectal neoplasia.

The pooled results in this study clearly showed that acromegalic patients are at a significantly increased risk of developing colorectal adenomatous and hyperplastic polyps as well as colorectal cancer compared with controls. This estimate is in agreement with the results of three larger epidemiological studies assessing the risk of colonic cancer in acromegaly<sup>[7,9,10]</sup>. According to these findings, therefore, it is beyond doubt that a suitable endoscopic approach, with early large bowel endoscopic screening and regular surveillance, is fully justified in acromegaly patients. Needless to say total colonoscopy is essential in acromegaly, as in several studies proximal colon pathology was found<sup>[13,33,34]</sup>. This requirement is made more difficult by the increased length and capacity of the acromegalic colon<sup>[13,42]</sup>. To overcome this and the prolonged colonic transit time in acromegalic patients, rigorous bowel preparation, in excess of that usually used, is needed<sup>[42]</sup>.

One possible weakness of this meta-analysis might be the inclusion of underpowered studies of small sizes. Another weakness might be the fact that five of the nine meta-analysed studies come from the same country, i.e. Italy. However, all possible weaknesses are compensated by the lack of heterogeneity among the meta-analysed studies and also by the lack of publication bias.

Why should acromegaly patients be at an increased risk of colorectal neoplasia? The answer to this question is difficult, but it seems that the increased risk for several cancers among acromegaly patients may be due to the elevated proliferative and anti-apoptotic activity associated with increased circulating levels of insulin-like growth factor- I (IGF- I). There is an increasing body of evidence suggesting that adults with high concentrations of IGF- I are at increased risk of colorectal cancer<sup>[45-47]</sup>. In some of these studies, high IGF binding protein-3 (IGFBP-3) levels were associated with a lower risk of cancer<sup>[45,46]</sup>. In acromegaly, GH excess increases serum IGF- I and, to a lesser extent, IGFBP-3 concentrations, with the IGF- I to IGFBP-3 ratio being greater as GH concentrations increase<sup>[48,49]</sup>. Thus, an elevated IGF- I to IGFBP-3 ratio is expected to increase cancer risk in acromegaly<sup>[45,50]</sup>. On the other hand, IGF- I receptors and mRNAs for IGF- I have been identified in human colorectal cells<sup>[51]</sup>. IGF- I is a known mitogen that may stimulate, by autocrine and paracrine actions, the proliferation of intestinal epithelial cells and their

migration<sup>[52]</sup>. In fact, increased proliferation of colonic epithelium, proportional to circulating IGF- I levels, has been demonstrated in acromegaly<sup>[53]</sup>. Moreover, IGF- I is able to stimulate the growth of colorectal cancer cells *in vitro*, whereas blockade of IGF- I receptors inhibits cell growth in the same model<sup>[51,54,55]</sup>.

In conclusion, this study showed that acromegalic patients are at an increased risk of colorectal adenomatous and hyperplastic polyps as well as colorectal cancer. Therefore, acromegaly should be characterized as a disorder carrying a high risk for the development of colorectal neoplasia where an aggressive approach to endoscopic management with early screening and regular surveillance is justified.

## COMMENTS

### Background

Patients with acromegaly may be at an increased risk for malignancies in several systems including the digestive tract. However, the reported higher indices of colorectal neoplasia in acromegalics have not been a universal finding.

### Research frontiers

We evaluated the risk of colorectal neoplasm in acromegalic patients by meta-analyzing all relevant controlled studies.

### Innovations and breakthroughs

We made a comprehensive search of studies dealing with colorectal neoplasm in acromegalic patients. The studies were analyzed to determine the risk of colorectal neoplasm in these patients.

### Applications

Based on this evaluation, we concluded that acromegalic patients are at an increased risk of colorectal adenomatous and hyperplastic polyps as well as colorectal cancer. Therefore, acromegaly should be characterized as a disorder carrying a high risk for the development of colorectal neoplasia where an aggressive approach to endoscopic management with early screening and regular surveillance is justified.

### Peer review

The authors explored the risk of colorectal neoplasm in acromegalic patients. It was concluded that acromegalic patients are at an increased risk of colorectal adenomatous and hyperplastic polyps as well as colorectal cancer.

## REFERENCES

- 1 Wright AD, Hill DM, Lowy C, Fraser TR. Mortality in acromegaly. *Q J Med* 1970; **39**: 1-16
- 2 Alexander L, Appleton D, Hall R, Ross WM, Wilkinson R. Epidemiology of acromegaly in the Newcastle region. *Clin Endocrinol (Oxf)* 1980; **12**: 71-79
- 3 Ritchie CM, Atkinson AB, Kennedy AL, Lyons AR, Gordon DS, Fannin T, Hadden DR. Ascertainment and natural history of treated acromegaly in Northern Ireland. *Ulster Med J* 1990; **59**: 55-62
- 4 Bengtsson BA, Eden S, Ernest I, Oden A, Sjogren B. Epidemiology and long-term survival in acromegaly. A study of 166 cases diagnosed between 1955 and 1984. *Acta Med Scand* 1988; **223**: 327-335
- 5 Etxabe J, Gaztambide S, Latorre P, Vazquez JA. Acromegaly: an epidemiological study. *J Endocrinol Invest* 1993; **16**: 181-187
- 6 Pines A, Rozen P, Ron E, Gilat T. Gastrointestinal tumors in acromegalic patients. *Am J Gastroenterol* 1985; **80**: 266-269
- 7 Ron E, Gridley G, Hrubec Z, Page W, Arora S, Fraumeni JF Jr. Acromegaly and gastrointestinal cancer. *Cancer* 1991; **68**: 1673-1677
- 8 Barzilay J, Heatley GJ, Cushing GW. Benign and malignant tumors in patients with acromegaly. *Arch Intern Med* 1991; **151**: 1629-1632
- 9 Orme SM, McNally RJ, Cartwright RA, Belchetz PE. Mortality and cancer incidence in acromegaly: a retrospective cohort study. United Kingdom Acromegaly Study Group. *J Clin Endocrinol Metab* 1998; **83**: 2730-2734
- 10 Baris D, Gridley G, Ron E, Weiderpass E, Møller M, Ekbom A, Olsen JH, Baron JA, Fraumeni JF Jr. Acromegaly and cancer risk: a cohort study in Sweden and Denmark. *Cancer Causes Control* 2002; **13**: 395-400
- 11 Ladas SD, Thalassinou NC, Ioannides G, Raptis SA. Does acromegaly really predispose to an increased prevalence of gastrointestinal tumours? *Clin Endocrinol (Oxf)* 1994; **41**: 597-601
- 12 Ortego J, Vega B, Sampedro J, Escalada J, Boixeda D, Varela C. Neoplastic colonic polyps in acromegaly. *Horm Metab Res* 1994; **26**: 609-610
- 13 Renehan AG, Bhaskar P, Painter JE, O'Dwyer ST, Haboubi N, Varma J, Ball SG, Shalet SM. The prevalence and characteristics of colorectal neoplasia in acromegaly. *J Clin Endocrinol Metab* 2000; **85**: 3417-3424
- 14 Mantel N, Haenszel W. Statistical aspects of the analysis of data from retrospective studies of disease. *J Natl Cancer Inst* 1959; **22**: 719-748
- 15 DerSimonian R, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177-188
- 16 Cochran WG. The Combination of Estimates from Different Experiments. *Biometrics* 1954; **8**: 101-129
- 17 Higgins JP, Thompson SG. Quantifying heterogeneity in a meta-analysis. *Stat Med* 2002; **21**: 1539-1558
- 18 Sutton AJ, Abrams KR, Jones DR, Sheldon TA, Song F. Methods for meta-analysis in medical research. New York: John Wiley & Sons, 2000
- 19 Begg CB, Mazumdar M. Operating Characteristics of a Rank Correlation Test for Publication Bias. *Biometrics* 1994; **50**: 1088-1101
- 20 Klein I, Parveen G, Gavalier JS, Vanthiel DH. Colonic polyps in patients with acromegaly. *Ann Intern Med* 1982; **97**: 27-30
- 21 Ituarte EA, Petrini J, Hershtman JM. Acromegaly and colon cancer. *Ann Intern Med* 1984; **101**: 627-628
- 22 Ezzat S, Strom C, Melmed S. Colon polyps in acromegaly. *Ann Intern Med* 1991; **114**: 754-755
- 23 Jenkins PJ, Fairclough PD, Richards T, Lowe DG, Monson J, Grossman A, Wass JA, Besser M. Acromegaly, colonic polyps and carcinoma. *Clin Endocrinol (Oxf)* 1997; **47**: 17-22
- 24 Brunner JE, Johnson CC, Zafar S, Peterson EL, Brunner JF, Mellinger RC. Colon cancer and polyps in acromegaly: increased risk associated with family history of colon cancer. *Clin Endocrinol (Oxf)* 1990; **32**: 65-71
- 25 Cheung NW, Boyages SC. Increased incidence of neoplasia in females with acromegaly. *Clin Endocrinol (Oxf)* 1997; **47**: 323-327
- 26 Jenkins PJ, Frajese V, Jones AM, Camacho-Hubner C, Lowe DG, Fairclough PD, Chew SL, Grossman AB, Monson JP, Besser GM. Insulin-like growth factor I and the development of colorectal neoplasia in acromegaly. *J Clin Endocrinol Metab* 2000; **85**: 3218-3221
- 27 Fukuda I, Hizuka N, Murakami Y, Itoh E, Yasumoto K, Sata A, Takano K. Clinical features and therapeutic outcomes of 65 patients with acromegaly at Tokyo Women's Medical University. *Intern Med* 2001; **40**: 987-992
- 28 Matyja V, Kos-Kudla B, Foltyn W, Strzelczyk J, Latos W, Marek B, Kajdaniuk D, Karpe J, Ostrowska Z, Sieron-Stoltny K, Sieron A. Detection of colorectal lesions by using autofluorescence colonoscopy in acromegalics and their relation to serum growth hormone and insulin-like growth factor-1 levels. *Neuro Endocrinol Lett* 2006; **27**: 639-643
- 29 Colao A, Pivonello R, Auriemma RS, Galdiero M, Ferone D, Minuto F, Marzullo P, Lombardi G. The association of fasting insulin concentrations and colonic neoplasms in acromegaly: a colonoscopy-based study in 210 patients. *J Clin Endocrinol Metab* 2007; **92**: 3854-3860
- 30 Larijani B, Aliannejad R, Khaleghnejad-Tabari N, Baradar-Jalili R, Ansari R, Tavangar SM, Bandarian F. The prevalence



- of polyp in colon of patients with acromegaly. *Arch Iran Med* 2007; **10**: 236-238
- 31 **Terzolo M**, Tappero G, Borretta G, Asnaghi G, Pia A, Reimondo G, Boccuzzi A, Cesario F, Rovero E, Paccotti P. High prevalence of colonic polyps in patients with acromegaly. Influence of sex and age. *Arch Intern Med* 1994; **154**: 1272-1276
  - 32 **Vasen HF**, van Erpecum KJ, Roelfsema F, Raue F, Koppeschaar H, Griffioen G, van Berge Henegouwen GP. Increased prevalence of colonic adenomas in patients with acromegaly. *Eur J Endocrinol* 1994; **131**: 235-237
  - 33 **Delhougne B**, Deneux C, Abs R, Chanson P, Fierens H, Laurent-Puig P, Duysburgh I, Stevenaert A, Tabarin A, Delwaide J. The prevalence of colonic polyps in acromegaly: a colonoscopic and pathological study in 103 patients. *J Clin Endocrinol Metab* 1995; **80**: 3223-3226
  - 34 **Colao A**, Balzano A, Ferone D, Panza N, Grande G, Marzullo P, Bove A, Iodice G, Merola B, Lombardi G. Increased prevalence of colonic polyps and altered lymphocyte subset pattern in the colonic lamina propria in acromegaly. *Clin Endocrinol (Oxf)* 1997; **47**: 23-28
  - 35 **Martino A**, Cammarota G, Cianci R, Bianchi A, Sacco E, Tilaro L, Marzetti E, Certo M, Pirozzi G, Fedeli P, Pandolfi F, Pontecorvi A, Gasbarrini G, De Marinis L. High prevalence of hyperplastic colonic polyps in acromegalic subjects. *Dig Dis Sci* 2004; **49**: 662-666
  - 36 **Bhansali A**, Kochhar R, Chawla YK, Reddy S, Dash RJ. Prevalence of colonic polyps is not increased in patients with acromegaly: analysis of 60 patients from India. *J Gastroenterol Hepatol* 2004; **19**: 266-269
  - 37 **Matano Y**, Okada T, Suzuki A, Yoneda T, Takeda Y, Mabuchi H. Risk of colorectal neoplasm in patients with acromegaly and its relationship with serum growth hormone levels. *Am J Gastroenterol* 2005; **100**: 1154-1160
  - 38 **Terzolo M**, Reimondo G, Gasperi M, Cozzi R, Pivonello R, Vitale G, Scillitani A, Attanasio R, Cecconi E, Daffara F, Gaia E, Martino E, Lombardi G, Angeli A, Colao A. Colonoscopic screening and follow-up in patients with acromegaly: a multicenter study in Italy. *J Clin Endocrinol Metab* 2005; **90**: 84-90
  - 39 **Bogazzi F**, Cosci C, Sardella C, Costa A, Manetti L, Gasperi M, Rossi G, Bartalena L, Martino E. Identification of acromegalic patients at risk of developing colonic adenomas. *J Clin Endocrinol Metab* 2006; **91**: 1351-1356
  - 40 **Jenkins PJ**, Besser M. Clinical perspective: acromegaly and cancer: a problem. *J Clin Endocrinol Metab* 2001; **86**: 2935-2941
  - 41 **Melmed S**. Acromegaly and cancer: not a problem? *J Clin Endocrinol Metab* 2001; **86**: 2929-2934
  - 42 **Jenkins PJ**, Fairclough PD. Colorectal neoplasia in acromegaly. *Clin Endocrinol (Oxf)* 2001; **55**: 727-729
  - 43 **Atkin WS**. Risk of colorectal neoplasia in acromegaly: an independent view. *Clin Endocrinol (Oxf)* 2001; **55**: 723-725
  - 44 **Renahan AG**, Odwyer ST, Shalet SM. Screening colonoscopy for acromegaly in perspective. *Clin Endocrinol (Oxf)* 2001; **55**: 731-733
  - 45 **Ma J**, Pollak MN, Giovannucci E, Chan JM, Tao Y, Hennekens CH, Stampfer MJ. Prospective study of colorectal cancer risk in men and plasma levels of insulin-like growth factor (IGF)-I and IGF-binding protein-3. *J Natl Cancer Inst* 1999; **91**: 620-625
  - 46 **Giovannucci E**, Pollak MN, Platz EA, Willett WC, Stampfer MJ, Majeed N, Colditz GA, Speizer FE, Hankinson SE. A prospective study of plasma insulin-like growth factor-1 and binding protein-3 and risk of colorectal neoplasia in women. *Cancer Epidemiol Biomarkers Prev* 2000; **9**: 345-349
  - 47 **Kaaks R**, Toniolo P, Akhmedkhanov A, Lukanova A, Biessy C, Dechaud H, Rinaldi S, Zeleniuch-Jacquotte A, Shore RE, Riboli E. Serum C-peptide, insulin-like growth factor (IGF)-I, IGF-binding proteins, and colorectal cancer risk in women. *J Natl Cancer Inst* 2000; **92**: 1592-1600
  - 48 **Juul A**, Pedersen SA, Sorensen S, Winkler K, Jørgensen JO, Christiansen JS, Skakkebaek NE. Growth hormone (GH) treatment increases serum insulin-like growth factor binding protein-3, bone isoenzyme alkaline phosphatase and forearm bone mineral content in young adults with GH deficiency of childhood onset. *Eur J Endocrinol* 1994; **131**: 41-49
  - 49 **Ghigo E**, Aimaretti G, Maccario M, Fanciulli G, Arvat E, Minuto F, Giordano G, Delitala G, Camanni F. Dose-response study of GH effects on circulating IGF-I and IGFBP-3 levels in healthy young men and women. *Am J Physiol* 1999; **276**: E1009-E1013
  - 50 **Giovannucci E**, Pollak M. Risk of cancer after growth-hormone treatment. *Lancet* 2002; **360**: 268-269
  - 51 **Lahm H**, Amstad P, Wyniger J, Yilmaz A, Fischer JR, Schreyer M, Givel JC. Blockade of the insulin-like growth-factor-I receptor inhibits growth of human colorectal cancer cells: evidence of a functional IGF-II-mediated autocrine loop. *Int J Cancer* 1994; **58**: 452-459
  - 52 **Simmons JG**, Pucilowska JB, Lund PK. Autocrine and paracrine actions of intestinal fibroblast-derived insulin-like growth factors. *Am J Physiol* 1999; **276**: G817-G827
  - 53 **Cats A**, Dullaart RP, Kleibeuker JH, Kuipers F, Sluiter WJ, Hardonk MJ, de Vries EG. Increased epithelial cell proliferation in the colon of patients with acromegaly. *Cancer Res* 1996; **56**: 523-526
  - 54 **Durrant LG**, Watson SA, Hall A, Morris DL. Co-stimulation of gastrointestinal tumour cell growth by gastrin, transforming growth factor alpha and insulin like growth factor-I. *Br J Cancer* 1991; **63**: 67-70
  - 55 **Lahm H**, Suardet L, Laurent PL, Fischer JR, Ceyhan A, Givel JC, Odartchenko N. Growth regulation and co-stimulation of human colorectal cancer cell lines by insulin-like growth factor I, II and transforming growth factor alpha. *Br J Cancer* 1992; **65**: 341-346

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## VIRAL HEPATITIS

# Replication of clinical hepatitis B virus isolate and its application for selecting antiviral agents for chronic hepatitis B patients

Yin-Ping Lu, Tao Guo, Bao-Ju Wang, Ji-Hua Dong, Jian-Fang Zhu, Zhao Liu, Meng-Ji Lu, Dong-Liang Yang

Yin-Ping Lu, Tao Guo, Ji-Hua Dong, Jian-Fang Zhu, Zhao Liu, Department of Virology, Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China

Yin-Ping Lu, Bao-Ju Wang, Dong-Liang Yang, Division of Clinical Immunology, Tongji Hospital of Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

Meng-Ji Lu, Institute of Virology, Duisburg-Essen University, Essen 45122, Germany

**Author contributions:** Lu YP and Guo T contributed equally to this work; Lu YP, Guo T and Yang DL designed the research; Lu YP, Guo T, Wang BJ, Dong JH and Zhu JF performed the research; Lu YP and Guo T analyzed the data; Lu MJ provided reagents/analytic tools; and Yang DL offered financial support.

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**Correspondence to:** Dr. Yin-Ping Lu, Department of Virology, Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, 1277 Jiefang Avenue, Wuhan 430022, Hubei Province, China. [yinpinglu@163.com](mailto:yinpinglu@163.com)  
Telephone: +86-27-85726121 Fax: +86-27-85776343

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were obtained from the sera of 8 patients, each patient had at least two isolates. One isolate from each individual was selected and subcloned into pHY106 vector, including 5 isolates with YVDD mutation and 3 isolates with YIDD mutation. All recombinant plasmids harboring HBV isolates were transfected into Huh7 cells. The results indicated that HBV genome carried in HBV replicons of clinical HBV isolates could effectively replicate and express in Huh7 cells. Adefovir, but not lamivudine, inhibited HBV replication both *in vitro* and *in vivo*, and *in vitro* inhibition was dose-dependent.

**CONCLUSION:** The novel method described herein enables individualized selection of anti-HBV agents in clinic and is useful in future studies of antiviral therapy for CHB.

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**Key words:** Hepatitis B virus; Chronic hepatitis B; Hepatitis B virus isolate; Antiviral agents

**Peer reviewer:** Vasiliy I Reshetnyak, MD, PhD, Professor, Scientific Research Institute of General Reanimatology, 25-2, Petrovka Str., 107031, Moscow, Russia

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## Abstract

**AIM:** To establish a cell model harboring replicative clinical hepatitis B virus (HBV) isolates and evaluate its application in individualized selection of anti-HBV agents for chronic hepatitis B (CHB) patients.

**METHODS:** The full-length HBV genomic DNA from 8 CHB patients was amplified by polymerase chain reaction (PCR). All the patients were treated with lamivudine for at least seven months and finally became resistant to lamivudine. The amplified HBV DNA fragments were inserted into pHY106 vectors by *Sap* I digestion. The recombinant plasmids containing 1.1 copies of HBV genome were transiently transfected into Huh7 cell line, and the levels of HBsAg, HBeAg and intercellular HBV replicative intermediates were determined by ELISA and Southern blot analysis, respectively, with or without lamivudine and adefovir treatment. The antiviral treatment with adefovir was administered to the patients and analyzed in parallel.

**RESULTS:** A total of 25 independent HBV isolates

## INTRODUCTION

Hepatitis B virus (HBV) infection may lead to acute liver disease, chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC). Over 350 million people worldwide are estimated to be infected chronically by HBV and are, therefore, at risk of liver failure, cirrhosis, or HCC<sup>[1-4]</sup>. The principal treatment for chronic hepatitis B (CHB) involves the use of interferon alpha (IFN- $\alpha$ ) or nucleoside analogs.

Although the use of IFN- $\alpha$  and nucleoside analogs (such as lamivudine and adefovir) has improved the treatment of chronically infected HBV patients, an

effective reduction in virus load is only observed in less than 40% of treated patients<sup>[5-10]</sup>. The molecular basis for resistance to antiviral therapy is not clearly defined. However, studies have suggested that HBV genome mutations may play a direct role in the development of resistance to antiviral agents<sup>[11-15]</sup>.

*In vitro* analysis of clinical HBV isolates is difficult due to the lack of HBV cell culture model<sup>[16-19]</sup>. Fortunately, the lack of *in vitro* infectivity can be conquered by transfecting recombinant plasmids encoding over-length HBV genome into human liver cell lines. Following transfection, HBV pre-genomic and messenger RNA are transcribed from the plasmids and progeny virus is replicated and released from cells. The present study is to develop a more efficient method for selecting anti-HBV agents using a highly efficient HBV expression vector. It is supposed to be useful for future studies on resistance surveillance and novel drug discovery.

## MATERIALS AND METHODS

### Patients

Eight patients with CHB were selected according to the diagnostic criteria of CHB<sup>[20]</sup>, and the infection of hepatitis A, C, E and G viruses, human immunodeficiency virus, cytomegalovirus and the use of alcohol were excluded. All patients were treated with lamivudine for more than seven months, and were found to be resistant to lamivudine treatment in the Department of Infectious Diseases of Union Hospital of Tongji Medical College, Huazhong University of Science and Technology, from January 2005 to August 2005. All the patients were HBsAg and HBeAg positive, with HBV DNA levels above 10<sup>6</sup> copies/mL and elevated serum ALT ( $\geq 1.5$  times that of the upper normal limit) at the time of sample collection. Access to the materials complied with the Helsinki Declaration and approved by the local ethics committee, and the informed consents were obtained from all the patients.

### Plasmids and bacteria

HBV cloning and expression vector pHY106 was kindly provided by Dr. Lu MJ (Institute of Virology, Duisburg-Essen University, Germany), which contains a cytomegalovirus (CMV) promoter upstream of a short, recombinant HBV sequence that allows the in-frame insertion of a full-length HBV genome by *Sap* I digestion (Figure 1). The HBV replicon, pHBV1.3, containing 1.3-fold full length genome of HBV (*ayw* subtype), was conducted in our laboratory as described previously<sup>[21]</sup>. Plasmid pUC19 and *E.coli* JM109 strain were maintained in our laboratory and stored at -80°C.

### Cell culture

Human hepatoblastoma cell line, Huh7, was maintained in MEM medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a moist atmosphere containing 5% CO<sub>2</sub>.

### Primers design

Primers were designed according to Gunther's method<sup>[22]</sup>, P1 (1803-1821): 5'-CCGGAAGCTTGAGCTCTTC-TTTTTCACCTCTGCCTAATCA-3' and P2 (1841-1822): 5'-CCGGAAGCTTGAGCTCTTCAAA AAGTTGCATGGTGCTGG-3' for amplifying HBV full-length genome. The italics denoted *Hind* III, *Sac* I and *Sap* I sites for inserting HBV genome into pUC19 and PHY106.

### Amplification of full-length HBV genomic DNA

HBV DNA was isolated from 200  $\mu$ L sera using QIAamp Blood Kit (Qiagen Co., Germany) according to manufacturer's instructions. Polymerase chain reaction (PCR) amplification of full-length HBV genomes was performed using primers P1 and P2 according to the methods of Gunther *et al.*<sup>[22]</sup>. If the initial PCR reactions were negative, a second round of PCR was performed under identical conditions using 5  $\mu$ L of 1:10 dilution of the first round reaction products as template.

### Construction of recombinant plasmids harboring full-length HBV genome

Following PCR amplification, PCR product was purified using a QIAquick Gel Kit (Qiagen Co., Germany) according to manufacturer's instructions. Then PCR products and plasmid pUC19 were digested with the restriction enzyme *Sac* I, and the 3.2 kb PCR products were inserted into the linear pUC19 vector (Figure 2). Recombinant plasmids were identified by restriction enzyme cleavage and termed as pUC19-HBV1.0.

### Construction of HBV replicons harboring 1.1-fold length HBV genome

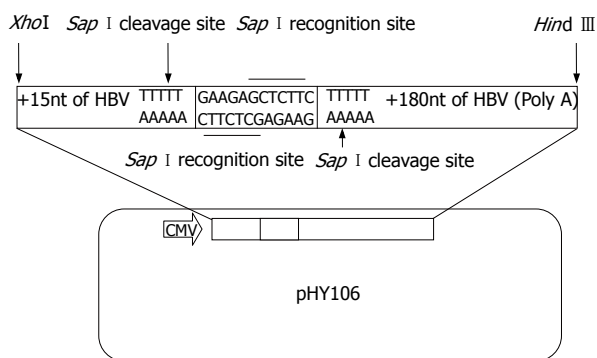
pUC19-HBV1.0 was cleaved with the restriction enzyme *Sap* I, and a 3.2 kb fragment was recovered and subcloned into pPHY106 vector (Figure 2). Recombinant plasmids were identified by *Hind* III and *Nsi* I digestion and termed as pPHY106-HBV1.1. HBV polymerase gene of pPHY106-HBV1.1 was analyzed by sequencing.

### Replication of clinical HBV replicon in Huh7 cell line

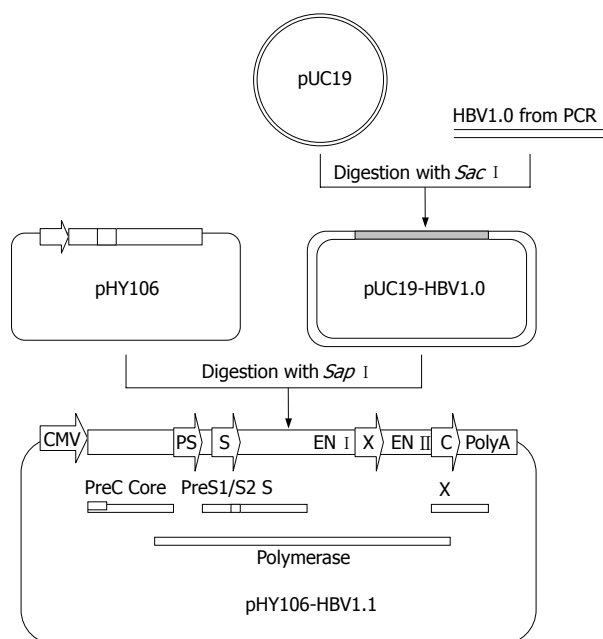
The plasmid pPHY106-HBV1.1 was transiently transfected into Huh7 cells, and the replication of clinical HBV replicons was measured. Huh7 cells were seeded with  $5 \times 10^5$ /well and cultured in six-well plates. Sixteen hours later, the cells were transfected with 5  $\mu$ g of plasmid pPHY106-HBV1.1 using 5  $\mu$ L lipofectamine reagent (Invitrogen Co., USA) according to the manufacturer's instructions. Following transfection, cultures were fed with fresh media and incubated for 96 h. The levels of HBsAg, HBeAg and HBV DNA in supernatant were determined by ELISA and quantitative PCR everyday. Meanwhile, the total cellular DNA was isolated and HBV replicative intermediates were detected by Southern blot hybridization.

### Southern blot analysis of HBV replicative intermediates

Five  $\mu$ g of total DNA of transfected cells was separated



**Figure 1** The schematic map of HBV expression vector of pHY106.

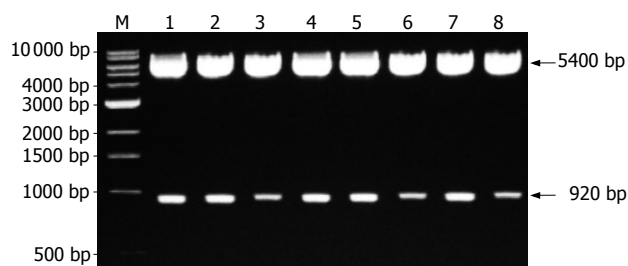


**Figure 2** Construction of replicons of clinical HBV isolates using pHY106. Full-length HBV genomes from PCR application can be digested with *Sap* I and inserted into *Sap* I-digested pHY106 to produce HBV replicon. HBV open reading frames (ORFs) for the pre-core (PC), core, preS1 (PS1), preS2 (2) surface (S), polymerase, and X genes are indicated by square frame. Promoter sequences for the preS (PS), surface (S) and core (C) genes are indicated by arrows.

on 1.5% agarose gels, and blot onto Hybond-N nylon membranes (Amersham Bioscience Inc, UK) using standard Southern blot procedures. Membranes were hybridized with a  $^{32}$ P-labeled full-length HBV DNA fragment and viral DNA was analyzed using a storage phosphor system (Cyclone, Packard Bioscience Co.).

#### Antiviral treatment in Huh7 cells transfected with clinical HBV replicons

Plasmid pHY106-HBV1.1 was transiently transfected into Huh7 cells as described above. The next day, cells were fed with fresh medium containing 0.01, 0.1, 1 and 10  $\mu$ mol/L of adefovir (Gilead Sciences, USA) and 0.01, 0.1, 1 and 10  $\mu$ mol/L of lamivudine, respectively, for 72 h, after which intracellular HBV replicative intermediates were isolated and quantified as described above.



**Figure 3** Identification of recombinant expression plasmids by restriction endonuclease digestion analysis. M: 1 kb DNA ladder marker; Lane 1-8: Recombinant plasmids digested with *Hind* III and *Nsi* I.

#### Statistical analysis

Data were presented as mean  $\pm$  SD error of the mean (SEM). Statistical significance of differences between the measured parameters was determined by Mann-Whitney's test. Statistical analyses were performed using the SPSS13.0 software. The values were statistically significant at  $P < 0.05$ .

## RESULTS

#### Amplification and cloning of full-length clinical HBV isolates

Using the method of Gunther *et al*, full-length HBV DNA was amplified from the sera of 6 patients following a single round of PCR. For the other two cases, a second round of PCR was performed to amplify HBV full-length genome successfully. In general, it was more difficult to amplify full-length HBV genome from patients with low levels of viremia. The PCR products were cloned into pUC19 vector, and a total of 25 independent HBV clones were obtained from the sera of the 8 patients and each patient had at least two clones.

#### Construction of HBV replicons harboring 1.1-fold length HBV genome

One clone was selected from each patient, and was subcloned into pHY106 vector. The directions of inserted clones were identified by digestion with restriction enzyme *Hind* III and *Nsi* I (*Nsi* I is a single restriction site at nt1064 of HBV genome). If HBV clones were forwardly inserted, the recombinant plasmid should be cleaved into two fragments with a molecular weight of 5400 bp and 920 bp (Figure 3). Polymerase gene of 5 HBV isolates showed YVDD mutation and the other 3 isolates showed YIDD mutation.

#### Replication of clinical HBV replicons in Huh7 cell line

Eight of the HBV isolates cloned into the PHY106 vector were analyzed for their ability to replicate *in vitro*. Recombinant plasmids were transfected into Huh7 cells and allowed to replicate for 96 h, after which HBsAg, HBeAg and HBV DNA were detected, and intracellular replicative intermediates were extracted and analyzed by Southern blot hybridization. A marked variation was observed in the levels of HBsAg, HBeAg, HBV DNA and intracellular replicative intermediates among



Table 1 HBsAg and HBeAg levels of clinical HBV isolates in culture supernatants (mean ± SD)

Isolates	24 h		48 h		72 h		96 h	
	HBsAg	HBeAg	HBsAg	HBeAg	HBsAg	HBeAg	HBsAg	HBeAg
1	0.236 ± 0.028	0.158 ± 0.018	0.792 ± 0.055	0.435 ± 0.039	1.892 ± 0.168	1.276 ± 0.138	1.725 ± 0.258	1.212 ± 0.237
2	0.225 ± 0.019	0.149 ± 0.014	0.646 ± 0.043	0.453 ± 0.044	1.737 ± 0.132	1.321 ± 0.215	1.722 ± 0.633	1.121 ± 0.153
3	0.214 ± 0.038	0.152 ± 0.015	0.756 ± 0.075	0.421 ± 0.054	1.654 ± 0.156	1.232 ± 0.342	1.563 ± 0.742	1.133 ± 0.217
4	0.195 ± 0.022	0.122 ± 0.012	0.533 ± 0.057	0.349 ± 0.033	1.278 ± 0.132	1.133 ± 0.142	1.236 ± 0.571	1.123 ± 0.342
5	0.189 ± 0.028	0.112 ± 0.013	0.636 ± 0.062	0.352 ± 0.024	0.996 ± 0.114	0.982 ± 0.211	1.006 ± 0.263	1.012 ± 0.411
6	0.188 ± 0.033	0.132 ± 0.013	0.537 ± 0.075	0.384 ± 0.042	0.987 ± 0.132	0.952 ± 0.156	0.945 ± 0.154	0.943 ± 0.125
7	0.179 ± 0.018	0.122 ± 0.012	0.639 ± 0.031	0.298 ± 0.027	1.139 ± 0.163	0.892 ± 0.142	0.997 ± 0.218	0.885 ± 0.218
8	0.236 ± 0.043	0.155 ± 0.015	0.836 ± 0.061	0.475 ± 0.039	1.754 ± 0.174	1.312 ± 0.111	1.825 ± 0.318	1.212 ± 0.323
Positive	0.336 ± 0.048	0.217 ± 0.028	0.956 ± 0.078	0.552 ± 0.066	1.868 ± 0.218	1.202 ± 0.324	1.922 ± 0.525	1.189 ± 0.305
Negative	0.046 ± 0.012	0.032 ± 0.011	0.044 ± 0.018	0.040 ± 0.015	0.047 ± 0.020	0.038 ± 0.012	0.042 ± 0.013	0.039 ± 0.018

Plasmid pHBV 1.3 is positive control; Plasmid pHY106 is negative control.

Table 2 Viral loads in supernatants of Huh7 cells transfected with HBV replicon (copies/mL)

Replicons	Transfected time (h)			
	24	48	72	96
1	$5.6 \times 10^2$	$2.5 \times 10^3$	$3.6 \times 10^5$	$3.9 \times 10^5$
2	$5.2 \times 10^2$	$2.0 \times 10^3$	$2.8 \times 10^5$	$2.7 \times 10^5$
3	$4.5 \times 10^2$	$1.8 \times 10^3$	$2.4 \times 10^5$	$2.6 \times 10^5$
4	$< 10^2$	$1.1 \times 10^3$	$7.5 \times 10^4$	$9.7 \times 10^4$
5	$< 10^2$	$1.2 \times 10^3$	$8.2 \times 10^4$	$9.1 \times 10^4$
6	$< 10^2$	$1.5 \times 10^3$	$7.8 \times 10^4$	$8.9 \times 10^4$
7	$< 10^2$	$1.3 \times 10^3$	$9.5 \times 10^4$	$1.2 \times 10^5$
8	$5.5 \times 10^2$	$2.3 \times 10^3$	$2.8 \times 10^5$	$2.9 \times 10^5$
pHBV1.3	$6.5 \times 10^2$	$2.8 \times 10^3$	$2.5 \times 10^5$	$2.7 \times 10^5$
pHY106	$< 10^2$	$< 10^2$	$< 10^2$	$< 10^2$

pHBV1.3 is positive control; pHY106 is negative control.

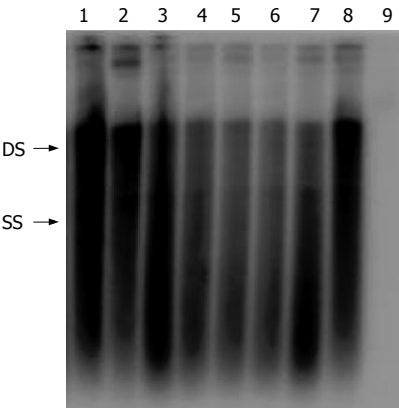


Figure 4 Analysis of intracellular replication of clinical HBV isolates. HBV isolates were cloned into the vector pHY106 and transfected into Huh2 cells. Seventy-two hours after transfection, intracellular replicative intermediates were isolated and analyzed by Southern blot. Double-stranded (DS) and single-stranded (SS) intermediates are indicated. Lane 1-8: eight replicons of clinical HBV isolates; Lane 9: pHY106 negative control.

individual clones (Tables 1 and 2, Figure 4). The HBsAg and HBeAg levels rapidly reached to a plateau within 72 h. Further incubation did not increase the concentration of HBsAg and HBeAg in the culture supernatants. There was also a consistency of the HBsAg, HBeAg and HBV DNA levels in supernatants (Tables 1 and 2).

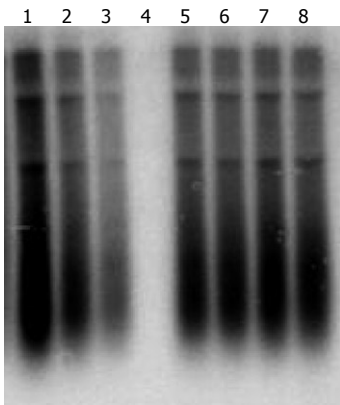


Figure 5 Antiviral susceptibility of an HBV isolate cloned from a patient with lamivudine resistance. An HBV isolate amplified from the sera of patients failing in lamivudine therapy was cloned into the pHY106 vector and analyzed *in vitro*. Lane 1-4: transfected cells treated with 0.01, 0.1, 1 and 10 μmol/L of adefovir; Lane 5-8: transfected cells treated with 0.01, 0.1, 1 and 10 μmol/L of lamivudine.

**Antiviral treatment in Huh7 cells transfected with clinical HBV replicons**

A cell-based antiviral assay was used to test the lamivudine and adefovir susceptibility of the 8 clinical isolates that replicated efficiently in cell culture. The results indicated that adefovir but not lamivudine, efficiently inhibited the replication of clinical HBV isolates *in vitro*, furthermore, the inhibition effect depended upon the concentration of adefovir. The viral replication was completely inhibited in the transfected cells treated with 10 μmol/L adefovir (Figure 5).

**Antiviral treatment in vivo**

All chronic HBV infected patients who had resistance to lamivudine were treated with adefovir for 6 mo. These patients were sensitive to adefovir therapy, and their HBV viral load in sera dropped at least  $10^3$ - $10^5$  copies/mL. Together with the results of antiviral test of adefovir *in vitro* as described above, it indicated that antiviral susceptibility of clinical HBV isolates was well coincident between *in vivo* and *in vitro*.

**DISCUSSION**

HBV contains a 3.2-kb, circular, double-stranded DNA genome and causes acute and chronic hepatitis B, cirrhosis, and eventually HCC<sup>[23]</sup>. Investigation of the expression and replication of the HBV genome as well as the full viral life cycle is hampered by the lack

of an *in vitro* tissue culture system in which HBV is propagated<sup>[16-19]</sup>. The HBV cell model system has been the subject of many studies in the last four decades. Unfortunately, no study has reported the identification of an appropriate tissue culture system to propagate HBV. This has greatly hindered the progress of HBV studies in many fields, such as virology, molecular biology, immunology and antiviral therapy. In an attempt to overcome this obstacle, several researchers transduced HBV genome into human hepatocyte *via* plasmids, in which HBV gene could replicate, express, and even assemble infectious virions<sup>[21,24-26]</sup>. Because the HBV genome is very dense and genes overlap with each other, HBV genome was inserted into plasmids in a head-to-tail linkage manner.

Recently, many groups have transfected over-length HBV genome into hepatocytes for viral replication and anti-HBV studies<sup>[21,24-26]</sup>. It is well known that the longest HBV transcript is a 3.5 kb mRNA; therefore, several plasmids carrying HBV multiple genomes were constructed, such as 2.0 copies and 1.3 copies of HBV genome, these over-length HBV genome contains complete HBV replication units, such as Enh I, Enh II, DR1, DR2, the transcription origin site of viral pre-genome, promoters and ORFs. Although these artificially constructed HBV replicons played an important role in anti-HBV studies, they were not suitable for the individualized selection of antiviral agents. Recently many scientists working in HBV fields considered that HBV replicated as quasi-species *in vivo* and individual species are likely to vary<sup>[27,28]</sup>. HBV genome mutation is mostly caused by immune and drug pressures, for example, CHB patients treated with lamivudine, when mutation appeared in polymerase gene of HBV, will be resistant to lamivudine therapy. As a result, HBV replicon can be used for the individualized selection of antiviral agents *in vitro* based on clinical HBV isolates.

In this study, we established a novel method to assess *in vitro* replication of clinical HBV isolate and investigated its application for selecting antiviral agents for CHB patients. We employed pHY106 vector to construct HBV replicons of clinical isolates. The pHY106 contains a CMV promoter, followed by a 15 nucleotide HBV sequence encoding the pre-core initiation site (plus the next two amino acids of the pre-core protein), a short heterologous linker sequence that contains two *Sap* I sites, and finally a 180 nucleotide region that encodes the carboxy terminus of the HBV X gene (five amino acids) and the polyadenylation signal for HBV mRNA. Following digestion of pHY106 and full-length HBV genomes with *Sap* I (Figure 2), HBV genomes can be inserted in-frame, resulting in the generation of replication-competent HBV clones, 95% of which are derived from patient virus (Figure 2). The CMV promoter upstream of the pre-core initiation site allows efficient transcription of the 3.5 kb pre-genomic RNA after transfection of liver cell lines. The minimal 5' and 3' sequence derived from the wild type HBV

strain in pHY106 is highly conserved among published HBV isolates. Thus, HBV isolates cloned into the pHY106 vector should be representative of patient virus with little or no genotypic changes.

We obtained 8 clinical HBV replicons from CHB patients with lamivudine resistance, and found that these replicons were not susceptible to lamivudine treatment *in vitro* because of YMDD mutation of polymerase gene. Sequence analysis of clinical HBV isolates with established drug resistant mutations will provide useful information for selecting the existing or novel antiviral agents or the combination of antiviral agents for further treatment. At least four major mutational patterns are associated with lamivudine resistance (rtM204I, rtL180M + rtM204I, rtL180M + M204V, and rtV173L + rtL180M + M204V)<sup>[29]</sup>. Many mutations enhance viral replication, e.g., the pre-core mutation G1896A has been shown to greatly enhance the replication capacity of HBV encoding the rtM204I (but not rtM204V) lamivudine resistant mutation<sup>[30]</sup>. Adefovir demonstrated an excellent therapeutic effect for CHB patients who failed in lamivudine therapy<sup>[31-35]</sup>. Together with the results of antiviral treatment of adefovir *in vitro* and *in vivo*, our results confirmed that adefovir inhibited the replication of HBV with lamivudine resistance *in vitro* and *in vivo*, and indicated that antiviral susceptibility of clinical HBV isolates was well coincident between *in vivo* and *in vitro*. Therefore, the antiviral treatment *in vitro* will provide useful information for individualized selection of antiviral agents *in vivo*.

Several antiviral agents including lamivudine, adefovir and entecavir are available for CHB treatment, and several novel antiviral agents for CHB treatment are being developed. *In vitro* analysis of drug susceptibility based on clinical HBV isolates is useful for personalized selection of the existing or novel antiviral agents or combinations of antiviral agents for treatment. However, there are still some problems to be resolved. Firstly, HBV replicates as quasi-species *in vivo*, and the replication capacity of individual species are likely to vary; therefore the predominant HBV isolate should be selected in this study. Secondly, the replication of HBV *in vitro* is significantly different from that of *in vivo*, and the influence of the difference between *in vitro* and *in vivo* on antiviral agent selection should be considered. Finally, despite the use of a high fidelity DNA polymerase for HBV genome amplification, artificial mutation may occur in HBV genome, and the replication of HBV isolates may be affected.

Our study described a novel strategy and method for developing an *in vitro* system for selection of antiviral agents based on clinical HBV isolates, and evaluated the application in individualized selection of antiviral agents for chronic hepatitis B patients. The results indicated that *in vitro* analysis of drug susceptibility based on clinical HBV isolates is useful for individualized selection of antiviral agents for patients with CHB.

## COMMENTS

### Background

Hepatitis B virus (HBV) is the leading cause of chronic hepatitis. Antiviral treatment of chronic hepatitis B (CHB) relies on interferon alpha and nucleoside analogs that inhibit activity of HBV polymerase. However, resistance of HBV to antiviral nucleoside analogs, caused by mutation of HBV polymerase gene, has become a major clinical problem. Moreover, the lack of reliable *in vitro* infection system and convenient models has hindered the new treatment options. Therefore, establishment of new strategies for studying *in vitro* the drug susceptibility of clinical isolates of HBV is extremely important for antiviral therapy for CHB patients.

### Research frontiers

The phenotypic assays have first been developed for monitoring drug susceptibility of human immunodeficiency virus viral populations. Some of them are used currently in clinical practice to monitor drug resistance. As compared with the human immunodeficiency virus phenotypic assay, the development and clinical use of the HBV phenotypic assay are still at an early stage. This study demonstrated the relevance of this type of assay for longitudinal monitoring of the drug susceptibility status of the viral population isolated from clinical samples.

### Innovations and breakthroughs

This study describes a novel strategy and method for the cloning of HBV genomes isolated from CHB patients into plasmidic vectors and the basis of another phenotypic assay capable of assessing HBV drug susceptibility *in vitro* and evaluates the effects of antiviral agents in circulating viral isolates from chronic HBV.

### Applications

This work may help determine the drug susceptibility of HBV quasi-species to antivirals and select antiviral agents for CHB patients.

### Peer review

In this study the authors established a novel method to assess *in vitro* replication of clinical HBV isolate and investigated its application for selecting antiviral agents for CHB patients. It will be very useful in future studies of antiviral therapy for CHB.

## REFERENCES

- Maddrey WC. Hepatitis B: an important public health issue. *J Med Virol* 2000; **61**: 362-366
- Lai CL, Ratziu V, Yuen MF, Poynard T. Viral hepatitis B. *Lancet* 2003; **362**: 2089-2094
- Villeneuve JP. The natural history of chronic hepatitis B virus infection. *J Clin Virol* 2005; **34** Suppl 1: S139-S142
- Cougot D, Neuveut C, Buendia MA. HBV induced carcinogenesis. *J Clin Virol* 2005; **34** Suppl 1: S75-S78
- Farrell GC, Teoh NC. Management of chronic hepatitis B virus infection: a new era of disease control. *Intern Med J* 2006; **36**: 100-113
- Loomba R, Liang TJ. Novel approaches to new therapies for hepatitis B virus infection. *Antivir Ther* 2006; **11**: 1-15
- Craxi A, Antonucci G, Camma C. Treatment options in HBV. *J Hepatol* 2006; **44**: S77-S83
- Zoulim F. Entecavir: a new treatment option for chronic hepatitis B. *J Clin Virol* 2006; **36**: 8-12
- Liu CJ, Lai MY, Chao YC, Liao LY, Yang SS, Hsiao TJ, Hsieh TY, Lin CL, Hu JT, Chen CL, Chen PJ, Kao JH, Chen DS. Interferon alpha-2b with and without ribavirin in the treatment of hepatitis B e antigen-positive chronic hepatitis B: a randomized study. *Hepatology* 2006; **43**: 742-749
- Leemans WF, Flink HJ, Janssen HL, Niesters HG, Schalm SW, de Man RA. The effect of pegylated interferon-alpha on the treatment of lamivudine resistant chronic HBsAg positive hepatitis B virus infection. *J Hepatol* 2006; **44**: 507-511
- Das K, Xiong X, Yang H, Westland CE, Gibbs CS, Sarafianos SG, Arnold E. Molecular modeling and biochemical characterization reveal the mechanism of hepatitis B virus polymerase resistance to lamivudine (3TC) and emtricitabine (FTC). *J Virol* 2001; **75**: 4771-4779
- Buti M, Rodriguez-Frias F, Jardi R, Esteban R. Hepatitis B virus genome variability and disease progression: the impact of pre-core mutants and HBV genotypes. *J Clin Virol* 2005; **34** Suppl 1: S79-S82
- Liaw YF. The current management of HBV drug resistance. *J Clin Virol* 2005; **34** Suppl 1: S143-S146
- Tong S. Mechanism of HBV genome variability and replication of HBV mutants. *J Clin Virol* 2005; **34** Suppl 1: S134-S138
- Pawlotsky JM. The concept of hepatitis B virus mutant escape. *J Clin Virol* 2005; **34** Suppl 1: S125-S129
- Paran N, Geiger B, Shaul Y. HBV infection of cell culture: evidence for multivalent and cooperative attachment. *EMBO J* 2001; **20**: 4443-4453
- Yang J, Ding X, Zhang Y, Bo X, Zhang M, Wang S. Fibronectin is essential for hepatitis B virus propagation in vitro: may be a potential cellular target? *Biochem Biophys Res Commun* 2006; **344**: 757-764
- Guha C, Mohan S, Roy-Chowdhury N, Roy-Chowdhury J. Cell culture and animal models of viral hepatitis. Part I: hepatitis B. *Lab Anim* (NY) 2004; **33**: 37-46
- Tang H, Raney AK, McLachlan A. Replication of the wild type and a natural hepatitis B virus nucleocapsid promoter variant is differentially regulated by nuclear hormone receptors in cell culture. *J Virol* 2001; **75**: 8937-8948
- Chinese Society of Hepatology and Chinese Society of infectious Diseases, Chinese Medical Association. The guideline of prevention and treatment for chronic hepatitis B. *Zhonghua Ganzangbing Zazhi* 2005; **13**: 881-891
- Lu YP, Wang BJ, Dong JH, Liu Z, Guan SH, Lu MJ, Yang DL. Construction and Characterization of a Hepatitis B Virus Replicon. *Virologica Sinica* 2007; **22**: 8-13
- Gunther S, Li BC, Miska S, Kruger DH, Meisel H, Will H. A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J Virol* 1995; **69**: 5437-5444
- Seeger C, Mason WS. Hepatitis B virus biology. *Microbiol Mol Biol Rev* 2000; **64**: 51-68
- Sells MA, Chen ML, Acs G. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc Natl Acad Sci USA* 1987; **84**: 1005-1009
- Yaginuma K, Shirakata Y, Kobayashi M, Koike K. Hepatitis B virus (HBV) particles are produced in a cell culture system by transient expression of transfected HBV DNA. *Proc Natl Acad Sci USA* 1987; **84**: 2678-2682
- Yang PL, Althage A, Chung J, Chisari FV. Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. *Proc Natl Acad Sci USA* 2002; **99**: 13825-13830
- Xu H, Peng M, Qing Y, Ling N, Lan Y, Liang Z, Cai D, Li Y, Ren H. A Quasi species of the pre-S/S gene and mutations of enhancer II/core promoter/pre-C in mothers and their children infected with hepatitis B virus via mother-to-infant transmission. *J Infect Dis* 2006; **193**: 88-97
- Burda MR, Gunther S, Dandri M, Will H, Petersen J. Structural and functional heterogeneity of naturally occurring hepatitis B virus variants. *Antiviral Res* 2001; **52**: 125-138
- Westland CE, Yang H, Delaney WE 4th, Wulfssohn M, Lama N, Gibbs CS, Miller MD, Fry J, Brosgart CL, Schiff ER, Xiong S. Activity of adefovir dipivoxil against all patterns of lamivudine-resistant hepatitis B viruses in patients. *J Viral Hepat* 2005; **12**: 67-73
- Chen RY, Edwards R, Shaw T, Colledge D, Delaney WE 4th, Isom H, Bowden S, Desmond P, Locarnini SA. Effect of the G1896A precore mutation on drug sensitivity and replication yield of lamivudine-resistant HBV in vitro. *Hepatology* 2003; **37**: 27-35
- Suzuki F, Kumada H, Nakamura H. Changes in viral loads of lamivudine-resistant mutants and evolution of HBV sequences during adefovir dipivoxil therapy. *J Med Virol* 2006; **78**: 1025-1034

- 32 **Zeng M**, Mao Y, Yao G, Wang H, Hou J, Wang Y, Ji BN, Chang CN, Barker KF. A double-blind randomized trial of adefovir dipivoxil in Chinese subjects with HBeAg-positive chronic hepatitis B. *Hepatology* 2006; **44**: 108-116
- 33 **Schildgen O**, Sirma H, Funk A, Olotu C, Wend UC, Hartmann H, Helm M, Rockstroh JK, Willems WR, Will H, Gerlich WH. Variant of hepatitis B virus with primary resistance to adefovir. *N Engl J Med* 2006; **354**: 1807-1812
- 34 **Marcellin P**, Chang TT, Lim SG, Tong MJ, Sievert W, Shiffman ML, Jeffers L, Goodman Z, Wulfsohn MS, Xiong S, Fry J, Brosgart CL. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med* 2003; **348**: 808-816
- 35 **Hadziyannis SJ**, Tassopoulos NC, Heathcote EJ, Chang TT, Kitis G, Rizzetto M, Marcellin P, Lim SG, Goodman Z, Wulfsohn MS, Xiong S, Fry J, Brosgart CL. Adefovir dipivoxil for the treatment of hepatitis B e antigen-negative chronic hepatitis B. *N Engl J Med* 2003; **348**: 800-807

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# Disease activity and cancer risk in inflammatory bowel disease associated with primary sclerosing cholangitis

Harry Sokol, Jacques Cosnes, Olivier Chazouilleres, Laurent Beaugerie, Emmanuel Tiret, Raoul Poupon, Philippe Seksik

Harry Sokol, Jacques Cosnes, Laurent Beaugerie, Philippe Seksik, Gastroenterology and Nutrition Department, Hôpital Saint-Antoine, Université Pierre et Marie Curie-Paris6, AP-HP, Paris 75571, France

Olivier Chazouilleres, Raoul Poupon, Hepatology Department, Hôpital Saint-Antoine, Université Pierre et Marie Curie-Paris6, AP-HP, Paris 75571, France

Emmanuel Tiret, Department of Abdominal Surgery, Hôpital Saint-Antoine, Université Pierre et Marie Curie-Paris6, AP-HP, Paris 75571, France

**Author contributions:** Cosnes J, Seksik P and Sokol H designed research; Sokol H performed research; Sokol H, Cosnes J, Chazouilleres O, Beaugerie L, Tiret E, Poupon R and Seksik P analyzed data; Sokol H wrote the paper.

**Correspondence to:** Harry Sokol, MD, Gastroenterology and Nutrition Department, Hôpital Saint-Antoine 184, rue du faubourg Saint-Antoine, cedex 12, Paris 75571, France. [sokol\\_harry@yahoo.fr](mailto:sokol_harry@yahoo.fr)

Telephone: +33-1-49283162 Fax: +33-1-49283188

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**CONCLUSION:** This study confirms that patients with PSC-IBD have a particular disease phenotype independent of the initial disease location. Although their disease is less active and they use more 5-aminosalicylates, they present a higher risk of colorectal cancer.

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**Key words:** Primary sclerosing cholangitis; Inflammatory bowel disease; Colorectal cancer; Ulcerative colitis; Crohn's disease

**Peer reviewer:** Andrew Ukleja, MD, Assistant Professor, Clinical Assistant Professor of Medicine, Director of Nutrition Support Team, Director of Esophageal Motility Laboratory, Cleveland Clinic Florida, Department of Gastroenterology, 2950 Cleveland Clinic Blvd., Weston, FL 33331, United States

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## Abstract

**AIM:** To investigate the phenotype of inflammatory bowel disease associated with primary sclerosing cholangitis (PSC-IBD).

**METHODS:** Data from 75 PSC-IBD patients evaluated in our tertiary center between 1963 and 2006 were collected and compared to 150 IBD patients without PSC, matched for sex, birth date, IBD diagnosis date and initial disease location regarding ileal, different colonic segments, and rectum, respectively.

**RESULTS:** While PSC-IBD patients received more 5-aminosalicylates (8.7 years/patient *vs* 2.9 years/patient,  $P < 0.001$ ), they required less immunosuppressors (24% *vs* 46% at 10 years;  $P < 0.001$ ) and less intestinal resection (10% *vs* 44% at 10 years,  $P < 0.001$ ). The 25-year cumulative rate of colectomy was 25.1% in PSC-IBD and 37.3% in controls ( $P = 0.004$ ). The 25-year cumulative rate of colorectal cancer was 23.4% in PSC-IBD *vs* 0% in controls ( $P = 0.002$ ). PSC was the only independent risk factor for the development of colorectal cancer (OR = 10.8; 95% CI, 3.7-31.3). Overall survival rate without liver transplantation was reduced in PSC-IBD patients (67% *vs* 91% in controls at 25 years,  $P = 0.001$ ).

## INTRODUCTION

The association of primary sclerosing cholangitis (PSC) with ulcerative colitis (UC) was first described by Smith and Loe in 1965<sup>[1]</sup>. The association with Crohn's disease (CD) was suggested by Atkinson and Carroll in 1964<sup>[2]</sup>, but was found less common than with UC. PSC may appear many years after proctocolectomy for colitis, and onset of IBD can be seen many years after liver transplantation for complicated PSC<sup>[3-7]</sup>. There is no evident correlation between the severity of UC and that of the associated PSC<sup>[4,5]</sup>.

Backwash ileitis, rectal sparing and low disease activity seem to characterize IBD when associated to PSC<sup>[8,9]</sup>. These characteristics may be partly linked as a proximal colonic disease and is usually less symptomatic than a distal one. Another crucial point regarding colitis is the increased risk of colorectal carcinoma. The two major risk factors for this complication are long duration of disease and the extent

of colitis. Recently, family history of sporadic colorectal cancer, active inflammation within the colonic mucosa and presence of PSC have been shown to increase the risk of colorectal cancer or dysplasia<sup>[10-14]</sup>. In a meta-analysis involving 116 studies<sup>[15]</sup>, the cumulative probability of cancer in UC patients regardless of disease extent or PSC association was 18% at 30 years. In a study from Broomé *et al*<sup>[16]</sup>, the cumulative risk of colorectal neoplasia (cancer or dysplasia) after 25 years of disease duration was 50% in the PSC-IBD group and 10% in the group of patients with UC without PSC. Nevertheless, conflicting results have been published<sup>[17-20]</sup>, but these studies had different sample size, end points and comparison groups. Moreover, patients with PSC may be misdiagnosed for IBD due to usual quiescent colitis in this sub-group of patients.

The patients with PSC associated UC are more likely to have extensive disease or pancolitis than those without PSC<sup>[8]</sup>. Moreover, the rectum seems to be less involved by inflammatory lesions<sup>[8]</sup>. Thus, comparing the colorectal cancer risk of PSC-UC patients with that of all coming UC patients without PSC may be debatable. Moreover, CD can also be associated with PSC, and there is no reason to restrain this comparison exclusively to UC patients. Besides the disease duration, the extent of disease is the most important matching criteria for comparing the colorectal cancer risk of PSC-IBD patients to IBD patients without PSC.

The aim of this study was to describe the clinical setting and outcome, in particular the risk of neoplasia, of a cohort of PSC associated IBD patients and to compare them to a group of IBD patients without PSC matched for disease location and extent at diagnosis. The patients were not matched for the type of IBD (i.e. CD or UC) because we considered that PSC-IBD patients represent a third IBD phenotype which have to be regarded without presumption<sup>[8]</sup>.

## MATERIAL AND METHODS

### Patients

Patients in this study were obtained from the MICISTA Registry, a tertiary clinical database of all IBD patients evaluated by the same staff of physicians at Rothschild Hospital (1974-2002) and then at Saint-Antoine Hospital (since 2003 to present time). The registry was built during the year 1994, with data before 1994 collected retrospectively. Data were collected prospectively in patients entering the database after 1994. There were 51 patients with additional diagnosis of PSC in the MICISTA registry ( $n = 51$  out of 5274, 0.97%). In addition, PSC-IBD patients followed in the Hepatology Department of the Saint-Antoine Hospital in the same period were also included ( $n = 24$ ). In all patients, diagnosis of PSC was defined by the following criteria: persistently elevated serum gamma glutamyl transferase (GGT) or alkaline phosphatase levels for at least 3 mo, characteristic radiographic appearance of sclerosing cholangitis and/or histologic features consistent with PSC on liver biopsy and absence of conditions associated with secondary sclerosing cholangitis<sup>[21]</sup>. The diagnosis of CD, UC, indeterminate colitis (IC) or

unclassified IBD (IBDu) were based on Lennard-Jones criteria and Montreal classification<sup>[22,23]</sup>.

### Matching

Controls were chosen randomly within the MICISTA registry to match to the identified cases (2 controls for one PSC case). In order to focus the analysis on colorectal cancer risk, we chose the following matching criteria: gender, birth year ( $\pm 2.5$  years), IBD diagnosis calendar year ( $\pm 2.5$  years) and initial disease location. Digestive tract was divided into the following seven segments: upper gastrointestinal tract, jejunum, ileum, caecum and ascending colon, transverse and descending colon, sigmoid colon, and rectum. Controls were matched to case considering each gastrointestinal tract segment.

### Data collection

Data concerning demographic information, IBD clinical settings (duration, initial and cumulative extent, year-by-year disease activity (assessed prospectively over one year according to a score taking into account flares: score 0 to 3, hospitalisations: score 4, excision surgery: score 5) from 1995 to 2005, extra intestinal lesions, medical and surgical treatment), morphological examination results (endoscopy, radiology), histopathology findings (digestive and liver biopsies) and outcome information (including colorectal neoplasia, colectomy, PSC complications and death) were abstracted from medical files. Colorectal neoplasia included flat low or high grade dysplasia and adenocarcinoma. Low and high grade dysplasia occurring in non-flat lesion was not taken into consideration.

### Statistical analysis

Comparisons between the two groups were made using the Student's *t* test or Mann Whitney test when appropriate. A *P* value under 0.05 was considered significant. The cumulative risks for colorectal neoplasia, CRC, treatment by immunosuppressors, ileal or colonic surgery and colectomy were calculated using Kaplan-Meier survival curves. The calculation of the cumulative risk for colorectal neoplasia and CRC were done after censoring observations at the time of proctocolectomy or death or at the end of follow up. Risk factors for colorectal neoplasia and CRC were established on the entire population of the study (composed by the two groups). Seventeen variables were tested by univariate analysis using the log rank test. Those variables with *P* values below 0.20 were further tested in a logistic multivariate regression model using a backward stepwise procedure (Cox model). In addition, two therapeutic variables assessing a prolonged treatment with aminosaliclates and immunosuppressors (treatment of more than two years) was entered in the model. The final step retained independent factors with *P* values below 0.05 in a 2-tailed test. Results are given as odds ratios (OR)  $\pm$  95% confidence intervals (CI).

## RESULTS

### Characteristics of the cases and controls

Seventy five IBD patients with concomitant PSC were

Table 1 Demographical and clinical characteristics of the two groups

	PSC-IBD ( <i>n</i> = 75)		IBD without PSC ( <i>n</i> = 150)		<i>P</i>	
Male (%)	53.3		53.3		1	
Age at IBD diagnosis (yr) (mean + SD)	27.8 (14)		28.5 (14)		0.75	
Age at PSC diagnosis (yr) (mean + SD)	35.8 (15.6)		NA			
IBD Follow up (mo) (mean + SD)	172 (133)		132 (123)		0.025	
Initial/Cumulative IBD location (%)						
Pancolitis	41 (55.4)	49 (66.2)	74 (49.3)	91 (60.7)	0.39	0.65
Rectal sparing	18 (24.3)	15 (20.3)	30 (20)	20 (13.3)	0.46	0.18
Sigmoid colon involvement	64 (86.5)	68 (91.9)	132 (88.6)	138 (92.6)	0.65	0.85
Left colon involvement	60 (81.1)	68 (91.9)	113 (75.8)	130 (87.2)	0.38	0.3
Right colon involvement	55 (74.3)	61 (82.4)	93 (62.4)	109 (72.7)	0.076	0.13
Ileum involvement	9 (12)	14 (18.7)	19 (12.7)	36 (24)	0.88	0.36
Jejunum involvement	0	1 (1.3)	0	3 (2)	1	0.59
Upper gastrointestinal tract involvement	1 (1.3)	1 (1.3)	4 (2.7)	8 (5.3)	0.46	0.14
Extra-intestinal manifestations (%)						
Articular	16 (21.3)		34 (22.7)		0.82	
Oral aphtae	3 (4)		4 (2.7)		0.58	
Skin	8 (10.7)		7 (4.7)		0.09	
Muscular	0		1 (0.7)		0.67	
Ophthalmological	0		4 (2.7)		0.19	
At least one extra intestinal lesion	22 (29.3)		39 (26)		0.59	
Initial/Final diagnosis (%)						
CD	19 (25.3)	21 (28)	43 (28.7)	62 (41.3)	0.6	0.051
UC	40 (53.3)	42 (56)	94 (62.7)	80 (53.3)	0.18	0.7
IC	1 (1.3)	1 (1.3)	1 (0.7)	3 (2)	0.61	0.72
IBDu	15 (20)	11 (14.7)	12 (8)	5 (3.3)	0.009	0.002
Smoking At IBD diagnosis/After IBD diagnosis	4 (5.3)	6 (8)	45 (30)	49 (32.7)	< 0.001	< 0.001
Colorectal neoplasia (%)						
Dysplasia	2 (2.7)		1 (0.7)		0.2	
Carcinoma	8 (10.7)		1 (0.7)		< 0.001	
Appendectomy (%)	9 (12.7)		30 (20.3)		0.25	

PSC-IBD: Inflammatory bowel disease associated with primary sclerosing cholangitis; CD: Crohn's disease; UC: Ulcerative colitis; IC: Indeterminate colitis; IBDu: Unclassified IBD; NA: Not applicable.

Table 2 Clinical features and outcomes of PSC-IBD patients

Clinical features	<i>n</i> = 75	
Age at PSC diagnosis (yr) (SD)	35.8 (15.6)	
Age at IBD diagnosis (yr) (SD)	27.8 (14)	
Disease topography	Intrahepatic	42%
	Intra- and extrahepatic	55.10%
	Extrahepatic	2.90%
Morphologic examinations	ERCP	50.70%
	MRCP	77.30%
	Endoscopic ultrasound	17.30%
	Liver biopsy	81.30%
Delay between diagnosis and UDCA treatment (mo) (SD)	13.5 (38.8)	
Complications	Acute cholangitis	15.10%
	Cholangiocarcinoma	2.80%
	Cirrhosis	20.80%
	Ascites	9.70%
	Gastrointestinal bleeding	5.70%
Non UDCA treatments	Corticosteroid	12.70%
	immunosuppressors	7%
	Liver transplantation	17.30%
Death	8.50%	

PSC-IBD: Inflammatory bowel disease associated with primary sclerosing cholangitis; UDCA: Ursodeoxycholic acid; ERCP: Endoscopic retrograde cholangiopancreatography; MRCP: Magnetic resonance cholangiopancreatography.

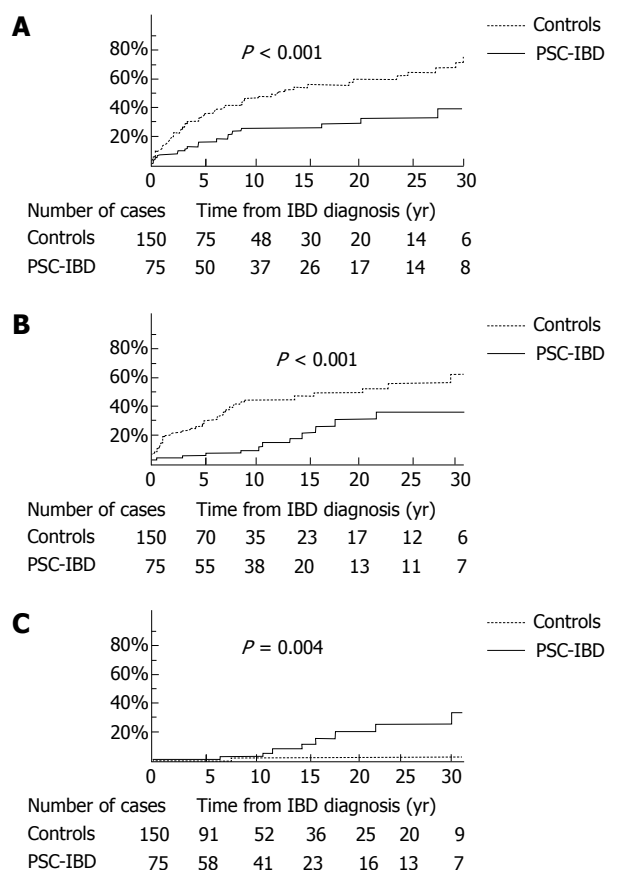
identified and were matched to 150 IBD patients without PSC. Demographic and clinical characteristics

of the two groups are described in Table 1. The mean follow up was longer in the PSC-IBD group. Rectal sparing and ileal involvement were observed in 20% and 19% of PSC-IBD patients (cumulative topography). Systemic manifestations were equally reported in the two groups. The final IBD diagnosis differs between the two groups. Final diagnosis of CD was made more often in the control group than in the PSC-IBD group ( $P = 0.05$ ). Although UC was finally diagnosed in the same proportion in the two groups, the proportion of IBD unclassified was significantly higher in the PSC-IBD group. PSC-IBD patients were significantly less likely to be current smokers (whether before or after IBD diagnosis) than controls without PSC.

Characteristics of patients with PSC and IBD are presented in Table 2. The mean time from onset of IBD to discovery of associated PSC was  $8 \pm 15.6$  years. 94.7% of PSC-IBD patients received ursodeoxycholic acid (UDCA), and the mean delay between PSC diagnosis and UDCA treatment was 13.5 mo. Thirteen patients underwent orthotopic liver transplantation (17.3%).

### IBD severity

**IBD year-by-year activity:** In controls, IBD was active during 351 out of 739 patient-years (47.5%) and hospitalization was required in 90 patient-years (12.2%), whereas PSC-IBD patients exhibited active IBD in



**Figure 1** Cumulative probability of colorectal cancer or dysplasia (A), cumulative incidence of immunosuppressors use (B), cumulative incidence of colonic or ileal surgical resection (C), from the IBD diagnosis date in cases (those with inflammatory bowel disease -associated with primary sclerosing cholangitis) vs controls (those with IBD without PSC).

143 out of 529 patient-years (27%;  $P < 0.001$ ) and hospitalization in 30 patient-years (5.7%;  $P < 0.001$ ).

**Medical treatment:** PSC-IBD patients received significantly more 5-ASA (8.5 years/patient *vs* 3.0 years/patient in controls,  $P < 0.0001$ ). In the PSC-IBD group, 48.6% of the patients required systemic steroids or immunosuppressors, as compared to 85.3% in the control group ( $P < 0.001$ ; OR = 0.15; 95%CI, 0.07-0.31). In fact, in the PSC-IBD group and in the control group, 21.6% and 38% of patients, respectively, had at least once enteral nutrition or systemic steroid therapy ( $P = 0.014$ ; OR = 0.5; 95% CI, 0.2-0.9). Moreover, 27% of PSC-IBD patients *vs* 47.3% of controls received immunosuppressors ( $P = 0.004$ ; OR = 0.4; 95%CI, 0.2-0.8). Finally, cumulative incidence of immunosuppressors use at 10 years after the diagnosis of IBD was 24% in the PSC-IBD group and 46% in the control group ( $P < 0.001$ , Figure 1A).

**Surgery requirement:** A colonic or a small bowel surgical resection was performed more frequently in the control group than in the PSC-IBD group (36.7% *vs* 17.3%;  $P = 0.003$ ). The cumulative incidence was also lower. At 10 years, 10% of patients from the PSC-IBD group and 44% from the control group underwent surgical resection ( $P < 0.001$ , Figure 1B). This difference

between the two groups was essentially due to colonic resection difference as cumulative incidence of small bowel resection was not different between the two groups (log rank test:  $P = 0.14$ ), whereas cumulative incidence of colonic resection showed major differences (log rank test:  $P = 0.0004$ ). Cumulative incidence of colectomy was significantly lower in the PSC-IBD group than in the control group (25.1% *vs* 37.3% at 25 years;  $P = 0.004$ ). The most common indication of colectomy was failure of medical treatment in both groups (55.9% in the control group and 62.5% in the PSC-IBD group;  $P = 0.7$ ). In the PSC-IBD group, the second indication was colorectal neoplasia (37.5%). In the control group the second and third indications were fulminant colitis (38.2%) and colorectal neoplasia (5.9%), respectively.

**Overall survival:** Six deaths were observed in the PSC-IBD group and five in the control group. Global actuarial survival rate at 25 years was similar in the two groups: 91.2% in the control group and 88.4% in the PSC-IBD group ( $P = 0.4$ ). Nevertheless, when censoring observation at liver transplantation, the survival was shorter in PSC-IBD group with a 25-year survival rate of 91.2% in control group and 67.6% in PSC-IBD group ( $P < 0.001$ , Figure 2). In the PSC-IBD group, causes of death were most of the time (5/6) related to chronic liver disease complications including cholangiocarcinoma (2 cases), hepatocellular carcinoma (1 case), decompensated cirrhosis (1 case), and immediate post transplantation complication in one case. Death was of cardiac origin in the last case. In the control group, causes of death were never related to IBD and were due to tuberculosis, lung cancer, ischemic cardiac events and septic shock.

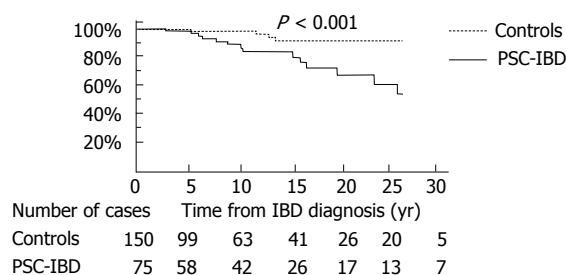
### Colorectal neoplasia risk

**Colorectal dysplasia or cancer risk:** Crude incidence of colorectal neoplasia (dysplasia and cancer) was higher in the PSC-IBD group ( $n = 10$ , 13.3%) than in controls ( $n = 2$ , 1.3%). The cumulative rate of colorectal neoplasia was also higher in PSC-IBD group (25.6% *vs* 1.5% in control group at 25 years;  $P = 0.004$ ; Figure 2). When restricted to cancer only, results were similar with colorectal cancer observed in 10.7% of patients in the PSC-IBD group *versus* 0.7% in the control group, and cumulative rates of colorectal cancer of 23.4 and 0% at 25 years in cases and controls, respectively ( $P = 0.002$ ). The mean IBD duration at cancer diagnosis was 209 mo  $\pm$  93 mo in PSC-IBD group, whereas the only case of colorectal cancer in control group was diagnosed 534 mo after the IBD onset. Nevertheless, duration of the colitis in PSC-IBD patients may be underestimated due to the mild course of the disease.

UDCA exposition were 78.3  $\pm$  23.7 mo and 71.2  $\pm$  8.2 mo in PSC-IBD patients with and without neoplasia, respectively ( $P = 0.75$ ). When taking into account only carcinoma, UDCA exposition were 74.6  $\pm$  25.9 mo and 71.8  $\pm$  8.1 mo in PSC-IBD patients with and without colorectal cancer, respectively ( $P = 0.91$ ).

None of the patients with colorectal cancer had





**Figure 2** Overall survival from IBD diagnosis date in cases (those with inflammatory bowel disease associated with primary sclerosing cholangitis) vs controls (those with chronic ulcerative colitis). Follow up was censored for those who underwent orthotopic liver transplantation.

a familial history of colorectal cancer. Among the 8 colorectal cancers diagnosed in PSC-IBD group (6 PSC-UC and 2 PSC-CD patients), 7 had neither nodal involvement nor metastasis and were treated by proctocolectomy. The remaining patient had a nodal invasion and was treated with adjuvant chemotherapy (5FU and folinic acid). For one patient who underwent a proctocolectomy for uncontrolled active UC, colon cancer was unexpectedly diagnosed on histological examination of the colon. The only case of cancer diagnosed in the control group was localized (T4N0M0) and required colectomy with no adjuvant treatment. The 8 colorectal cancers in PSC-IBD group were located in the right colon, whereas the only case of colorectal cancer in the control group was located in the left colon. Among the 8 cases of colorectal cancer in the PSC-IBD group, 3 patients underwent orthotopic liver transplantation (2 liver transplantations after colorectal cancer diagnosis). Until the end of follow up, no death related to colon cancer was observed.

**Risk factor of neoplasia and colorectal cancer:** The univariate analyses for the risk of colorectal neoplasia and colorectal cancer were established on the entire population of the study and the variables tested are described in Table 3. Five variables were further tested in the multivariate regression model: initial sigmoid colon involvement, extraintestinal manifestations, association with PSC, treatment with 5-ASA for more than two years, and treatment with immunosuppressors for more than two years. After analysis, the only independent variable associated with colorectal neoplasia was association with PSC (OR = 6.9; 95% CI, 3.2-14.9). Similarly, PSC was the only independent variable associated with colorectal cancer (OR = 10.8; 95% CI, 3.7-31.3).

## DISCUSSION

In the present study, PSC-IBD patients had a less active IBD than patients without PSC, and PSC was the only independent risk factor for CRC (Odds Ratio 10.8). Our study was performed in a large cohort and displays a methodological design to address the role of PSC as a risk factor of colorectal cancer in IBD. This case-control study involves 75 PSC-IBD patients. Each case was matched to two IBD controls without PSC. Initial

**Table 3** Univariate analysis for the risk of colorectal neoplasia *n* (%)

	Neoplasia ( <i>n</i> = 12)	No neoplasia ( <i>n</i> = 213)	Log rank <i>P</i>
Male gender	8 (66.7)	112 (52.6)	> 0.20
Age at IBD diagnosis below 16 yr	3 (25)	52 (24.4)	> 0.20
Age at IBD diagnosis below 40 yr	9 (75)	165 (77.5)	> 0.20
Initial diagnosis of CD	2 (16.7)	60 (28.2)	> 0.20
IBD familial history	1 (8.3)	27 (12.7)	> 0.20
Initial IBD location			
Ileum involvement	1 (8.3)	27 (12.7)	> 0.20
Right colon involvement	9 (75)	139 (65.3)	> 0.20
Left colon involvement	9 (75)	164 (77)	> 0.20
Sigmoid colon involvement	12 (100)	184 (86.4)	0.12
Rectum involvement	10 (83.3)	166 (77.9)	> 0.20
Anoperineal involvement	0	21 (9.9)	> 0.20
5 ASA during more than 2 yr	6 (54.6)	79 (42.5)	> 0.20
Immunosuppressant during more than 2 yr	3 (25)	69 (32.4)	> 0.20
Appendectomy before IBD onset	2 (16.7)	37 (17.4)	> 0.20
Current smoking	2 (16.7)	53 (24.9)	> 0.20
Extra-intestinal manifestations	2 (16.7)	61 (28.6)	0.09
PSC	10 (83.3)	65 (30.5)	0.004

IBD: Inflammatory bowel disease associated; PSC: Primary sclerosing cholangitis; CD: Crohn's disease.

disease location accounts for a matching criterion. This matching procedure was chosen to limit confounding factors which could bias colorectal neoplasia risk evaluation. It is, therefore, noticeable that the cumulative disease location was not statistically different between the two groups. In a study from the Mayo Clinic published in 2005<sup>[8]</sup>, a trend to an increase of colorectal neoplasia was observed, but the two groups compared had different rates of pancolitis : 87% in the PSC-IBD group *vs* 54% in the control group ( $P < 0.001$ ). Thus, in this study, the trend observed could be due to the difference in colitis extent. In our study, this bias has been avoided by the matching procedure. Moreover, in the study of Loftus *et al*<sup>[8]</sup>, matching criteria included the year of first visit at the Mayo clinic  $\pm 10$  years and the IBD duration before the first visit at the Mayo clinic  $\pm 5$  years, meaning that two matched patients could have a difference of 15 years regarding the IBD diagnosis date. In our study, this difference was at maximum of 5 years. Finally, our study is the only one to use the birth date as a matching criterion.

The increased risk of colorectal cancer in PSC-IBD patients has been discussed in the literature. Although some studies did not retrieve an excessive risk<sup>[17-20]</sup>, many recent studies are in agreement with our results<sup>[14-16]</sup>. The fact that 33% of patients in the study by definition had PSC could hold back others factors. Nevertheless, our multivariate analysis showed that PSC remained an independent factor. In our study, cumulative incidences of colorectal neoplasia and cancer in the control group were low compared to recent data of the literature. In the study of Rutter *et al* focusing on ulcerative colitis<sup>[24]</sup>, the cumulative incidences of colorectal neoplasia and cancer were higher than in our control group (7.7% and 2.5% *vs* 1.5% and 0% at 20 years). Furthermore, in our

IBD group without PSC, CD was finally diagnosed in 40% of the cases and cumulative incidence of colectomy was higher than in the study of Rutter *et al*<sup>[24]</sup> (22.2% *vs* 9.5% at 20 years). High colectomy rates could play a protective role regarding colonic neoplasia. Indeed, in a middle 1980's study on UC<sup>[25]</sup>, with high colectomy rate (31% at 18 years), the cumulative incidence of colorectal cancer was closer to our results (1.4% at 18 years). More recently, in a population based cohort study from Copenhagen county<sup>[26]</sup>, the overall cumulative probability for development of CRC in UC patients was 1.1% at 20 years with an overall cumulative colectomy probability after 20 years of 27.9%.

To assess IBD activity in the two groups, we took into account the number of active patient-years and the requirement of medical or surgical treatment. Our results pointed out a less active disease in PSC-IBD patients. Ten years after IBD diagnosis, 46% of the patients from the control group had required immunosuppressors (*versus* only 24% in the PSC-IBD group). The lesser severity of PSC associated IBD was also reflected by a lower surgery rate. Moreover, in the control group colectomy was indicated in 94.1% for uncontrolled active disease (with more than one third of severe acute colitis), whereas in PSC-IBD group, colectomy was performed for this indication in only 62.5% of cases (with no case of severe acute colitis). Five-aminosalicylates might prevent colorectal cancer in IBD<sup>[27]</sup> and difference between the two study groups regarding the 5-ASA consumption could have skew our conclusions on colorectal neoplasia and cancer risk. In fact, PSC-IBD patients received more 5-ASA than controls and despite larger use of 5-ASA, this assumed protective factor did not decrease the risk of colorectal neoplasia in this group. The larger use of 5-ASA in PSC-IBD patients can be explained by their weak IBD activity. While patients with PSC-IBD have often a quiescent IBD course and carry on their 5-ASA treatment lengthily, IBD patients without PSC can have a more active disease requiring immunosuppressors. Until recently, 5-ASA was not known to have a preventive effect on colorectal cancer risk in IBD<sup>[27]</sup>, and patients in this situation stopped their 5-ASA treatment.

The reasons of the increased risk of colorectal neoplasia in PSC-IBD patients are still not well understood. The role of endogenous bile acids has been hypothesized, but remains speculative. The high frequency of colorectal cancer and of several other cancers in PSC patients suggest that other factors are involved in the carcinogenesis<sup>[28,29]</sup>. Moreover, two recent studies have suggested that UDCA treatment decrease the colorectal neoplasia risk in PSC-IBD patients<sup>[30, 31]</sup>. As a consequence, it may be hypothesized that our PSC-IBD patients could have presented a higher rate of colorectal neoplasia in the absence of UDCA treatment.

In our patients with PSC, as in other series<sup>[8]</sup>, the most frequent presentation was pancolitis. On the contrary, rectal sparing and backwash ileitis were retrieved in only 20% and 19%, respectively, whereas it was seen more often in several studies (52% and 51%,

respectively, in the recent study by Loftus *et al*<sup>[8]</sup>). In our study, current smoker status was less frequent in PSC-IBD group than in control group. This has already been described and is probably emphasized by a strong association between PSC and UC compared to CD<sup>[32,33]</sup>, and by the protective effect of smoking regarding the onset of UC and PSC<sup>[19,34-36]</sup>.

In our study, most of the deaths in PSC-IBD patients were related to complications of a chronic liver disease. In both groups, death was directly linked neither to an IBD complication nor to a colorectal neoplasia or cancer.

In conclusion, this study confirms that PSC-IBD patients have a higher risk of colorectal neoplasia and cancer than IBD patients without PSC, despite a lower disease activity and a larger use of 5-ASA. It demonstrates that this higher risk is unrelated to disease location or extent. It suggests that a factor directly linked to PSC may increase the risk of neoplasia. Our finding strongly support that these patients require a close colorectal neoplasia tracking by endoscopy, and may be by other methods.

## COMMENTS

### Background

Inflammatory bowel disease associated with primary sclerosing cholangitis (PSC-IBD) may have a distinct IBD phenotype, with a low activity and a high risk of colorectal cancer. However, lower activity may be due to more proximal disease location and rectal sparing, and the high risk of colorectal cancer to the high frequency of pancolitis.

### Research frontiers

IBD patients with colonic involvement have an increase risk of colorectal neoplasia. Some studies suggest that PSC-IBD patients might have an increased risk of colorectal neoplasia compared to patients with IBD alone. However, no previous studies comparing colorectal cancer risk between PSC-IBD and IBD patients and using rigorous matching criteria are available.

### Innovations and breakthroughs

In this study, we confirmed that patients with PSC-IBD have a particular disease phenotype, with a high rate of pancolitis and a lower disease activity compared to IBD matched controls without PSC. Despite a weaker disease activity and a higher use of 5-aminosalicylates, we showed that PSC-IBD patients have a higher risk of colorectal cancer. Contrariwise to previous studies, we used a matching procedure taking into account the disease extent and the diagnosis date. Thus we avoided these major biases.

### Applications

This study confirms that PSC-IBD patients have a higher risk of colorectal neoplasia and cancer than IBD patients without PSC, despite a lower disease activity and a larger use of 5-aminosalicylates. It demonstrates that this higher risk is unrelated to disease location or extent. It suggests that a factor directly linked to PSC may increase the risk of neoplasia. These results strongly support that PSC-IBD patients require a close colorectal neoplasia tracking by endoscopy, and may be by other methods.

### Peer review

The paper by Sokol and co-workers investigated disease activity and cancer risk in inflammatory bowel disease associated with primary sclerosing cholangitis. This paper is interesting and it has clearly stated aims, the sample size and the overall designs of the study are fair, the results adequate to provide experimental evidence and to support valid conclusions. The authors conclusions support previous literature about increased risk of colon cancer in PSC-IBD patients, compared to patients with PSC alone.

## REFERENCES

- 1 Smith MP, Loe RH. Sclerosing Cholangitis; Review Of

- Recent Case Reports And Associated Diseases And Four New Cases. *Am J Surg* 1965; **110**: 239-246
- 2 **Atkinson AJ**, Carroll WW. Sclerosing Cholangitis. Association With Regional Enteritis. *Jama* 1964; **188**: 183-184
  - 3 **Aadland E**, Schrupf E, Fausa O, Elgjo K, Heilo A, Aakhus T, Gjone E. Primary sclerosing cholangitis: a long-term follow-up study. *Scand J Gastroenterol* 1987; **22**: 655-664
  - 4 **Chapman RW**, Arborgh BA, Rhodes JM, Summerfield JA, Dick R, Scheuer PJ, Sherlock S. Primary sclerosing cholangitis: a review of its clinical features, cholangiography, and hepatic histology. *Gut* 1980; **21**: 870-877
  - 5 **Fausa O**, Schrupf E, Elgjo K. Relationship of inflammatory bowel disease and primary sclerosing cholangitis. *Semin Liver Dis* 1991; **11**: 31-39
  - 6 **Riley TR**, Schoen RE, Lee RG, Rakela J. A case series of transplant recipients who despite immunosuppression developed inflammatory bowel disease. *Am J Gastroenterol* 1997; **92**: 279-282
  - 7 **Wiesner RH**, LaRusso NF. Clinicopathologic features of the syndrome of primary sclerosing cholangitis. *Gastroenterology* 1980; **79**: 200-206
  - 8 **Loftus EV Jr**, Harewood GC, Loftus CG, Tremaine WJ, Harmsen WS, Zinsmeister AR, Jewell DA, Sandborn WJ. PSC-IBD: a unique form of inflammatory bowel disease associated with primary sclerosing cholangitis. *Gut* 2005; **54**: 91-96
  - 9 **Moayyeri A**, Daryani NE, Bahrami H, Haghpanah B, Nayyer-Habibi A, Sadatsafavi M. Clinical course of ulcerative colitis in patients with and without primary sclerosing cholangitis. *J Gastroenterol Hepatol* 2005; **20**: 366-370
  - 10 **Brentnall TA**, Haggitt RC, Rabinovitch PS, Kimmey MB, Bronner MP, Levine DS, Kowdley KV, Stevens AC, Crispin DA, Emond M, Rubin CE. Risk and natural history of colonic neoplasia in patients with primary sclerosing cholangitis and ulcerative colitis. *Gastroenterology* 1996; **110**: 331-338
  - 11 **Broome U**, Lindberg G, Lofberg R. Primary sclerosing cholangitis in ulcerative colitis—a risk factor for the development of dysplasia and DNA aneuploidy? *Gastroenterology* 1992; **102**: 1877-1880
  - 12 **D'Haens GR**, Lashner BA, Hanauer SB. Pericholangitis and sclerosing cholangitis are risk factors for dysplasia and cancer in ulcerative colitis. *Am J Gastroenterol* 1993; **88**: 1174-1178
  - 13 **Kornfeld D**, Ekbom A, Ihre T. Is there an excess risk for colorectal cancer in patients with ulcerative colitis and concomitant primary sclerosing cholangitis? A population based study. *Gut* 1997; **41**: 522-525
  - 14 **Leidenius MH**, Farkkila MA, Karkkainen P, Taskinen EI, Kellokumpu IH, Hockerstedt KA. Colorectal dysplasia and carcinoma in patients with ulcerative colitis and primary sclerosing cholangitis. *Scand J Gastroenterol* 1997; **32**: 706-711
  - 15 **Eaden JA**, Abrams KR, Mayberry JF. The risk of colorectal cancer in ulcerative colitis: a meta-analysis. *Gut* 2001; **48**: 526-535
  - 16 **Broome U**, Lofberg R, Veress B, Eriksson LS. Primary sclerosing cholangitis and ulcerative colitis: evidence for increased neoplastic potential. *Hepatology* 1995; **22**: 1404-1408
  - 17 **Choi PM**, Nugent FW, Rossi RL. Relationship between colorectal neoplasia and primary sclerosing cholangitis in ulcerative colitis. *Gastroenterology* 1992; **103**: 1707-1709
  - 18 **Gurbuz AK**, Giardiello FM, Bayless TM. Colorectal neoplasia in patients with ulcerative colitis and primary sclerosing cholangitis. *Dis Colon Rectum* 1995; **38**: 37-41
  - 19 **Loftus EV Jr**, Sandborn WJ, Tremaine WJ, Mahoney DW, Zinsmeister AR, Offord KP, Melton LJ 3rd. Risk of colorectal neoplasia in patients with primary sclerosing cholangitis. *Gastroenterology* 1996; **110**: 432-440
  - 20 **Nuako KW**, Ahlquist DA, Sandborn WJ, Mahoney DW, Siems DM, Zinsmeister AR. Primary sclerosing cholangitis and colorectal carcinoma in patients with chronic ulcerative colitis: a case-control study. *Cancer* 1998; **82**: 822-826
  - 21 **Chapman RW**, Arborgh BA, Rhodes JM, Summerfield JA, Dick R, Scheuer PJ, Sherlock S. Primary sclerosing cholangitis: a review of its clinical features, cholangiography, and hepatic histology. *Gut* 1980; **21**: 870-877
  - 22 **Lennard-Jones JE**. Classification of inflammatory bowel disease. *Scand J Gastroenterol Suppl* 1989; **170**: 2-6; discussion 16-19
  - 23 **Silverberg MS**, Satsangi J, Ahmad T, Arnott ID, Bernstein CN, Brant SR, Caprilli R, Colombel JF, Gasche C, Geboes K, Jewell DP, Karban A, Loftus Jr EV, Pena AS, Riddell RH, Sachar DB, Schreiber S, Steinhart AH, Targan SR, Vermeire S, Warren BF. Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 2005; **19** Suppl A: 5-36
  - 24 **Rutter MD**, Saunders BP, Wilkinson KH, Rumbles S, Schofield G, Kamm MA, Williams CB, Price AB, Talbot IC, Forbes A. Thirty-year analysis of a colonoscopic surveillance program for neoplasia in ulcerative colitis. *Gastroenterology* 2006; **130**: 1030-1038
  - 25 **Hendriksen C**, Kreiner S, Binder V. Long term prognosis in ulcerative colitis—based on results from a regional patient group from the county of Copenhagen. *Gut* 1985; **26**: 158-163
  - 26 **Winther KV**, Jess T, Langholz E, Munkholm P, Binder V. Long-term risk of cancer in ulcerative colitis: a population-based cohort study from Copenhagen County. *Clin Gastroenterol Hepatol* 2004; **2**: 1088-1095
  - 27 **Eaden J**, Abrams K, Ekbom A, Jackson E, Mayberry J. Colorectal cancer prevention in ulcerative colitis: a case-control study. *Aliment Pharmacol Ther* 2000; **14**: 145-153
  - 28 **Bergquist A**, Ekbom A, Olsson R, Kornfeldt D, Loof L, Danielsson A, Hultcrantz R, Lindgren S, Prytz H, Sandberg-Gertzen H, Almer S, Granath F, Broome U. Hepatic and extrahepatic malignancies in primary sclerosing cholangitis. *J Hepatol* 2002; **36**: 321-327
  - 29 **Stiehl A**. Primary sclerosing cholangitis: neoplastic potential in bile ducts, colon and the pancreas? *J Hepatol* 2002; **36**: 433-434
  - 30 **Pardi DS**, Loftus EV Jr, Kremers WK, Keach J, Lindor KD. Ursodeoxycholic acid as a chemopreventive agent in patients with ulcerative colitis and primary sclerosing cholangitis. *Gastroenterology* 2003; **124**: 889-893
  - 31 **Tung BY**, Emond MJ, Haggitt RC, Bronner MP, Kimmey MB, Kowdley KV, Brentnall TA. Ursodiol use is associated with lower prevalence of colonic neoplasia in patients with ulcerative colitis and primary sclerosing cholangitis. *Ann Intern Med* 2001; **134**: 89-95
  - 32 **Olsson R**, Danielsson A, Jarnerot G, Lindstrom E, Loof L, Rolny P, Ryden BO, Tysk C, Wallerstedt S. Prevalence of primary sclerosing cholangitis in patients with ulcerative colitis. *Gastroenterology* 1991; **100**: 1319-1323
  - 33 **Rasmussen HH**, Fallingborg JF, Mortensen PB, Vyberg M, Tage-Jensen U, Rasmussen SN. Hepatobiliary dysfunction and primary sclerosing cholangitis in patients with Crohn's disease. *Scand J Gastroenterol* 1997; **32**: 604-610
  - 34 **Beaugerie L**, Massot N, Carbonnel F, Cattin S, Gendre JP, Cosnes J. Impact of cessation of smoking on the course of ulcerative colitis. *Am J Gastroenterol* 2001; **96**: 2113-2116
  - 35 **Mitchell SA**, Thyssen M, Orchard TR, Jewell DP, Fleming KA, Chapman RW. Cigarette smoking, appendectomy, and tonsillectomy as risk factors for the development of primary sclerosing cholangitis: a case control study. *Gut* 2002; **51**: 567-573
  - 36 **van Erpecum KJ**, Smits SJ, van de Meeberg PC, Linn FH, Wolfhagen FH, vanBerge-Henegouwen GP, Algra A. Risk of primary sclerosing cholangitis is associated with nonsmoking behavior. *Gastroenterology* 1996; **110**: 1503-1506



CLINICAL RESEARCH

## Endorectal ultrasonography *versus* phased-array magnetic resonance imaging for preoperative staging of rectal cancer

Ahmet Mesrur Halefoglu, Sadik Yildirim, Omer Avlanmis, Damlanur Sakiz, Adil Baykan

Ahmet Mesrur Halefoglu, Department of Radiology, Sisli Etfal Training and Research Hospital, Sisli 34360, Istanbul, Turkey

Sadik Yildirim, Omer Avlanmis, Adil Baykan, Department of General Surgery, Sisli Etfal Training and Research Hospital, Sisli 34360, Istanbul, Turkey

Damlanur Sakiz, Department of Pathology, Sisli Etfal Training and Research Hospital, Sisli 34360, Istanbul, Turkey

**Author contributions:** Halefoglu AM and Baykan A designed study concept; Halefoglu AM, Avlanmis O and Baykan A performed research; Avlanmis O and Sakiz D collected data; Yildirim S, Baykan A and Avlanmis O performed surgery; Halefoglu AM contributed on statistical analysis, Halefoglu AM wrote the paper.

**Correspondence to:** Ahmet Mesrur Halefoglu, Department of Radiology, Sisli Etfal Training and Research Hospital, Sisli 34360, Istanbul, Turkey. [halefoglu@hotmail.com](mailto:halefoglu@hotmail.com)

Telephone: +90-212-2795643 Fax: +90-212-2415015

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phased-array MRI gave an accuracy of 74.50% (21 out of 34). The sensitivity and specificity was found to be 61.76% and 80.88%, respectively. By using ERUS in the detection of lymph node metastases, an accuracy of 76.47% (18 out of 34) was obtained. The sensitivity and specificity were found to be 52.94% and 84.31%, respectively.

**CONCLUSION:** ERUS and phased-array MRI are complementary methods in the accurate preoperative staging of rectal cancer. In conclusion, we can state that phased-array MRI was observed to be slightly superior in determining the depth of transmural invasion (T stage) and has same value in detecting lymph node metastases (N stage) as compared to ERUS.

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**Key words:** Endoscopic ultrasonography; Magnetic resonance imaging; Pelvic phased-array coil; Preoperative staging; Rectal cancer

**Peer reviewer:** Rene Lambert, Professor, International Agency for Research on Cancer, 150 Cours Albert Thomas, Lyon 69372 cedex 8, France

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### Abstract

**AIM:** To compare the diagnostic accuracy of pelvic phased-array magnetic resonance imaging (MRI) and endorectal ultrasonography (ERUS) in the preoperative staging of rectal carcinoma.

**METHODS:** Thirty-four patients (15 males, 19 females) with ages ranging between 29 and 75 who have biopsy proven rectal tumor underwent both MRI and ERUS examinations before surgery. All patients were evaluated to determine the diagnostic accuracy of depth of transmural tumor invasion and lymph node metastases. Imaging results were correlated with histopathological findings regarded as the gold standard and both modalities were compared in terms of predicting preoperative local staging of rectal carcinoma.

**RESULTS:** The pathological T stage of the tumors was: pT1 in 1 patient, pT2 in 9 patients, pT3 in 21 patients and pT4 in 3 patients. The pathological N stage of the tumors was: pN0 in 19 patients, pN1 in 9 patients and pN2 in 6 patients. The accuracy of T staging for MRI was 89.70% (27 out of 34). The sensitivity was 79.41% and the specificity was 93.14%. The accuracy of T staging for ERUS was 85.29% (24 out of 34). The sensitivity was 70.59% and the specificity was 90.20%. Detection of lymph node metastases using

### INTRODUCTION

In recent years, the treatment of rectal carcinoma has been improved by the introduction of new surgical techniques and neoadjuvant therapies. Surgery is still the method of choice for the treatment of rectal carcinoma. The depth of tumor infiltration into the rectal wall and involvement of the regional lymph nodes are the major factors in determining prognosis<sup>[1,2]</sup>. Therefore, assessment of the invasion depth (T stage) and lymph node involvement (N stage) are vital components of preoperative staging. The three main techniques currently used are computed tomography (CT), endorectal ultrasonography (ERUS), and magnetic resonance



imaging (MRI) using various coils. Positron emission tomography (PET) is useful adjunct to systemic and regional staging, especially of a recurrent rectal cancer, but is rarely used as part of loco-regional staging of rectal cancer preoperatively<sup>[3,4]</sup>. The utility of ERUS and MRI for preoperative local staging of rectal carcinoma has been widely demonstrated<sup>[5-7]</sup>. In our study, we compared the ability of ERUS and pelvic phased-array MRI for preoperative local staging of rectal carcinoma. The imaging results obtained by both examinations were correlated with the histopathological gold standard evaluations of the surgical specimens.

## MATERIALS AND METHODS

Between June 2005-July 2007, 34 consecutive patients (15 male and 19 female), with a mean age of 58.7 (ranging from 29 to 75 years) who had biopsy proven rectal carcinoma were included in this study. All of the patients had underwent colonoscopic examination in which a biopsy procedure was also performed preoperatively. Following histopathological analysis of the endoscopic biopsy specimens, patients were diagnosed as having rectal adenocarcinoma. Patients who previously underwent chemotherapy or radiotherapy were excluded from the study. Regarding the location of the rectal tumors, the rectum is considered starting from the anal verge and extending to the rectosigmoid junction. Five cancers (14.71%) were in the upper third of the rectum, 17 cancers (50%) were in the middle third of the rectum and 12 cancers (35.29%) were in the lower third of the rectum.

Fifty patients (44.12%) underwent an abdomino-perineal resection and 19 patients (55.88%) a low-anterior resection. Following surgery, operative specimens were analysed by a pathologist (D.S.) who was unaware of ERUS and MRI results. The sections were evaluated microscopically in terms of determining the depth of transmural tumor invasion and lymph node metastases according to TNM criteria<sup>[8]</sup>.

### ERUS

ERUS examinations were performed by an experienced colorectal endoscopist (A.B.) on this area using a B-K Falcon 2101 ultrasound machine with a 7 and 10 MHz rotating superficial endoprobes. All patients were given enema the day before the examination. Informed consents were obtained from all of the patients prior to the examination.

Endorectal ultrasound was carried out with the patients in the left lateral decubitus position without needing any sedation. The tip of the transducer was covered with a latex balloon filled with degassed water.

The bowel wall is represented in five sonographic layers as a result of differences in acoustic impedance<sup>[9]</sup>. Beginning with the lumen, the five layers are: (1) hyperechoic layer from the interface between mucosa and ultrasound probe; (2) hypoechoic layer produced from the mucosa and muscularis mucosae; (3) hyperechoic layer corresponding to the submucosa; (4) hypoechoic layer corresponding to the muscularis propria; and

(5) hyperechoic layer being the interface between the muscularis propria and perirectal fat/serosa<sup>[9,10]</sup>.

Ultrasonographic staging of tumor depth is denoted by the prefix "u". The ultrasonographic staging corresponds to the TNM classification: (1) uT1, tumor confined to mucosa and submucosa; (2) uT2, tumor infiltrating muscularis propria; (3) uT3, tumor invading perirectal fat; and (4) uT4, tumor infiltrating surrounding organs<sup>[11]</sup>.

The sonographic criteria for identifying involved lymph nodes consist of size greater than 5 mm, mixed signal intensity, irregular margins and spherical rather than ovoid or flat shape.

### MRI

MRI examinations of the same patient group were performed by means of a 1.5 tesla superconducting magnet (GE, Signa, Milwaukee, Wisconsin, USA). All patients gave informed consent for the examination.

During the examination a pelvic coil was used which is a wrap-around surface coil around the pelvis. Patients did not undergo rectal air insufflation, nor did they receive bowel preparation or intravenous contrast. The patients were placed in head - first supine position in the magnet. We did not perform T1 weighted images. The imaging protocol included T2 weighted images obtained by acquiring a non-breath hold FSE sequence by using the following parameters: TR: 3700 ms; TE: 105 ms; Echo train length: 16; Matrix size: 512 × 256; Section thickness: 4 mm; Field of view: 26 cm × 26 cm. T2 weighted images were obtained in axial, sagittal and coronal planes.

A single specialized radiologist (A.M.H.) who was blinded to the ERUS examination results evaluated these images. The layers as showed by MRI are defined as follows: (1) mucosa; thin, low-signal intensity line; (2) submucosa; thicker, higher-signal intensity; (3) muscularis propria; low signal; (4) perirectal fat; high signal layer; and (5) mesorectal fascia; fine, low-signal intensity layer enveloping the perirectal fat and rectum<sup>[10]</sup>.

This fascia represents the plane of dissection during total mesorectal excision and the tumor proximity to within 1 mm of this fascia was taken as a marker of tumor involvement of the circumferential resection margin.

T2 weighted images are more useful for evaluating the rectal wall layers and revealing involvement of other pelvic structures. On these images both the depth of transmural tumor invasion (T staging) and lymph node involvement (N staging) were assessed.

The depth of tumor invasion (T stage) and lymph node involvement (N stage) were classified according to the TNM classification<sup>[8]</sup>. In this staging system: (1) T1 tumors are confined to mucosa and submucosa; (2) T2 tumors invade muscularis propria; (3) T3 tumors extend to mesorectal fat; (4) T4 tumors show adjacent organ invasion. N0: No nodal involvement; N1: One to three regional nodes positive for tumor; N2: Four or more regional nodes positive for tumor.

Spiculation from the tumor margin was considered to indicate malignant tumoral infiltration and, therefore, as

**Table 1** Depth of transmural tumor invasion (T staging): Comparison of phased-array MRI and histopathologic findings

	p-T <sub>1</sub>	p-T <sub>2</sub>	p-T <sub>3</sub>	p-T <sub>4</sub>
MR-T <sub>1</sub>	1	0	0	0
MR-T <sub>2</sub>	0	5	1	0
MR-T <sub>3</sub>	0	4	18	0
MR-T <sub>4</sub>	0	0	2	3
No	1	9	21	3

MR-T: T staging evaluation by phased-array MRI; p-T: Pathological T staging; No: Number of cases.

**Table 2** Depth of transmural tumor invasion (T staging): Comparison of ERUS and histopathologic findings

	p-T <sub>1</sub>	p-T <sub>2</sub>	p-T <sub>3</sub>	p-T <sub>4</sub>
ERUS-T <sub>1</sub>	0	0	0	0
ERUS-T <sub>2</sub>	1	4	3	0
ERUS-T <sub>3</sub>	0	5	18	1
ERUS-T <sub>4</sub>	0	0	0	2
No	1	9	21	3

ERUS-T: T staging evaluation by ERUS; p-T: Pathological T staging; No: Number of cases.

in the Maastricht study<sup>[12]</sup>, spiculated lesions (i.e. showing perirectal strandings) were classified as T3 disease.

Any discrete hypointense lesion detected in the mesorectal fat was interpreted as a lymph node. Lymph nodes of 5 mm diameter or greater were reported as nodal metastases while those lesser than 5 mm diameter were considered to be uninvolved<sup>[13]</sup>.

### Statistical analysis

The overall accuracy, sensitivity, specificity, positive predictive value and negative predictive value were calculated for ERUS and MRI to predict transmural tumor invasion and lymph node involvement using the histopathological findings as the gold standard.

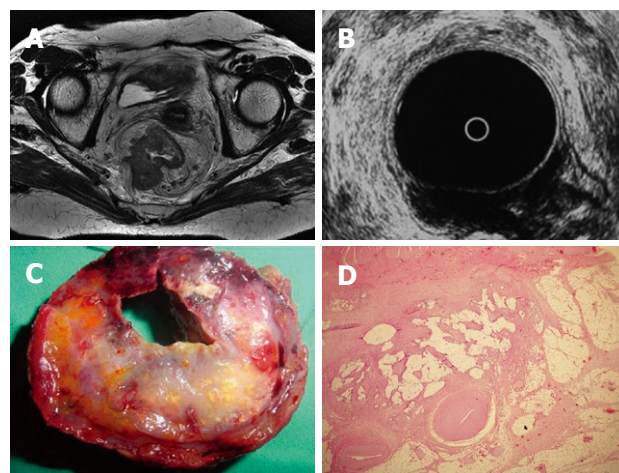
## RESULTS

All tumors could be detected by both ERUS and MRI. The histopathological evaluation of resected tumors revealed adenocarcinoma for all of the patients. Mean histological tumor size was 3.7 cm (range 1.5-6.8 cm). The pathological T stage of these adenocarcinomas was: pT1 in 1 patient, pT2 in 9 patients, pT3 in 21 patients and pT4 in 3 patients. The pathological N staging of these tumors was: pN0 in 19 patients, pN1 in 9 patients and pN2 in 6 patients.

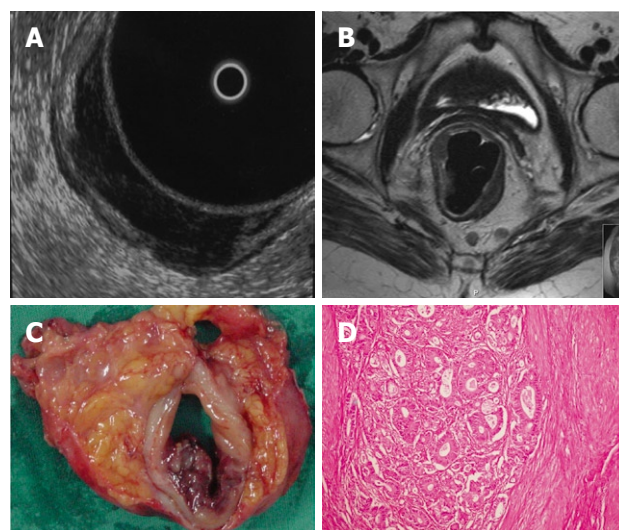
### T staging

Comparison of T staging results obtained with phased-array MRI and ERUS with the pathology is summarized in Tables 1 and 2.

Regarding MRI, one patient with pT3 tumor was understaged as T2 tumor. In 4 patients with pT2 tumors, MRI overestimated as T3 tumors and in 2 patients with pT3 tumors, MRI overestimated as T4 (Figure 1A, C and D). In the remaining cases, with



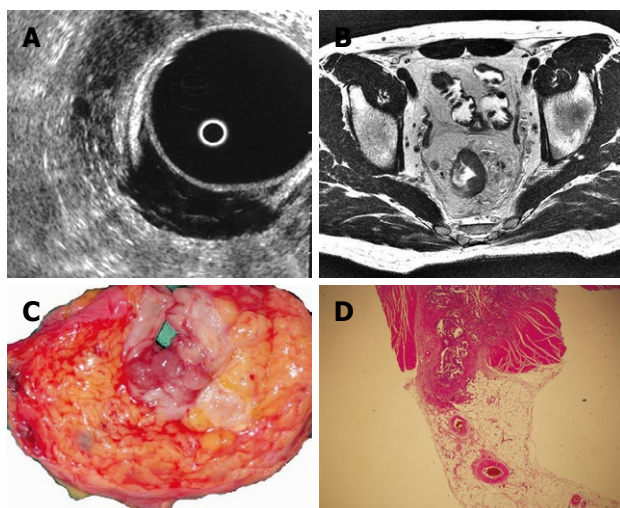
**Figure 1** A: MRI demonstrates a large tumor passing through the muscularis propria and invading the mesorectal fatty tissue within very close proximity to the mesorectal fascia; lymph nodes are also present; The MRI prediction was a T4 tumor; B: This was predicted as a T3 tumor by ERUS examination; C: Macroscopic specimen shows that the tumor has already filled all the mesorectal fatty tissue but the mesorectal fascia is still intact; D: Pathological examination reveals that this is a T3 stage tumor.



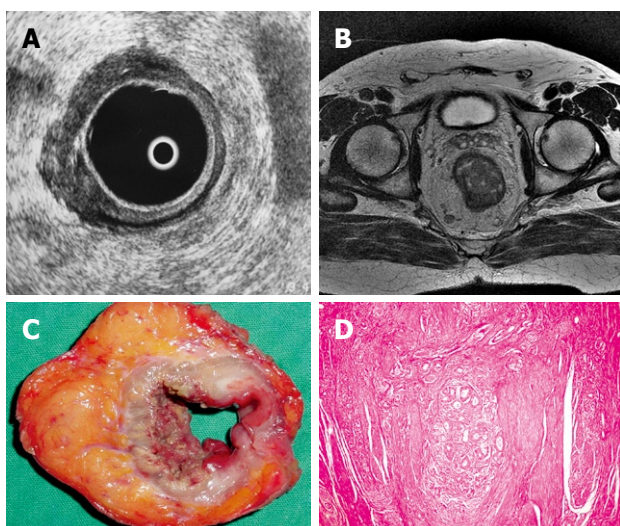
**Figure 2** A: ERUS shows the tumor invading the muscularis propria which can be regarded as a T2 tumor. A lymph node is also seen; B: MRI clearly demonstrates that the tumor is confined to the muscularis propria and does not invade the mesorectal fatty tissue. Lymph nodes are also seen; C: Macroscopic specimen reveals the tumor does not extend to the mesorectal fat; D: Pathology confirms that this is a T2 stage tumor.

a pT1 tumor in 1 patient, pT2 tumor in 5 patients (Figure 2B, C and D), pT3 tumor in 18 patients (Figure 3B, C and D) and pT4 tumor in 3 patients, MRI correctly assessed the stage of transmural tumor invasion. The accuracy of T staging was 89.70% (27 out of 34). The sensitivity was 79.41% and the specificity was 93.14%. MRI correctly predicted invasion in 23 patients and no invasion in 6 patients, thus the overall accuracy in terms of discriminating between pT1-pT2 and pT3-pT4 tumors was found to be 85.29% with a 95.8% sensitivity and 60% specificity. The positive and negative predictive values were calculated as 85.19% and 85.7%, respectively. With ERUS, 4 patients were understaged. In 3 patients with





**Figure 3** A: ERUS examination shows the tumor extend to the mesorectal fat by passing beyond the muscularis propria. A lymph node is also seen; B: MRI defines the tumor as violating the muscularis propria and extending to the mesorectal fatty tissue. Lymph nodes are seen; C: Operation specimen confirms mesorectal invasion; D: Pathology specimen demonstrates tumor cells invading the mesorectum which is indicative of a T3 tumor.



**Figure 4** A: ERUS shows perirectal fat invasion of the tumor and predicts it as T3; B: MRI demonstrates that the tumor is not invading the mesorectal fatty tissue and is confined to the rectum which is considered as a T2 tumor. The lymph nodes are also present in the mesorectal fatty tissue; C: Operation specimen reveals that the tumor does not extend beyond the muscularis propria; D: Pathology confirms that this is a T2 stage tumor.

pT3 tumors, ERUS staged as T2 and in 1 patient with pT4 tumor, ERUS staged as T3. In 6 patients, overestimation did occur where 5 pT2 tumors were overestimated as T3 (Figure 4A, C and D) and 1 pT1 tumor was overestimated as T2.

In the remaining 4 cases with pT2 (Figure 2A, C and D), 18 with pT3 (Figure 3A, C and D) and 2 with pT4 tumors, ERUS correctly predicted the T staging.

The accuracy of T staging was 85.29% (24 out of 34). The sensitivity was 70.59% and the specificity was 90.20%. ERUS was correctly predicted invasion in 21 patients and no invasion in 5 patients, thus the overall

**Table 3** Phased-array MRI and ERUS in detecting lymph node metastases

	Pathology		
	N0	N1	N2
Phased-array MRI			
N0	8	1	1
N1	11	8	0
N2	0	0	5
ERUS			
N0	7	2	2
N1	12	7	0
N2	0	0	4

**Table 4** Comparison of overstaged and understaged cases by MRI and ERUS

	T staged		N staged	
	Overstaged	Understaged	Overstaged	Understaged
MRI	6	1	11	2
ERUS	6	4	12	4

accuracy of ERUS in terms of discriminating between pT1-pT2 and pT3-pT4 tumors was found to be 76.47% with a 87.5% sensitivity and 50% specificity. The positive and negative predictive values were calculated as 80.77% and 62.50%, respectively.

### N staging

Comparison of N staging results obtained with phased-array MRI and ERUS with the pathology is summarized in Table 3. Detection of lymph node metastases using phased-array MRI gave an accuracy of 74.50% (21 out of 34). The sensitivity and specificity were found to be 61.6% and 80.88%, respectively. By using ERUS in the detection of lymph node metastases, an accuracy of 76.47% (18 out of 34) was obtained. The sensitivity and specificity were found to be 52.94% and 84.31%, respectively. Overstaging and understaging of phased-array MRI and ERUS in terms of predicting T and N stage are summarized in Table 4.

## DISCUSSION

Treatment options for rectal cancer depend on the stage at presentation<sup>[1]</sup>. Since staging of rectal cancer with digital rectal examination is unreliable, preoperative staging is mostly based on imaging<sup>[14]</sup>. Accurate staging is particularly important because stage 1 tumors are best treated with surgery alone, whereas stage 2 and 3 tumors require preoperative chemoradiotherapy<sup>[15]</sup>.

CT, ERUS and MRI are the imaging modalities predominantly utilized in the preoperative staging of rectal cancer. CT is unable to differentiate the different layers of the rectal wall and has lower overall predictive accuracy than ERUS and MRI. Initially, preoperative local staging of rectal carcinoma using body coil MRI was only 60% accurate in predicting the transmural tumor invasion<sup>[16]</sup>.

This poor result can be attributed to the use of body coil which suffers from low spatial resolution. But in the

recent years, the advent of endorectal MRI has made it possible to generate images with high signal-to-noise ratio (SNR) near the coil with better identification of the rectal wall. The reported accuracy ranging between 81% and 89%<sup>[17,18]</sup> compares favorably with that of ERUS. However, endorectal MRI does have the same limitations as ERUS. Major pitfalls include poor resolution of pelvic structures surrounding the rectum due to the small field of view, and failure to insert the coil in patients with stenosing tumors.

MRI with a pelvic phased-array coil provides slightly lower resolution of the rectal wall compared to the endorectal coil, but allows the entire pelvis to be imaged, and thus, more distant spread can be assessed. In addition, this coil is noninvasive and useful for all rectal tumors regardless of site and size.

ERUS can distinguish the different anatomic layers of the bowel and thus, it appears to have advantages over both CT or MRI in assessing mural penetration and is invaluable in assessing patients considered for local resection. However, it is highly operator dependent, has poor patient acceptability, has limited depth penetration and can not be performed in stenotic tumors or tumors in the upper rectum<sup>[19,20]</sup>. The assessment of the mesorectal fascia is also hampered by its limited field of view.

Kwok *et al*<sup>[21]</sup> concluded that ERUS was the most accurate technique for assessing wall penetration. However, in studies that compared MRI with an endorectal coil with ERUS, the former was found to be as effective as ERUS for assessing T stage and was more effective in assessing nodal involvement. They concluded that MRI using an endorectal coil was the most accurate technique for predicting the pathological stage of rectal cancer.

The meta-analysis of Bipat *et al*<sup>[22]</sup>, also found that ERUS was the best technique for assessing local invasion, but stressed its limitations: operator dependency, no assessment of stenotic tumor, inability to visualize with a rigid probe, tumors located in the upper rectum, inability to detect lymph nodes outside the range of the transducer, and inability to visualize mesorectal fascia. The authors emphasized that none of the techniques were able to identify involved lymph nodes with satisfactory accuracy.

Overall accuracy in the ERUS assessment of tumor depth ranges from 63%-96% with an average of 81.8% in 2718 patients<sup>[9,11,20]</sup>. In a review of cross-sectional studies investigating tumor depth in 873 patients, the overall accuracy was 85%, with sensitivity ranging from 84% in T1 to 76% in T4<sup>[23]</sup>.

Overstaging of tumor depth frequently occurs as a result of perineoplastic inflammation as ultrasound can not clearly differentiate between inflammatory and neoplastic tissue<sup>[11,24]</sup>. Similarly, preoperative biopsy causes hemorrhage and obliteration of sonographic layers<sup>[11]</sup>.

Phased-array coils or pelvic coils have improved spatial resolution with improved signal-to-noise ratio<sup>[7]</sup>, without the technical limitations of endorectal MRI<sup>[25]</sup>. They have the advantage of having a larger field of view of the mesorectal fascia. The role of circumferential

resection margin as an important prognostic indicator of local recurrence is evident and several MRI studies have shown a high accuracy in this regard<sup>[12,25,26]</sup>. Beets-Tan *et al*<sup>[12]</sup> used contrast-enhanced thin section MRI on a 1.5 tesla scanner with a quadrature phased-array spine coil and reported that the depth of transmural tumor invasion and mesorectal fascia involvement were predicted correctly in 83% and 100% of their patients, respectively. Although contrast enhancement may be helpful for differentiating reactive changes from true tumor invasion, they reported that MRI could not be used to distinguish reliably between fibrosis with and fibrosis without tumor cells.

On the other hand, Tatli *et al*<sup>[27]</sup> in their study using gadolinium-enhanced combined pelvic- phased array and endo-rectal coil MRI, surgical treatment groups (stage 1 vs stage 2/3) were accurately predicted in 33 out of 39 patients (85%). Overall, a 93% sensitivity, 86% specificity, and 88% accuracy were achieved in the identification of mesorectal fat invasion.

Brown *et al*<sup>[28]</sup> conducted a prospective study that found correct invasion depth assessment attained in 100% of their cases. Judging from the excellent results reported by this group who were able to differentiate between desmoplastic spiculation and true invasion, the best technique may be the one described by these authors and involves more precise image acquisition and administration of effective contrast material. Thus, thin section MRI performed on a 1.5 tesla scanner with a phased-array coil in general can be considered to provide moderate to good accuracy in the prediction of invasion depth and good accuracy in the prediction of mesorectal fascia involvement. These data are comparable to accuracy rates of 82%-88%<sup>[23,29]</sup> obtained with ERUS for the prediction of invasion depth.

Although overall T stage accuracies by MRI are similar to that of ERUS, MRI has higher accuracies when assessing T3 and T4 tumors as compared to early T stages (T1 and T2)<sup>[25,26]</sup>. When directly compared to ERUS for a T3 tumor, Blomqvist *et al*<sup>[30]</sup> had 11 false positives with ERUS compared to 8 for MRI. On the other hand, Akasu *et al*<sup>[31]</sup> in their series found that, two third of staging errors in invasion depth resulted from overstaging and were most common with pT2 tumors.

This data is consistent with our study in which most staging errors arised from overstaging with both modalities and these were mostly pT2 tumors (4 pT2 cases were overestimated by MRI and 5 pT2 cases were overestimated by ERUS).

Recent studies confirmed that ERUS can accurately stage the depth of tumor invasion particularly in T1 and T2 tumors<sup>[20,22,32,33]</sup>, whereas MRI seems superior in more locally advanced disease<sup>[25,26]</sup>.

Although our patient population is too small to make a final statement, we found that phased-array MRI had slightly better accuracy (89.70%), sensitivity (79.41%) and specificity (93.14%) as compared to ERUS (85.29%, 70.59% and 90.20%, respectively) for detecting the depth of transmural tumor invasion.

Also we obtained better results by phased-array MRI



in terms of predicting early T stages (6 out of 10) as compared to ERUS (4 out of 10).

The preoperative assessment of regional lymph node status forms part of the overall staging of any rectal tumor. The overall accuracy of assessing lymph node involvement ranges between 59% and 95%<sup>[17]</sup>. Nearly all published MR imaging studies of rectal cancer have used size as a criterion for predicting nodal involvement<sup>[17,34]</sup>, although there is no particular size cut-off that can be used to discriminate between benign and malignant lymph nodes.

Brown *et al*<sup>[35]</sup> confirmed that in mesorectal lymph nodes greater than 3 mm, morphological criteria such as an irregular border and mixed signal intensity is definitely a better predictor of lymph node status than size alone. More recent studies suggest that multiple criteria should be used to improve accuracy<sup>[18]</sup>.

Regarding the detection of lymph node metastases, we obtained an accuracy of 74.50% with phased-array MRI and 76.47% with ERUS. Sensitivity was slightly better with MRI than ERUS (61.76% and 52.94% respectively).

ERUS and phased-array MRI are complementary methods for accurate preoperative staging of rectal cancer. Neither ERUS nor MRI can accurately stage tumoral invasion for all T stages. Nodal staging, although better defined with phased-array MRI, is limited for both of the methods<sup>[10]</sup>.

In conclusion, in this study comparing those two modalities we can state that phased-array MRI is slightly superior in determining the depth of transmural tumor invasion (T stage) and has same value in detecting lymph node metastases (N stage) as compared to ERUS.

## COMMENTS

### Background

The preoperative staging of rectal cancer is very important in terms of planning appropriate therapy and determining prognosis. Therefore, the assessment of transmural tumor invasion depth and detection of lymph node metastases are of major importance for which purpose authors have investigated the accuracy of most recently used imaging techniques, namely endorectal ultrasonography (ERUS) and phased-array magnetic resonance imaging (MRI).

### Research frontiers

ERUS and MRI using various coils are currently used imaging modalities for the preoperative staging of rectal carcinoma. The utility of these techniques for staging of rectal carcinoma has been demonstrated well in the literature and both are regarded very useful. In this prospective study, authors' aim was to determine the diagnostic accuracy of each technique in the same cohort of patient population and then to postsurgically compare the obtained results to find out which modality was more effective in the preoperative staging of rectal cancer.

### Innovations and breakthroughs

The results suggested that phased-array MRI is slightly superior to ERUS in determining the transmural tumor invasion depth whereas both techniques seem to yield similar values in detecting lymph node metastases.

### Applications

Based on this study, authors can state that ERUS and phased-array MRI can be used for the preoperative staging of rectal carcinoma and both techniques are accurate determinants of the T and N stages of tumors in most cases. They can also be applied as complementary methods for accurate preoperative staging of rectal cancer.

### Terminology

Pelvic phased-array MR technique provides a full evaluation of rectal wall layers

with a large field of view compared with the standard MR techniques. It uses the pelvic phased-array coil which is a wrap-around surface coil around the pelvis. This coil has the advantages of the surface coil by obtaining higher signal but with greater coverage than a single surface coil and improved homogeneity resulting in higher spatial resolution images.

### Peer review

This is a very clear text, well presented and well written, the compared effectiveness of MRI and endoscopic ultrasound is compared in 34 patients having a colorectal cancer, with have a pathology control. The results are very clear and important.

## REFERENCES

- 1 **Lindmark G**, Gerdin B, Pahlman L, Bergstrom R, Glimelius B. Prognostic predictors in colorectal cancer. *Dis Colon Rectum* 1994; **37**: 1219-1227
- 2 **Moriya Y**, Sugihara K, Akasu T, Fujita S. Patterns of recurrence after nerve-sparing surgery for rectal adenocarcinoma with special reference to loco-regional recurrence. *Dis Colon Rectum* 1995; **38**: 1162-1168
- 3 **Heriot AG**, Hicks RJ, Drummond EG, Keck J, Mackay J, Chen F, Kalff V. Does positron emission tomography change management in primary rectal cancer? A prospective assessment. *Dis Colon Rectum* 2004; **47**: 451-458
- 4 **Dobos N**, Rubesin SE. Radiologic imaging modalities in the diagnosis and management of colorectal cancer. *Hematol Oncol Clin North Am* 2002; **16**: 875-895
- 5 **Ahmad NA**, Kochman ML, Ginsberg GG. Endoscopic ultrasound and endoscopic mucosal resection for rectal cancers and villous adenomas. *Hematol Oncol Clin North Am* 2002; **16**: 897-906
- 6 **Brown G**, Davies S, Williams GT, Bourne MW, Newcombe RG, Radcliffe AG, Blethyn J, Dallimore NS, Rees BI, Phillips CJ, Maughan TS. Effectiveness of preoperative staging in rectal cancer: digital rectal examination, endoluminal ultrasound or magnetic resonance imaging? *Br J Cancer* 2004; **91**: 23-29
- 7 **Gagliardi G**, Bayar S, Smith R, Salem RR. Preoperative staging of rectal cancer using magnetic resonance imaging with external phase-arrayed coils. *Arch Surg* 2002; **137**: 447-451
- 8 **Sobin LH**, Wittekind C, editors. International union against cancer (UICC). TNM classification of malignant tumors. 6th ed. Baltimore, New York: Wiley-Liss, 2002: 199-202
- 9 **Kumar A**, Scholefield JH. Endosonography of the anal canal and rectum. *World J Surg* 2000; **24**: 208-215
- 10 **Bartram C**, Brown G. Endorectal ultrasound and magnetic resonance imaging in rectal cancer staging. *Gastroenterol Clin North Am* 2002; **31**: 827-839
- 11 **Massari M**, De Simone M, Cioffi U, Rosso L, Chiarelli M, Gabrielli F. Value and limits of endorectal ultrasonography for preoperative staging of rectal carcinoma. *Surg Laparosc Endosc* 1998; **8**: 438-444
- 12 **Beets-Tan RG**, Beets GL, Vliegen RF, Kessels AG, Van Boven H, De Bruine A, von Meyenfeldt MF, Baeten CG, van Engelshoven JM. Accuracy of magnetic resonance imaging in prediction of tumour-free resection margin in rectal cancer surgery. *Lancet* 2001; **357**: 497-504
- 13 **McNicholas MM**, Joyce WP, Dolan J, Gibney RG, MacErlaine DP, Hyland J. Magnetic resonance imaging of rectal carcinoma: a prospective study. *Br J Surg* 1994; **81**: 911-914
- 14 **Nicholls RJ**, Mason AY, Morson BC, Dixon AK, Fry IK. The clinical staging of rectal cancer. *Br J Surg* 1982; **69**: 404-409
- 15 **Minsky BD**. Adjuvant radiation therapy for rectal cancer: is there finally an answer? *Lancet* 2001; **358**: 1285-1286
- 16 **Hodgman CG**, MacCarty RL, Wolff BG, May GR, Berquist TH, Sheedy PF 2nd, Beart RW Jr, Spencer RJ. Preoperative staging of rectal carcinoma by computed tomography and 0.15T magnetic resonance imaging. Preliminary report. *Dis Colon Rectum* 1986; **29**: 446-450

- 17 **Vogl TJ**, Pegios W, Mack MG, Hunerbein M, Hintze R, Adler A, Lobbeck H, Hammerstingl R, Wust P, Schlag P, Felix R. Accuracy of staging rectal tumors with contrast-enhanced transrectal MR imaging. *AJR Am J Roentgenol* 1997; **168**: 1427-1434
- 18 **Kim NK**, Kim MJ, Yun SH, Sohn SK, Min JS. Comparative study of transrectal ultrasonography, pelvic computerized tomography, and magnetic resonance imaging in preoperative staging of rectal cancer. *Dis Colon Rectum* 1999; **42**: 770-775
- 19 **Adams DR**, Blatchford GJ, Lin KM, Ternent CA, Thorson AG, Christensen MA. Use of preoperative ultrasound staging for treatment of rectal cancer. *Dis Colon Rectum* 1999; **42**: 159-166
- 20 **Garcia-Aguilar J**, Pollack J, Lee SH, Hernandez de Anda E, Mellgren A, Wong WD, Finne CO, Rothenberger DA, Madoff RD. Accuracy of endorectal ultrasonography in preoperative staging of rectal tumors. *Dis Colon Rectum* 2002; **45**: 10-15
- 21 **Kwok H**, Bissett IP, Hill GL. Preoperative staging of rectal cancer. *Int J Colorectal Dis* 2000; **15**: 9-20
- 22 **Bipat S**, Glas AS, Slors FJ, Zwinderman AH, Bossuyt PM, Stoker J. Rectal cancer: local staging and assessment of lymph node involvement with endoluminal US, CT, and MR imaging--a meta-analysis. *Radiology* 2004; **232**: 773-783
- 23 **Solomon MJ**, McLeod RS. Endoluminal transrectal ultrasonography: accuracy, reliability, and validity. *Dis Colon Rectum* 1993; **36**: 200-205
- 24 **Starck M**, Bohe M, Simanaitis M, Valentin L. Rectal endosonography can distinguish benign rectal lesions from invasive early rectal cancers. *Colorectal Dis* 2003; **5**: 246-250
- 25 **Beets-Tan RG**. MRI in rectal cancer: the T stage and circumferential resection margin. *Colorectal Dis* 2003; **5**: 392-395
- 26 **Mathur P**, Smith JJ, Ramsey C, Owen M, Thorpe A, Karim S, Burke C, Ramesh S, Dawson PM. Comparison of CT and MRI in the pre-operative staging of rectal adenocarcinoma and prediction of circumferential resection margin involvement by MRI. *Colorectal Dis* 2003; **5**: 396-401
- 27 **Tatli S**, Morteale KJ, Breen EL, Bleday R, Silverman SG. Local staging of rectal cancer using combined pelvic phased-array and endorectal coil MRI. *J Magn Reson Imaging* 2006; **23**: 534-540
- 28 **Brown G**, Richards CJ, Newcombe RG, Dallimore NS, Radcliffe AG, Carey DP, Bourne MW, Williams GT. Rectal carcinoma: thin-section MR imaging for staging in 28 patients. *Radiology* 1999; **211**: 215-222
- 29 **Akasu T**, Sugihara K, Moriya Y, Fujita S. Limitations and pitfalls of transrectal ultrasonography for staging of rectal cancer. *Dis Colon Rectum* 1997; **40**: S10-S15
- 30 **Blomqvist L**, Machado M, Rubio C, Gabrielsson N, Granqvist S, Goldman S, Holm T. Rectal tumour staging: MR imaging using pelvic phased-array and endorectal coils vs endoscopic ultrasonography. *Eur Radiol* 2000; **10**: 653-660
- 31 **Akasu T**, Iinuma G, Fujita T, Muramatsu Y, Tateishi U, Miyakawa K, Murakami T, Moriyama N. Thin-section MRI with a phased-array coil for preoperative evaluation of pelvic anatomy and tumor extent in patients with rectal cancer. *AJR Am J Roentgenol* 2005; **184**: 531-538
- 32 **Nesbakken A**, Lovig T, Lunde OC, Nygaard K. Staging of rectal carcinoma with transrectal ultrasonography. *Scand J Surg* 2003; **92**: 125-129
- 33 **Marusch F**, Koch A, Schmidt U, Zippel R, Kuhn R, Wolff S, Pross M, Wierth A, Gastinger I, Lippert H. Routine use of transrectal ultrasound in rectal carcinoma: results of a prospective multicenter study. *Endoscopy* 2002; **34**: 385-390
- 34 **Zerhouni EA**, Rutter C, Hamilton SR, Balfe DM, Megibow AJ, Francis IR, Moss AA, Heiken JP, Tempany CM, Aisen AM, Weinreb JC, Gatsonis C, McNeil BJ. CT and MR imaging in the staging of colorectal carcinoma: report of the Radiology Diagnostic Oncology Group II. *Radiology* 1996; **200**: 443-451
- 35 **Brown G**, Richards CJ, Bourne MW, Newcombe RG, Radcliffe AG, Dallimore NS, Williams GT. Morphologic predictors of lymph node status in rectal cancer with use of high-spatial-resolution MR imaging with histopathologic comparison. *Radiology* 2003; **227**: 371-377

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## Influence of dexamethasone on mesenteric lymph node of rats with severe acute pancreatitis

Xi-Ping Zhang, Hong-Miao Xu, Yi-Yu Jiang, Shuo Yu, Yang Cai, Bei Lu, Qi Xie, Tong-Fa Ju

Xi-Ping Zhang, Yang Cai, Bei Lu, Qi Xie, Tong-Fa Ju, Department of General Surgery, Hangzhou First People's Hospital, Hangzhou 310006, Zhejiang Province, China  
Hong-Miao Xu, Graduate at Department of Stomatology, The First Affiliated Hospital of College of Medicine, Zhejiang University, Hangzhou 310003, Zhejiang Province, China  
Yi-Yu Jiang, Zhejiang Traditional Chinese Medicine University, Hangzhou 310053, Zhejiang Province, China  
Shuo Yu, Class 01 of Clinical Medicine Department, Zhejiang University, Hangzhou 310029, Zhejiang Province, China  
Author contributions: Zhang XP designed research; Xu HM, Jiang YY and Yu S performed research; Zhang XP and Jiang YY wrote the paper; Cai Y, Lu B, Xie Q and Ju TF contributed the experiment determination.

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Correspondence to: Dr. Xi-Ping Zhang, Department of General Surgery, Hangzhou First People's Hospital, Hangzhou 310006, Zhejiang Province, China. [zxp99688@vip.163.com](mailto:zxp99688@vip.163.com)  
Telephone: +86-571-87065701 Fax: +86-571-87914773  
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nodes were lower in the treated than in the model group ( $P < 0.05$ ,  $P < 0.01$  or  $P < 0.01$ ). NF- $\kappa$ B protein expression was negative in all groups. Comparing P-selectin and caspase-3 expression levels among all three groups, there was no marked difference between the model and treated group.

**CONCLUSION:** Dexamethasone can protect mesenteric lymph nodes. The mechanism may be by reducing the content of inflammatory mediators in the blood and inducing lymphocyte apoptosis.

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**Key words:** Apoptosis; Dexamethasone; Lymph node; Rats; Severe acute pancreatitis; Tissue microarrays

**Peer reviewers:** James H Grendell, Professor of Medicine, Chief, Division of Gastroenterology, Hepatology & Nutrition, Winthrop University Hospital, 222 Station Plaza N. #429, Mineola, New York 11501, United States; Yoshiharu Motoo, MD, PhD, FACP, FACG, Professor and Chairman, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

Zhang XP, Xu HM, Jiang YY, Yu S, Cai Y, Lu B, Xie Q, Ju TF. Influence of dexamethasone on mesenteric lymph node of rats with severe acute pancreatitis. *World J Gastroenterol* 2008; 14(22): 3511-3517 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3511.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3511>

### Abstract

**AIM:** To study the influence and mechanisms of dexamethasone on mesenteric lymph node of rats with severe acute pancreatitis (SAP).

**METHODS:** The SAP rats were assigned to model, treated or sham-operated groups. The mortality, pathological changes of mesenteric lymph nodes, expression levels of NF- $\kappa$ B, P-selectin, Bax, Bcl-2 and caspase-3 protein and changes in apoptotic indexes in lymph nodes were observed at 3, 6 and 12 h after operation. The blood levels of endotoxin, superoxide dismutase (SOD), malondialdehyde (MDA), and endothelin-1 (ET-1) in blood were determined.

**RESULTS:** SOD content, expression of Bax protein and apoptotic index were significantly higher in the treated group than in the model group at different time points ( $P < 0.05$  or  $P < 0.01$ ). Other blood-detecting indexes and histopathological scores of mesenteric lymph

### INTRODUCTION

Severe acute pancreatitis (SAP) with a high incidence of complications and high mortality has been a difficult disease in medical research for many years. Recent studies have confirmed that the release of manifold inflammatory mediators is an important part of the inflammatory cascade reaction. Apart from anti-inflammatory and anti-allergic activity, dexamethasone can improve microcirculation and inhibit enzyme and inflammatory mediators. Dexamethasone has sound therapeutic effects on SAP<sup>[1]</sup>. The mesenteric lymph nodes are very important for the protection of abdominal organs from infection, and perform an important role in maintaining immune balance.

In this experiment, the influence of dexamethasone on changes in inflammatory mediator levels in the

blood, pathological changes of mesenteric lymph nodes, and on changes in expression level of NF- $\kappa$ B, P-selectin, Bax, Bcl-2 and caspase-3 proteins, as well as on apoptotic index, has been observed. This is to explore the protective mechanism of dexamethasone on SAP complicated with mesenteric lymph node injury. This study is believed to be the first to apply tissue microarrays for histopathological determination of mesenteric lymph node disease severity. With advantages such as time and energy saving and high efficiency, tissue microarrays can significantly improve pathological study efficiency.

## MATERIALS AND METHODS

### Materials

Clean grade, healthy male Sprague-Dawley (SD) rats weighing 250-300 g were purchased from the Experimental Animal Center of the Medical School, Zhejiang University. Sodium taurocholate and sodium pentobarbital were purchased from Sigma (USA). Dexamethasone (injection) was purchased from Zhejiang Xinchang Pharmaceutical Company (China). Malondialdehyde (MDA), superoxide dismutase (SOD) assay kits were purchased from Nanjing Jiancheng Bioengineering Research Institute (China), with calculation units of nmol/mL and U/mL, respectively. The serum Endothelin-1 ELA kit (ET-1) was purchased from Cayman Chemical Company (Catalog Number: 583151, USA) and the calculation unit for content was ng/L (or pg/mL). The NF- $\kappa$ B, Bax, Bcl-2 and P-selectin antibody were purchased from Santa Cruz (Santa Cruz, CA). Caspase-3 antibodies was purchased from NeoMarkers. The DNA *in situ* nick end-labeling (TUNEL) kit was purchased from Takara (Japan).

### Animal grouping and rat SAP model preparation

Ninety clean grade, healthy male SD rats were prepared into the SAP models and randomly divided into the model and treated groups (45 rats each). Another 45 were selected to be the sham-operated group. Next, the above groups were randomly divided into 3, 6 and 12-h groups, with 15 rats in each group. The treated group was injected with dexamethasone *via* the tail vein: 0.5 mg/100 g body weight, single dose, 15 min after successful preparation of the SAP model. The sham operation consisted of abdominal opening, pancreas and duodenum turning over, and finally abdominal closure. The sham-operated and model groups were injected with saline *via* the tail vein 15 min after the operation. SAP preparation was as follows. The rats were anesthetized by intraperitoneal injection of 2% sodium pentobarbital (0.25 mL/100 g). In the model group, we identified the duodenal papilla inside the duodenum duct wall, and then used a No. 5 needle to drill a hole in the mesenterium avascular area. After inserting a segmental epidural catheter into the duodenum cavity *via* the hole, it was inserted into the biliary-pancreatic duct, in the direction of the papilla, in a retrograde position. This was followed by retrograde transfusion of 3.5% sodium

Table 1 Pathological score standard of lymph node

Score	Observation indexes
1	Follicle Germinal center dilated, lymphatic sinus dilated, sinus cell hyperplasia or only lymphatic sinus dilated, sinus cell hyperplasia
2	Follicle Germinal center dilated, lymphatic sinus dilated, sinus cell hyperplasia, spotty necrosis in mantle zone and Germinal center or only lymphatic sinus dilated, and sinus cell hyperplasia, infiltration of neutrophil, eosinophile granulocyte and plasmocyte
3	Follicle Germinal center dilated, lymphatic sinus dilated, sinus cell hyperplasia, spotty necrosis in mantle zone and Germinal center, infiltration of neutrophil, eosinophile granulocyte and plasmocyte

taurocholate 0.1 mL/100 g by microinjection pump at the speed of 0.2 mL/min, and then the hole in the duodenum lateral wall was sutured<sup>[2-6]</sup>.

### Survival rate and pathological changes

Rat mortality was measured at 3, 6 and 12 h after operation, and survival was calculated. We observed the pathological changes in the mesenteric lymph nodes. After euthanasia with sodium pentobarbital, we collected mesenteric lymph node samples from the rats. The lymph node pathological score was determined according to a set of standards developed by Zhang (Table 1).

### Observation index

The levels of plasma endotoxin, and serum SOD, MDA and endothelin-1 (ET-1) were determined from blood taken from the heart. The above indexes were all detected according to the manufacturers' instructions.

### Tissue microarrays and staining

Prepared tissue microarrays of mesenteric lymph node and adopted SP (streptavidin-peroxidase) method for immunohistochemical staining, observed the NF- $\kappa$ B, Bax and Bcl-2 protein expression respectively. TUNEL Staining was carried out as follows<sup>[7]</sup>. NF- $\kappa$ B, P-selectin, Bax, Bcl-2 and Caspase-3 protein expression: We applied tissue microarrays to prepare microarray sections of mesenteric lymph node; Adopted SP (streptavidin peroxidase) method for immunohistochemical = staining. We observed protei expression in mesenteric lymph nodes under light microscope, as follows: (-) < 10% positive cells; (+) 10%-20% positive cells; (++) 20%-50% positive cells; and (+++) > 50% positive cells. We applied the tissue microarrays to prepare the microarray sections of mesenteric lymph node. TUNEL staining was used to visualize the number of apoptotic cells in the lymph node tissue microarrays, and the apoptotic index (%) was calculated.

### Statistical analysis

Statistical analysis was conducted by using SPSS 11.5 software (SPSS, USA). The Kruskal-Wallis test or analysis of variance was applied for comparison of the three groups. The Bonfferoni test was also applied.  $P \leq 0.05$  was considered statistically significant.



**Table 2** Comparison of different indexes level in blood (*M* (*Q<sub>R</sub>*))

Index	Sham-operated group (h)			Model group (h)			Treated group (h)		
	3	6	12	3	6	12	3	6	12
Endotoxin (EU/mL)	0.02 (0.01)	0.02 (0.01)	0.02 (0.01)	0.04 (0.02)	0.06 (0.03)	0.06 (0.02)	0.03 (0.01)	0.04 (0.01)	0.04 (0.02)
MDA (nmol/mL)	9.9 (9.9)	13.2 (6.6)	13.2 (9.9)	29.7 (6.6)	33.0 (9.9)	29.7 (14.9)	19.8 (9.9)	26.4 (13.2)	26.4 (13.2)
SOD (U/mL)	105.6 (8.3)	103.6 (6.2)	99.2 (16.2)	76.6 (13.0)	73.0 (24.5)	77.6 (12.6)	90.8 (13.4)	91.9 (11.6)	88.7 (13.7)
ET-1 (ng/L)	14.05 (1.78)	14.53 (2.082)	14.78 (2.28)	17.97 (5.57)	19.21 (7.02)	18.31 (5.06)	13.64 (1.54)	13.86 (2.64)	13.66 (2.47)

## RESULTS

### Survival

In the model group, the mortality was 0, 0 and 13.33% (2/15) at 3, 6 and 12 h, respectively. The sham-operated and treated groups showed 100% survival, while there was no significant difference between the model and treated groups.

### Comparison of serum MDA, SOD and ET-1 levels

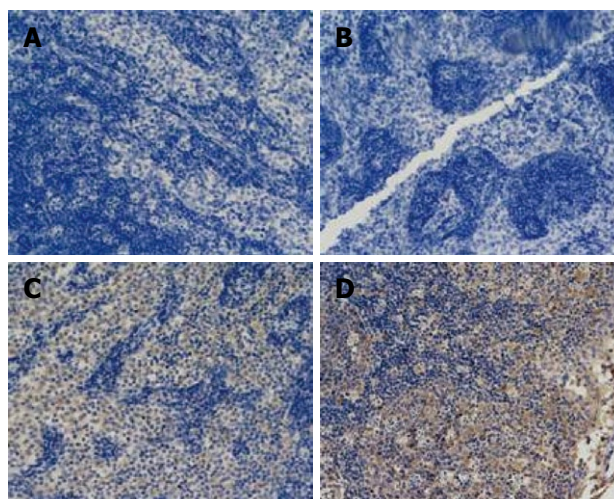
Serum MDA was significantly higher in the model and treated groups than in the sham-operated group at all time points ( $P < 0.01$ ). Serum MDA in the treated group was significantly lower than in the model group at 3 and 6 h ( $P < 0.01$ ) (Table 2).

Serum SOD in the model and treated groups was significantly lower than in the sham-operated group at different time points ( $P < 0.01$ ). Serum SOD in the treated group was significantly higher than in the model group at different time points ( $P < 0.01$ ) (Table 2).

Serum ET-1 differed significantly between the treated and sham-operated groups at various time points ( $P < 0.05$ ), and the level in the model group significantly higher than that in the sham-operated group at various time points ( $P < 0.01$ ). Serum ET-1 in the treated group was significantly lower than that in the model group at 3 and 12 h ( $P < 0.01$ ), and the level in the treated group was significantly lower than that in the model group at 6 h ( $P < 0.01$ ) (Table 2).

### Pathological changes in lymph nodes, seen by light microscopy

The mesenteric lymph nodes in the sham-operated group had normal morphology and structure. In the model and treated groups, swollen lymph nodes, dilated follicular germinal centers and expanded lymphatic sinuses were seen in most cases. Sinus cell hyperplasia and spotty necrosis in the mantle zone and germinal center of the lymph nodes were clearly visible, and neutrophil and plasmocyte infiltration was seen in a few cases. There were no marked differences in the pathological changes between the model and treated groups, but spotty necrosis in the mantle zone and germinal centers, as well as inflammatory cell infiltration only occurred in a few rats in the treated group.



**Figure 1** Tissue microarrays of mesenteric lymph nodes were prepared and stained for immunohistochemistry ( $\times 200$ ). (A) NF- $\kappa$ B expression in the treated group (6 h); (B) Bax protein expression in the sham-operated group (3 h); (C) Bax protein expression in the treated group (12 h); (D) Bcl-2 protein expression in the treated group (3 h).

### Comparison of lymph node pathological scores

The pathological score in the model group was significantly higher than in the sham-operated group at different time points (6 h,  $P < 0.05$ , 3 and 12 h,  $P < 0.01$ ). The score in the treated group was significantly higher than in the sham-operated group at 12 h ( $P < 0.01$ ). The pathological score was significantly lower in the treated group than in the model group at 6 h ( $P < 0.05$ ) (Table 3).

### Changes in lymph node expression of NF- $\kappa$ B, P-selectin, Bax, Bcl-2 and caspase-3

Expression of NF- $\kappa$ B was negative in all groups at all times (Figure 1A). Lymphocytes were stained positively for P-selectin. There was no significant difference in staining in any of the groups. Lymphocytes were stained positively for Bax. Expression of Bax was significantly higher in the treated group than in the model group at 12 h ( $P < 0.01$ ). There was no marked difference in the other groups (Tables 3 and 4, Figure 1B and C). Lymphocytes were stained positively for Bcl-2. There was no significant difference among any of the groups at different time points (Tables 3 and 4, Figure 1D).

### Comparison of caspase-3 expression level of lymph node

Lymphocytes were stained positive for caspase-3. There was no significant difference between the sham-operated and model groups at all time points. Caspase-3 expression in the sham-operated group was significantly lower than in the treated group at 12 h ( $P < 0.05$ ). There was no significant difference between the model and treated groups at all time-points (Tables 3 and 4).

### Comparison of apoptotic index in lymph nodes

The apoptotic cells in lymph nodes were the lymphocytes. Apoptotic cells appeared in three rats in the sham-operated group at 6 h, with an apoptotic index between

**Table 3** Comparison of different pathological indexes in the lymph node ( $M(Q_R)$ )

Indexes	Sham-operated group (h)			Model group (h)			Treated group (h)		
	3	6	12	3	6	12	3	6	12
Pathological score	2.0 (1.0)	2.0 (1.0)	1.0 (1.0)	2.0 (1.0)	2.0 (1.0)	2.0 (1.0)	2.0 (0.0)	2.0 (0.0)	2.0 (0.0)
P-selection	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Bax protein	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (1.0)	0.0 (1.0)
Bcl-2 protein	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)
Caspase-3 protein	0.0 (0.0)	0.0 (1.0)	0.0 (0.0)	0.0 (1.0)	0.0 (1.0)	0.0 (1.0)	0.0 (0.0)	0.0 (1.0)	0.0 (1.0)
Apoptotic index	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	0.0 (0.03)	0.0 (0.0)	0.0 (0.3)	0.0 (0.4)

**Table 4** Expression of Bax, Bcl-2 and P-selectin caspase-3 pathologic grade

Groups	Cases	Bax				Bcl-2			P-selectin		Caspase-3	
		-	+	++	+++	-	+	++	-	+	-	+
Sham-operated group-3 h	15	12	3			15			15		12	3
Sham-operated group-6 h	15	12	0	2		15			15		11	4
Sham-operated group-12 h	15	13		1	1	15			15		12	3
Model group-3 h	15	13	2			15			14	1	11	4
Model group-6 h	15	13	1	1		15			12	3	11	4
Model group-12 h	13	13				13			12	1	9	4
Treated group-3 h	15	12	3			12	1	2	15		12	3
Treated group-6 h	15	9	4	2		15			14	1	9	6
Treated group-12 h	15	8	5	1	1	15			14	1	5	10

10 and 20 per 10000 cells. Apoptotic cells appeared in four rats in the model group at 12 h, with an apoptotic index between 6 and 50 per 10000 cells. Apoptotic cells appeared in three, eight and nine rats at 3, 6 and 12 h, respectively, with an apoptotic index between 2 and 160 per 10000 cells in treated group. There was no significant difference among any of the groups at 3 h. The apoptotic index was significantly higher in the treated group than in the model group at 6 h ( $P < 0.05$ ). The apoptotic index was significantly higher in the treated group than in the sham-operated group at 12 h ( $P < 0.01$ ) (Table 4 and Figure 2).

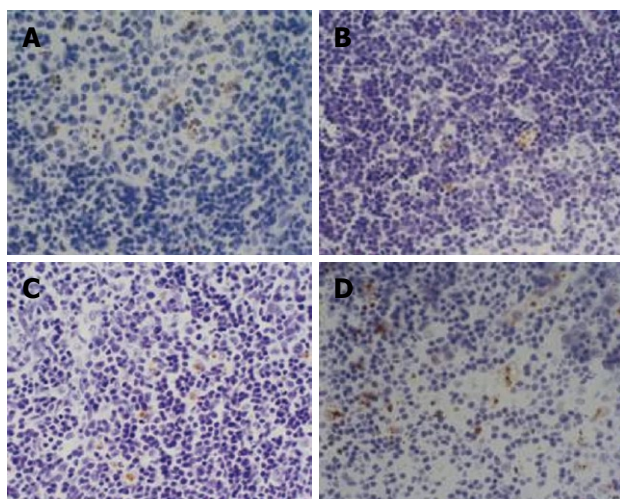
## DISCUSSION

At present, the pathogenesis of SAP has not been fully elucidated<sup>[7,8]</sup>. We are attracted to the barrier function of mesenteric lymph nodes during the onset and progression of SAP<sup>[9]</sup>. The normal mesenteric lymph node barrier can effectively prevent intestinal bacteria and endotoxin entering the body and maintain homeostasis<sup>[10-12]</sup>, while the function of the lymphocytes in the mesenteric lymph nodes reflects the function of the whole intestinal immune barrier<sup>[13]</sup>. If the function of the immune barrier is injured, the mesenteric lymph nodes are damaged and permeability of mucous membrane of small intestine is increased<sup>[14]</sup>, which reduces their capacity to phagocytose ectopic bacteria and endotoxin. The excessive release of inflammatory mediators during SAP is the main cause of mesenteric lymph node injury. According to the results of this experiment, after inducing SAP, the level of inflammatory mediators such as ET-1 and MDA in the blood was significantly higher in the treated group than in the sham-operated group, and the level of

inflammatory mediators was positively correlated with the severity of mesenteric lymph node injury.

Endotoxin can invade the body and cause gut-origin endotoxemia, increase permeability of mesenteric lymph nodes, and promote invasion of intestinal bacteria and endotoxin. As one of the important common mediators in inflammatory mediator cascade reaction of inflammatory reaction<sup>[15-17]</sup>, Nitric oxide (NO) can be regarded as an index of SAP. However, some researchers<sup>[18]</sup> believe that a small amount of endogenous NO can protect the body from ischemia-reperfusion injury, and prevent any increase in intestinal vasopermeability induced by endotoxemia and bacteria translocation.

NF- $\kappa$ B is a transcription factor that participates in the regulation of expression of inflammatory molecules. Its activation is a key initial step in the inflammatory reaction<sup>[19-21]</sup>. However, NF- $\kappa$ B was found to be negative in this experiment, which indicates that there is no direct relation between inflammatory reactions in the mesenteric lymph nodes and NF- $\kappa$ B. This study also showed that dexamethasone decreased the level of inflammatory mediators. It was demonstrated that dexamethasone exerted its anti-inflammatory effect by lowering the level of several inflammatory factors in the serum, inhibiting their production, and blocking the cascade reaction of the inflammatory mediators. P-Selectin is a member of the family of cell adhesion molecules. However, the content is very low and its expression is significantly increased with acute inflammation<sup>[22,23]</sup>. It is also an important indicator of inflammation<sup>[22,24]</sup>. In this study, there was no marked difference in P-selectin expression. Therefore, we suppose that P-selectin expression is not related to lymph node injury.



**Figure 2** TUNEL staining, showing the pathological changes in the mesenteric lymph nodes ( $\times 400$ ). (A) In the sham-operated group (6 h), there were no apoptotic cells; (B) In the model group (12 h), several apoptotic cells appeared; (C) treated group (6 h); (D) apoptotic cells were increased in the treated group (12 h).

Apoptosis also plays an important role during the onset, progression and prognosis of AP. It has been found<sup>[25,26]</sup> that when necrosis and apoptosis coexist and necrosis prevails, inducing apoptosis results in body protection. Both necrosis and apoptosis are mechanisms of death for injured cells<sup>[27]</sup>. Unlike necrosis, apoptosis does not release harmful substances in lysosomes or cause intense inflammatory reactions<sup>[28]</sup>. The present study showed that expression of Bax protein was significantly higher in the treated group than in the model group at 12 h. The expression of Bcl-2 protein was reduced. Bcl-2 expression was positively correlated with apoptosis level of mesenteric lymph nodes, and negatively correlated with SAP severity. The expression of Bax and Bcl-2 proteins was not significant in the model group at any time, with less apoptosis, which indicates that dexamethasone can protect lymph nodes by increasing Bax protein, inhibiting Bcl-2 protein, and inducing apoptosis of mesenteric lymph nodes. The apoptotic index was significantly higher in the treated group than in the model group, while the pathological score was significantly lower in the treated group than in the model group, which indicates that dexamethasone can promote the apoptosis of mesenteric lymph nodes and protect the lymph nodes. The massive apoptosis of lymph node cells certainly inhibits excessive inflammation, and protects several organs from SAP-related lymph node injury. Caspase-3 is one of the important proteases that can induce apoptosis, and is also the final effector in the caspase cascade effect, which is involved in apoptosis. Moreover, it has a pivotal position in the process of stopping the protease cascade. Caspase-3 is a marker of apoptosis and it is also involved in the process. It can destroy a variety of protease complexes in cells with the digestive way, activate intranuclear nuclease to cause DNA schizolysis, form DNA fragments, undermine cell calcium pump function, and lead to intracellular calcium overload<sup>[29,30]</sup>. Inhibiting caspase-3 activity can reduce the occurrence of

apoptosis<sup>[31]</sup>. In this study, there was no marked difference in caspase-3 expression level in the model and treated groups; therefore, we suppose that caspase-3 expression is not related to severity of lymph node injury.

Núñez *et al.*<sup>[32]</sup> found that apoptosis occurs with high concentrations of NO. Increasing NO in serum can alleviate the inflammatory reaction of AP. Apoptosis during SAP is the result of complex action of various factors whose relationships form a network structure. Since 1952, when Stephensen *et al.*<sup>[33]</sup> first reported the therapeutic effect of glucocorticoids on AP, many empirical studies have confirmed that glucocorticoids can improve survival of animals with pancreatitis<sup>[33-35]</sup>, although the precise mechanism is unclear. As a long-acting glucocorticoid, dexamethasone can be applied extensively in a clinical setting. It can regulate inflammatory mediators<sup>[36,37]</sup>, improve microcirculation<sup>[38]</sup>, eliminate OFR (oxygen free radical)<sup>[39]</sup>, and inhibit NF- $\kappa$ B<sup>[40,41]</sup>. There have now been several studies on the mechanism of dexamethasone in SAP, but there has still been no study on the effect of dexamethasone on mesenteric lymph nodes. We are of the opinion that a few apoptotic lymphocytes in lymph nodes mean that the function of lymphocytes has been slightly injured. However, the significance of lymphocyte apoptosis is that its immunological function is severely inhibited, which can have a protective effect.

Since it was first reported by Kononen *et al.*<sup>[42]</sup> in 1998, tissue microarray has been extensively used<sup>[43-45]</sup>, but there have been no reports on its application in the study of pancreatitis. In this experiment, the diameter of microarray tissue was only 2 mm. Super sensitive SP immunohistochemistry and TUNEL technique were used. The observation indexes of this experiment are satisfactory. This study demonstrates that tissue arrays of 2.0 mm diameter have advantages such as reliability, savings in time, energy and reagents, and convenient control<sup>[46,47]</sup>. This study provides a new theoretical basis for the application of tissue microarray in the pathological examination of AP.

## COMMENTS

### Background

The normal mesenteric lymph node barrier can effectively prevent intestinal bacteria and endotoxin entering the human body and maintain homeostasis. The function of lymphocytes in the mesenteric lymph nodes reflects the function of the whole intestinal immune barrier. If the function of the immune barrier is injured, the mesenteric lymph nodes will be damaged, which reduces the capacity for phagocytosis of ectopic bacteria and endotoxin. The excessive release of inflammatory mediators during SAP is the main cause of mesenteric lymph node injury.

### Research frontiers

SAP has a high incidence of complications and high mortality, and has been a difficult disease in medical research for many years. Recent studies have confirmed that the release of many inflammatory mediators is an important reason for the inflammatory cascade reaction. Apart from anti-inflammatory and anti-allergic activity, dexamethasone can improve microcirculation and inhibit enzymes and inflammatory mediators. Dexamethasone has sound therapeutic effects on SAP. In this study, the influence of dexamethasone on changes in content of inflammatory mediators in blood, pathological changes of mesenteric lymph nodes, and changes of expression of NF- $\kappa$ B, P-selectin, Bax, Bcl-2 and caspase-3 proteins, as well as apoptotic index, was investigated, to explore the



protective mechanism of dexamethasone on SAP complicated with injury of mesenteric lymph nodes.

### Innovations and breakthroughs

This study applied tissue microarrays for histopathological examination of mesenteric lymph nodes, which is believed to be the first report of the pathological category for severity of lymph nodes around the world.

### Applications

With advantages such as time and energy savings and high efficiency, tissue microarrays can significantly improve study efficiency. We applied tissue microarrays to pathological detection and obtained good results.

### Terminology

NF- $\kappa$ B is a transcription factor that participates in regulating expression of inflammatory molecules. Its activation is a key initial step in the inflammatory reaction. NF- $\kappa$ B can regulate the expression of inflammatory mediators.

### Peer review

This is a very interesting study. The authors studied the influences and mechanisms of dexamethasone on mesenteric lymph nodes in rats with SAP. It is believed to be the first report of the pathological classification of severity of lymph node injury around the world.

## REFERENCES

- Zhang XP, Zhang L, Chen LJ, Cheng QH, Wang JM, Cai W, Shen HP, Cai J. Influence of dexamethasone on inflammatory mediators and NF-kappaB expression in multiple organs of rats with severe acute pancreatitis. *World J Gastroenterol* 2007; **13**: 548-556
- Zhang XP, Chen L, Hu QF, Tian H, Xu RJ, Wang ZW, Wang KY, Cheng QH, Yan W, Li Y, Li QY, He Q, Wang F. Effects of large dose of dexamethasone on inflammatory mediators and pancreatic cell apoptosis of rats with severe acute pancreatitis. *World J Gastroenterol* 2007; **13**: 5506-5511
- Zhang XP, Zhang L, Xu HM, Xu YP, Cheng QH, Wang JM, Shen HP. Application of tissue microarrays to study the influence of dexamethasone on NF-kappaB expression of pancreas in rat with severe acute pancreatitis. *Dig Dis Sci* 2008; **53**: 571-580
- Xiping Z, Li C, Miao L, Hua T. Protecting effects of dexamethasone on thymus of rats with severe acute pancreatitis. *Mediators Inflamm* 2007; **2007**: 72361
- Zhang XP, Zhang L, Wang Y, Cheng QH, Wang JM, Cai W, Shen HP, Cai J. Study of the protective effects of dexamethasone on multiple organ injury in rats with severe acute pancreatitis. *JOP* 2007; **8**: 400-412
- Zhang XP, Tian H, Lu B, Chen L, Xu RJ, Wang KY, Wang ZW, Cheng QH, Shen HP. Tissue microarrays in pathological examination of apoptotic acinar cells induced by dexamethasone in the pancreas of rats with severe acute pancreatitis. *Hepatobiliary Pancreat Dis Int* 2007; **6**: 527-536
- Lankisch PG, Weber-Dany B, Doobe C, Finger T, Maisonneuve P, Lowenfels AB, Keim V. Pankrin: a new parameter for the diagnosis of acute pancreatitis in cases of late clinical presentation. *Pancreas* 2006; **32**: 330-331
- Sugimoto M, Takada T, Yasuda H. A new experimental pancreatitis by incomplete closed duodenal loop: the influence of pancreatic microcirculation on the development and progression of induced severe pancreatitis in rats. *Pancreas* 2004; **28**: e112-e119
- Gianotti L, Braga M, Alexander JW. [The intestine: a central organ in the pathogenesis of septic complications in acute pancreatitis] *Chir Ital* 1995; **47**: 14-24
- Garside P, Millington O, Smith KM. The anatomy of mucosal immune responses. *Ann N Y Acad Sci* 2004; **1029**: 9-15
- Harari Y, Weisbrodt NW, Moody FG. Ileal mucosal response to bacterial toxin challenge. *J Trauma* 2000; **49**: 306-313
- Kiyono H, Kweon MN, Hiroi T, Takahashi I. The mucosal immune system: from specialized immune defense to inflammation and allergy. *Acta Odontol Scand* 2001; **59**: 145-153
- Kiyono H, Kweon MN, Hiroi T, Takahashi I. The mucosal immune system: from specialized immune defense to inflammation and allergy. *Acta Odontol Scand* 2001; **59**: 145-153
- Lou TJ, Li N, Gao JZ. Intestinal barrier function of rats injured by glucocorticoid. *Zhonghua Qigian Yizhi Zazhi* 2005; **26**: 610-611
- Uehara S, Gothoh K, Handa H, Tomita H, Tomita Y. Immune function in patients with acute pancreatitis. *J Gastroenterol Hepatol* 2003; **18**: 363-370
- Gomez-Cambronero L, Camps B, de La Asuncion JG, Cerda M, Pellin A, Pallardo FV, Calvete J, Sweiry JH, Mann GE, Vina J, Sastre J. Pentoxifylline ameliorates cerulein-induced pancreatitis in rats: role of glutathione and nitric oxide. *J Pharmacol Exp Ther* 2000; **293**: 670-676
- Um SH, Kwon YD, Kim CD, Lee HS, Jeon YT, Chun HJ, Lee SW, Choi JH, Ryu HS, Hyun JH. The role of nitric oxide in experimental cerulein induced pancreatitis. *J Korean Med Sci* 2003; **18**: 520-526
- Schulz HU, Niederau C, Klonowski-Stumpe H, Halangk W, Luthen R, Lippert H. Oxidative stress in acute pancreatitis. *Hepatogastroenterology* 1999; **46**: 2736-2750
- Liu X, Nakano I, Yamaguchi H, Ito T, Goto M, Koyanagi S, Kinjoh M, Nawata H. Protective effect of nitric oxide on development of acute pancreatitis in rats. *Dig Dis Sci* 1995; **40**: 2162-2169
- Grisham MB. NF-kappaB activation in acute pancreatitis: protective, detrimental, or inconsequential? *Gastroenterology* 1999; **116**: 489-492
- Suk K, Yeou Kim S, Kim H. Regulation of IL-18 production by IFN gamma and PGE2 in mouse microglial cells: involvement of NF-kB pathway in the regulatory processes. *Immunol Lett* 2001; **77**: 79-85
- Vaquero E, Gukovsky I, Zaninovic V, Gukovskaya AS, Pandol SJ. Localized pancreatic NF-kappaB activation and inflammatory response in taurocholate-induced pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G1197-G1208
- Lundberg AH, Granger DN, Russell J, Sabek O, Henry J, Gaber L, Kotb M, Gaber AO. Quantitative measurement of P- and E-selectin adhesion molecules in acute pancreatitis: correlation with distant organ injury. *Ann Surg* 2000; **231**: 213-222
- Kameda H, Morita I, Handa M, Kaburaki J, Yoshida T, Mimori T, Murota S, Ikeda Y. Re-expression of functional P-selectin molecules on the endothelial cell surface by repeated stimulation with thrombin. *Br J Haematol* 1997; **97**: 348-355
- Ushiyama S, Laue TM, Moore KL, Erickson HP, McEver RP. Structural and functional characterization of monomeric soluble P-selectin and comparison with membrane P-selectin. *J Biol Chem* 1993; **268**: 15229-15237
- Pei HH, Liu RL, Jiang CQ, Ma LL, Fang XY. Influence of emodin on pancreatic acinar cell apoptosis in rats with acute pancreatitis. *Bengbu Yixueyuan Xuebao* 2005; **30**: 112-113
- Pei HH, Dai W, Zhou J. Effects of somatostatin on apoptosis of pancreatic acinar cell apoptosis in acute necrotizing pancreatitis in rats. *Guangdong Yixue Zazhi* 2004; **25**: 138-140
- Bhatia M. Apoptosis of pancreatic acinar cells in acute pancreatitis: is it good or bad? *J Cell Mol Med* 2004; **8**: 402-409
- Samuilov VD, Oleskin AV, Lagunova EM. Programmed cell death. *Biochemistry (Mosc)* 2000; **65**: 873-887
- Li H, Kolluri SK, Gu J, Dawson MI, Cao X, Hobbs PD, Lin B, Chen G, Lu J, Lin F, Xie Z, Fontana JA, Reed JC, Zhang X. Cytochrome c release and apoptosis induced by mitochondrial targeting of nuclear orphan receptor TR3. *Science* 2000; **289**: 1159-1164
- Henaff M, Antoine S, Mercadier JJ, Coulombe A, Hatem SN. The voltage-independent B-type Ca2+ channel modulates apoptosis of cardiac myocytes. *FASEB J* 2002; **16**: 99-101
- Nunez G, Benedict MA, Hu Y, Inohara N. Caspases: the



- proteases of the apoptotic pathway. *Oncogene* 1998; **17**: 3237-3245
- 33 **Stephenson HE Jr**, Pfeffer RB, Saupol GM. Acute hemorrhagic pancreatitis; report of a case with cortisone treatment. *AMA Arch Surg* 1952; **65**: 307-308
  - 34 **Kaneto H**, Fujii J, Seo HG, Suzuki K, Matsuoka T, Nakamura M, Tatsumi H, Yamasaki Y, Kamada T, Taniguchi N. Apoptotic cell death triggered by nitric oxide in pancreatic beta-cells. *Diabetes* 1995; **44**: 733-738
  - 35 **Schiller WR**, Duprez A, Iams WB, Suwa M, Anderson MC. Experimental pancreatitis. Treatment by colloid replacement and adrenocorticosteroid therapy combined with thoracic duct drainage. *Arch Surg* 1969; **98**: 698-702
  - 36 **Kimura T**, Zuidema GD, Cameron JL. Steroid administration and acute pancreatitis: studies with an isolated, perfused canine pancreas. *Surgery* 1979; **85**: 520-524
  - 37 **McKay CJ**, Gallagher G, Brooks B, Imrie CW, Baxter JN. Increased monocyte cytokine production in association with systemic complications in acute pancreatitis. *Br J Surg* 1996; **83**: 919-923
  - 38 **Barnes PJ**. Anti-inflammatory actions of glucocorticoids: molecular mechanisms. *Clin Sci (Lond)* 1998; **94**: 557-572
  - 39 **Yue MX**, Zhang GX, Li CL, Li XB, Zhang LC, Zhang SL, Xue L, Wang XM. The effect of anisodaminum and dexamethasone on microcirculation in rabbit with multiple organ dysfunction syndrome. *Wei xuanhuan Zazhi* 1997; **7**: 10-11
  - 40 **Liu JS**, Wei XG, Fu J, Liu Jin, Yuan YZ, Wu YL. Study of the relationship among endothelin, nitric oxide, oxygen free radical and acute pancreatitis. *Zhongguo Yishi Zazhi* 2003; **5**: 28-29
  - 41 **Meduri GU**. New rationale for glucocorticoid treatment in septic shock. *J Chemother* 1999; **11**: 541-550
  - 42 **Lanza L**, Scudeletti M, Monaco E, Monetti M, Puppo F, Filaci G, Indiveri F. Possible differences in the mechanism(s) of action of different glucocorticoid hormone compounds. *Ann N Y Acad Sci* 1999; **876**: 193-197
  - 43 **Kononen J**, Bubendorf L, Kallioniemi A, Barlund M, Schraml P, Leighton S, Torhorst J, Mihatsch MJ, Sauter G, Kallioniemi OP. Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 1998; **4**: 844-847
  - 44 **Li R**, Younes M, Frolov A, Wheeler TM, Scardino P, Ohori M, Ayala G. Expression of neutral amino acid transporter ASCT2 in human prostate. *Anticancer Res* 2003; **23**: 3413-3418
  - 45 **Zellweger T**, Ninck C, Mirlacher M, Annfeld M, Glass AG, Gasser TC, Mihatsch MJ, Gelmann EP, Bubendorf L. Tissue microarray analysis reveals prognostic significance of syndecan-1 expression in prostate cancer. *Prostate* 2003; **55**: 20-29
  - 46 **Wulfing P**, Diallo R, Muller C, Wulfing C, Poremba C, Heinecke A, Rody A, Greb RR, Bocker W, Kiesel L. Analysis of cyclooxygenase-2 expression in human breast cancer: high throughput tissue microarray analysis. *J Cancer Res Clin Oncol* 2003; **129**: 375-382
  - 47 **Parker RL**, Huntsman DG, Lesack DW, Cupples JB, Grant DR, Akbari M, Gilks CB. Assessment of interlaboratory variation in the immunohistochemical determination of estrogen receptor status using a breast cancer tissue microarray. *Am J Clin Pathol* 2002; **117**: 723-728

S- Editor Li DL L- Editor Kerr C E- Editor Lin YP



RAPID COMMUNICATION

## Prevalence of Barrett's esophagus in patients with moderate to severe erosive esophagitis

Nooman Gilani, Richard D Gerkin, Francisco C Ramirez, Shahina Hakim, Adam C Randolph

Nooman Gilani, Richard D Gerkin, Francisco C Ramirez, Shahina Hakim, Adam C Randolph, Department of Medicine and Research, Section of Gastroenterology, Carl. T. Hayden Veterans Administration Medical Center, Phoenix, Arizona 85012, United States

**Author contributions:** Gilani N designed research; Gilani N, Randolph AC and Hakim S performed research; Gilani N and Randolph AC analyzed data; Gerkin RD performed statistical analysis; Gilani N and Randolph AC wrote the paper; Gilani N, Ramirez FC, Randolph AC and Gerkin RD reviewed, edited and approved the final manuscript.

**Correspondence to:** Nooman Gilani, MD, FACP, FASGE, Chief of Endoscopy, Department of Gastroenterology (111G), Carl. T. Hayden VAMC, 650 E Indian School Road, Phoenix, Arizona 85012, United States. [ngilani@pol.net](mailto:ngilani@pol.net)

Telephone: +1-602-2775551 Fax: +1-602-2226562

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Even when suspected, BE and associated dysplasia can be missed in the presence of inflammation; therefore, repeat evaluation should be considered after complete healing of esophagitis.

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Gilani N, Gerkin RD, Ramirez FC, Hakim S, Randolph AC. Prevalence of Barrett's esophagus in patients with moderate to severe erosive esophagitis. *World J Gastroenterol* 2008; 14(22): 3518-3522 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3518.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3518>

### Abstract

**AIM:** To investigate the proportion of patients with moderate-severe erosive esophagitis (EE) who will have Barrett's esophagus (BE) after healing of inflammation.

**METHODS:** Patients with EE of Los Angeles (LA) class B, C and D who underwent follow-up endoscopy documenting complete mucosal healing.

**RESULTS:** A total of 86/169 patients were suspected of having BE (38 before healing and 48 after healing of EE) and, 46/86 eventually had the histological confirmation. At index esophago-gastro-duodenoscopy (EGD), BE was suspected in 38/169 (22%), and ultimately, histologically confirmed in 20 of these. In 11 patients where biopsies were performed in the presence of inflammation, BE was detected in 2 and missed in 5 (including 2 dysplasias). In 131/169 patients (77.5%), BE was not suspected at index EGD. After healing of EE though, 48 patients had suspicion of BE who underwent biopsies, and in 26 of these histology was positive for BE. The length of inflammation had a linear correlation with the length of BE ( $P = 0.01$ ). Out of multiple variables to predict BE, only the suspicion at index endoscopy was statistically significant ( $P = 0.01$ ).

**CONCLUSION:** BE was seen in 46/169 (27%) patients with EE of LA class B, C and D. The length of EE can predict the length of underlying BE segment.

### INTRODUCTION

Gastroesophageal reflux disease (GERD) affects 10-20 percent population in Western nations and may be complicated by erosive esophagitis (EE), Barrett's esophagus (BE), and esophageal adenocarcinoma among others<sup>[1]</sup>. In a large population-based cohort study, the estimated incidence of esophagitis was 2.4 per 1000 patient-years<sup>[2]</sup> and the importance of this complication rests in its potential for masking underlying BE, a condition clearly associated with adenocarcinoma<sup>[3]</sup>. BE appears to have a higher prevalence in middle-aged to elderly men<sup>[4-6]</sup>. In one study involving a population of veteran patients, the prevalence of BE was 13% in the setting of typical gastro-esophageal reflux symptoms<sup>[7]</sup>. The current opinion, once EE is found on endoscopy, is to provide effective anti-secretory therapy to eliminate the confounding factor (both visual and histological) of esophagitis, in order to correctly diagnose BE and any associated dysplasia. However, this practice is not universally accepted by the gastroenterology physicians due to limited data on the prevalence of BE in patients with EE<sup>[8]</sup>. We aimed to investigate the prevalence of BE in a group of patients with moderate-severe EE, after achieving complete healing on follow-up endoscopy.

## MATERIALS AND METHODS

### Patients

Records of all esophago-gastro-duodenoscopies (EGDs) performed at our institution between January 1998 and June 2006 were retrospectively reviewed from our endoscopy database. Our standardized, electronic report protocol contains all the essential elements included in the analysis (length of EE, Los Angeles classification of EE, length of suspected BE segment, size of hiatus hernia, *etc*). Other required information was obtained from patient's electronic medical records. All EGDs were either performed independently by a board certified, faculty gastroenterologist or by an in-training fellow under direct supervision of a faculty member. Photo documentation was obtained in all cases. Our department mandates completion of all elements of endoscopy reporting and adherence to the standardized biopsy protocol. In addition, patients with EE of class C and D are routinely (irrespective of the suspicion of BE) followed endoscopically, while class B patients undergo a follow-up EGD only if a normal Z-line cannot be clearly identified. For follow-up purposes, patients are routinely given written instructions at the conclusion of the initial visit in addition to a mailed letter, and a reminder phone call by the department clerk the day before their follow-up appointment. The patient population using our department's services comprises of 80% Caucasians, 15% Hispanics and 5% African-Americans.

LA classification of EE defines class A as one or more mucosal breaks confined to the mucosal folds, each no longer than 5 mm; class B, at least one mucosal break > 5 mm, confined to the mucosal folds, not contiguous between the tops of 2 folds; class C, at least one mucosal break contiguous between the tops of 2 or more mucosal folds but not circumferential ( $\leq 75\%$  of the luminal circumference) and class D, one or more circumferential mucosal breaks (comprising > 75% of esophageal luminal circumference). Inclusion criteria: all patients with EE of LA classes B, C, or D, who after receiving intensive anti-secretory therapy using double dose proton pump inhibitors (institutional formulary restricted to the use of omeprazole, lansoprazole and rabeprazole) had achieved complete mucosal healing, documented on at least one follow-up EGD. Exclusion criteria: patients with EE of LA class A, those without EGD follow up, those who failed to achieve complete mucosal healing after PPI therapy, those with known BE, and those with an EGD performed at our institution prior to the index examination. Demographic data including age, race, sex, and body mass index were noted. At index EGD length and LA class of EE, presence/absence of hiatus hernia and its estimated size were recorded. If biopsy specimens were obtained at index procedure due to suspected BE, the results of these were noted and compared with results of biopsies taken at follow-up endoscopy. Finally, if BE was endoscopically suspected, its length, and results of four quadrant biopsies taken at 2 cm intervals (a uniform practice in our department), when applicable, were documented.

For non-circumferential BE (tongue like salmon colored projections above the esophago-gastric junction), the mucosal sampling was obtained in a similar fashion from the involved area. BE was suspected endoscopically by the presence of salmon-colored columnar-appearing mucosa in the tubular esophagus, and confirmed by the presence of specialized intestinal metaplasia containing goblet cells, on histology. BE was labeled as short segment (SSBE) or long segment (LSBE) based on the length of the columnar appearing mucosal segment of < 3 cm, or  $\geq 3$  cm, respectively. The presence of any grade of dysplasia on histology was recorded. This study was approved by our institutional review board (IRB).

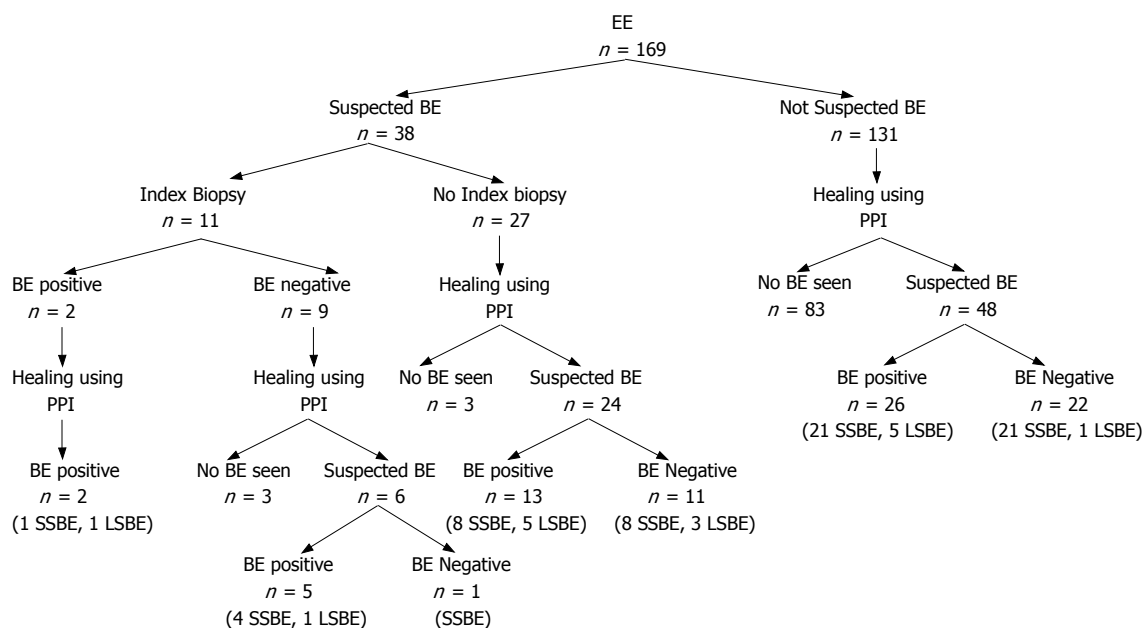
### Statistical analysis

Continuous variables were reported as mean  $\pm$  standard error of the mean (SEM). Categorical data were listed as percentages (%). For continuous outcomes, analysis of variance (ANOVA) was used to determine differences between groups. Logistic regression was performed to identify variables predictive of BE. These variables included age, length and severity of EE at index endoscopy, size of hiatus hernia, body mass index, and suspicion of BE at index endoscopy. Also, linear regression was used to determine predictors of the length of BE found after healing. A two-tailed  $P < 0.05$  was considered statistically significant.

## RESULTS

A total of 546 patients had the endoscopic diagnosis of EE at our institution between January, 1998 and June, 2006. Of these, 377 were excluded from the study (EE class A,  $n = 168$ ; prior EGD or history of BE,  $n = 91$ ; EE class B with visibly normal Z-line,  $n = 68$ ; failure to follow up,  $n = 18$ ; incomplete healing,  $n = 18$ ; insufficient documentation,  $n = 14$ ). After excluding above, 169 patients met the inclusion criteria of LA class B (36/169, 21.3%), C (83/169, 49.1%), or D (50/169, 29.6%) EE, and subsequently demonstrated complete healing on at least one follow-up EGD (mean duration: 12 wk; range: 2-52 wk; only 2 patients underwent FU EGD at 52 wk). The indications for the index EGD varied as listed in Table 1. The demographic information of the study population is listed in Table 2. The characteristics of the excluded patients were similar to the patients included in the study. Patients initially diagnosed with EE were mostly (98%) Caucasian men, and those diagnosed with BE were all men. The average age, BMI, and size of hiatus hernia were similar between BE and non-BE groups (Table 2). A hiatus hernia was more prevalent in patients diagnosed with BE than non-BE patients (90.2% *vs* 72.6%, respectively;  $P < 0.001$ ). The mean length of EE segment in the study population was  $4.44 \pm 0.35$  cm; the mean length of EE in class B was  $2.94 \pm 0.56$  cm; class C,  $3.83 \pm 0.38$  cm and class D,  $6.30 \pm 0.77$  cm. Interestingly, the mean length of EE segment increased as the severity of LA class increased (D > B/C;  $P = 0.002$ ).

A total of 86/169 (50.9%) patients were suspected



**Figure 1** Subsequent course of 169 patients with EE undergoing a repeat EGD. SSBE: Short segment Barrett's; LSBE: Long segment Barrett's.

**Table 1** Procedural indications for the 169 study patients at index endoscopy

Indications	Number of patients
HB	65
HB + dysphagia	18
HB + abd. Pain	5
HB + hematemesis	2
HB + N/V	1
HB + anemia	1
Dysphagia	29
Hematemesis	16
N/V	6
Dyspepsia	6
Anemia	5
Melena	4
Abdominal pain	3
Odynophagia	2
Globus sensation	1
Chest pain	1
Cough/pyrosis	1
Variceal screening	1
Hemoccult positive stools	1
PEG	1

HB: Heartburn; N: Nausea; V: Vomiting; PEG: Percutaneous endoscopic gastrostomy.

of BE (either before or after healing of EE), and 80 of these who underwent repeat biopsies, histological confirmation was obtained in 46 (57.5%). Histological confirmation of BE in patients with suspected LSBE was 12/16 (75%) and in patients with suspected SSBE was 34/64 (53.1%).

In 38 (22.5%) patients, BE was visually suspected at index EGD and in 11 of these biopsies were performed during the same procedure. Interestingly, BE could initially be confirmed only in 2 of these 11; however, after healing, 6 still had suspicion of BE and repeat biopsies confirmed specialized intestinal metaplasia in 5 of them (two patients also had a low grade and a high grade dys-

**Table 2** Demographic information of the patients undergoing index endoscopy (n = 169)

Patient demographics	BE group (n = 46)	Non BE group (n = 123)
Mean age (yr)	57.2	58.6
Men (%)	100	95.9
Race		
Caucasian (%)	87	87
Hispanic (%)	10.9	10.6
Black (%)	2.2	2.4
BMI (kg/m <sup>2</sup> )	28.6	28.8
Hiatus hernia size <sup>1</sup> (mean, cm)	3	2.5
% HH (P < 0.001)	90.2	72.6

<sup>1</sup>5 Patients with unknown size HH.

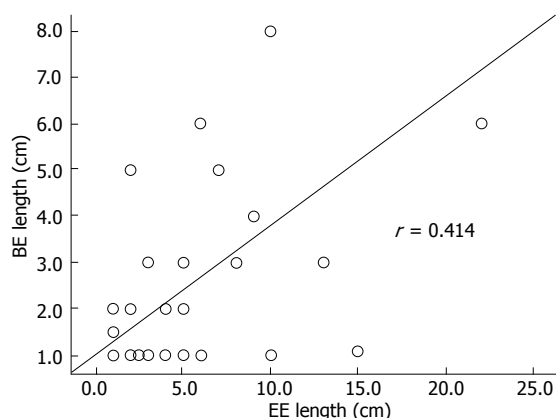
plasia each). In the remaining 27 with suspicion of BE at index EGD who did not undergo index biopsies, 24 were still suspected of BE after healing of EE, and in 13/24 histology was positive for BE on repeat biopsies (Figure 1).

A total of 131 EE patients were not suspected of having BE at index EGD. After healing of EE though, BE was suspected in 48/131 (who underwent biopsies). Of these, 26 were histologically confirmed as having BE (Figure 1).

Overall, 46/169 (27%) from the study population had BE confirmed by histology. The breakdown by LA class included 8 (17.4%) from B (6 SSBE, 2 LSBE), 25 (54.3%) from C (20 SSBE, 5 LSBE) and 13 (28.3%) from class D (8 SSBE, 5 LSBE) EE. This represented 22.2% of all patients from class B, 30.1% from C and 26% from class D esophagitis.

Logistic regression performed to identify variables that could predict the presence of BE (including age, BMI, length and severity of EE, size of hiatus hernia, and suspicion of BE at index) found only "suspicion of BE at index" to be significant. Patients in whom BE was





**Figure 2** In patients with EE the length of inflammation had a linear relationship with the length of BE segment.

suspected at index EGD were 4.5 times more likely to have histological confirmation than those in whom it was not suspected at index EGD ( $P = 0.01$ ). Linear regression was also performed and it was found that the length of EE correlated with the length of BE (found after healing of esophagitis); there appeared to be a linear relationship between these two variables ( $P = 0.009$ ) as shown in Figure 2.

## DISCUSSION

BE is an important, potentially pre-malignant complication of gastro-esophageal reflux disease. The major reason to evaluate patients with longstanding GERD is to recognize BE<sup>[9]</sup>. The possible role of GERD induced EE leading to BE has not been clearly established, but possible cellular injury and subsequent healing with columnar epithelium has been hypothesized<sup>[10]</sup>. On the other hand, this is also possible that the pathogenic mechanisms for the two entities (i.e., EE and BE), are different, but are often seen together due to shared patient characteristics and risk factors. Interestingly though, BE has also been identified in up to 25% of asymptomatic or minimally symptomatic individuals<sup>[11]</sup>.

In a multi-center study of non-veteran patients (60% men, 78% Caucasians) presenting for colonoscopy, the prevalence of BE was 6.8% in those with or without the symptoms of heartburn, and rose up to 15% if they had EE on the endoscopy<sup>[12]</sup>. In this study, patients with minor grades of esophagitis were not re-evaluated after medical therapy. In a study by Hanna *et al*<sup>[13]</sup>, 176 patients with EE but without apparent BE were followed for a mean of 11 wk while on a standard dose of PPI. At follow-up 116 (67%) showed complete healing of EE. Of these, 32 (27.6%) were suspected of having BE, but histology was confirmatory only in 16 (50%). Overall, BE was seen in 21 cases (12%), most of which had short-segment BE. In contrast, our study shows 27% prevalence of BE in association with higher grades of EE (some requiring more than one follow-up endoscopy to document complete healing). Again, in the study by Hanna *et al*, patients with visually suspected BE were excluded, and furthermore, 33% of their patients did

not achieve complete healing at follow up EGD and biopsies. These two factors could have accounted for the relatively low proportion of BE patients in their study. Interestingly, in our study, if we exclude patients with suspected BE (left arm of Figure 1) at index EGD, the prevalence of BE in the remaining patients (right arm of Figure 1) drops to 19.84% with most of these having SSBE (80.76%). In our study, the histological confirmation rate for BE was 18.18% (2/11) when biopsies were performed in the presence of inflammation and 56.4% (44/78) when performed after complete healing of EE. In two patients, BE with dysplasia was missed when biopsies were taken in the presence of inflammation. This does raise additional concerns that sampling for BE can be more difficult in those with EE and the potential for missing dysplastic areas could be higher. In our study, a significant number of patients with class B esophagitis did not undergo a follow-up EGD, as endoscopists felt confident that BE was not present.

In our analysis, as the severity of EE increased, so did the average length of erosions. Our data also suggest that the length of BE (if found at follow-up) can be predicted by the length of inflammation seen at the index examination. The strongest predictor for the presence of BE was the suspicion of it at index endoscopy. When visual diagnosis of BE was made, the final diagnosis of BE could be established only in 53% (46/86); although a sampling error cannot be ruled out, similar findings have also been reported by other investigators<sup>[14,15]</sup>. It will be interesting to see if newly available mucosal enhancing techniques (chromoendoscopy, high definition/magnification, narrow band imaging or confocal endomicroscopy) with the ability of targeted biopsies could increase the yield of diagnosing BE with or without the presence of EE.

The limitations of this study include the retrospective nature of the analysis and the fact that the patient population comprises mostly of older men who are at increased risk of EE<sup>[16]</sup> and BE<sup>[17]</sup>. Therefore, these data might not be applicable to populations who have higher preponderance of women and relatively younger patients. Furthermore, in the study, the possibility of higher inter-observer variability of EE grading cannot be ruled out.

In conclusion, BE was seen in 27% of patients with moderate-severe grades of EE. Esophageal inflammation can mask underlying BE or dysplasia, and make biopsies less accurate. The length of EE at index endoscopy may predict the length of BE at follow-up endoscopy. Therefore, it is suggested that follow-up endoscopy be performed, and evaluation for potential BE made once complete endoscopic healing of moderate-severe EE is achieved.

## COMMENTS

### Background

Gastro-esophageal reflux disease may be complicated by erosive esophagitis (EE) and Barrett's esophagus (BE). BE is clearly associated with esophageal adenocarcinoma.

### Research frontiers

To investigate whether patients with moderate-severe EE are at increased risk of BE and whether the presence of inflammation affects detection of underlying BE.

### Innovations and breakthroughs

Present study shows higher prevalence of BE with advanced grades of EE, once complete healing of inflammation is achieved. There is potential for missing Barrett's and even dysplasia if biopsies are obtained in the presence of active inflammation.

### Applications

BE appeared to be more prevalent in patients with moderate-severe EE. Endoscopic biopsies (if needed) at a follow-up examination should only be performed once complete mucosal healing is documented.

### Peer review

This is an interesting study, where the length of EE at index endoscopy may predict the length of BE at follow-up endoscopy. It will be some value for clinical practice.

## REFERENCES

- 1 Dent J, El-Serag HB, Wallander MA, Johansson S. Epidemiology of gastro-oesophageal reflux disease: a systematic review. *Gut* 2005; **54**: 710-717
- 2 Lassen A, Hallas J, de Muckadell OB. Esophagitis: incidence and risk of esophageal adenocarcinoma--a population-based cohort study. *Am J Gastroenterol* 2006; **101**: 1193-1199
- 3 Anderson LA, Watson RG, Murphy SJ, Johnston BT, Comber H, Mc Guigan J, Reynolds JV, Murray LJ. Risk factors for Barrett's oesophagus and oesophageal adenocarcinoma: results from the FINBAR study. *World J Gastroenterol* 2007; **13**: 1585-1594
- 4 Kubo A, Corley DA. Marked multi-ethnic variation of esophageal and gastric cardia carcinomas within the United States. *Am J Gastroenterol* 2004; **99**: 582-588
- 5 Spechler SJ. Barrett's esophagus. *Semin Gastrointest Dis* 1996; **7**: 51-60
- 6 Musana AK, Resnick JM, Torbey CF, Mukesh BN, Greenlee RT. Barrett's esophagus: incidence and prevalence estimates in a rural Mid-Western population. *Am J Gastroenterol* 2008; **103**: 516-524
- 7 Westhoff B, Brotze S, Weston A, McElhinney C, Cherian R, Mayo MS, Smith HJ, Sharma P. The frequency of Barrett's esophagus in high-risk patients with chronic GERD. *Gastrointest Endosc* 2005; **61**: 226-231
- 8 Veldhuyzen van Zanten SJ, Thomson AB, Barkun AN, Armstrong D, Chiba N, White RJ, Escobedo S, Sinclair P. The prevalence of Barrett's oesophagus in a cohort of 1040 Canadian primary care patients with uninvestigated dyspepsia undergoing prompt endoscopy. *Aliment Pharmacol Ther* 2006; **23**: 595-599
- 9 DeVault KR, Castell DO. Updated guidelines for the diagnosis and treatment of gastroesophageal reflux disease. The Practice Parameters Committee of the American College of Gastroenterology. *Am J Gastroenterol* 1999; **94**: 1434-1442
- 10 Hamilton SR, Yardley JH. Regenerative of cardiac type mucosa and acquisition of Barrett mucosa after esophagogastrectomy. *Gastroenterology* 1977; **72**: 669-675
- 11 Gerson LB, Shetler K, Triadafilopoulos G. Prevalence of Barrett's esophagus in asymptomatic individuals. *Gastroenterology* 2002; **123**: 461-467
- 12 Rex DK, Cummings OW, Shaw M, Cumings MD, Wong RK, Vasudeva RS, Dunne D, Rahmani EY, Helper DJ. Screening for Barrett's esophagus in colonoscopy patients with and without heartburn. *Gastroenterology* 2003; **125**: 1670-1677
- 13 Hanna S, Rastogi A, Weston AP, Totta F, Schmitz R, Mathur S, McGregor D, Cherian R, Sharma P. Detection of Barrett's esophagus after endoscopic healing of erosive esophagitis. *Am J Gastroenterol* 2006; **101**: 1416-1420
- 14 Eloubeidi MA, Provenzale D. Does this patient have Barrett's esophagus? The utility of predicting Barrett's esophagus at the index endoscopy. *Am J Gastroenterol* 1999; **94**: 937-943
- 15 Jago M, Volant A, Faycal J, Doucet L, Andlauer E, Delalande AH, Cholet F, Noursbaum JB, Gouerou H, Robaszkievicz M. Prevalence and topography of intestinal metaplasia in columnar lined esophagus. *Gastroenterol Clin Biol* 2007; **31**: 601-606
- 16 Du J, Liu J, Zhang H, Yu CH, Li YM. Risk factors for gastroesophageal reflux disease, reflux esophagitis and non-erosive reflux disease among Chinese patients undergoing upper gastrointestinal endoscopic examination. *World J Gastroenterol* 2007; **13**: 6009-6015
- 17 Guardino JM, Khandwala F, Lopez R, Wachsberger DM, Richter JE, Falk GW. Barrett's esophagus at a tertiary care center: association of age on incidence and prevalence of dysplasia and adenocarcinoma. *Am J Gastroenterol* 2006; **101**: 2187-2193

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## Can 5-aminosalicylic acid suppository decrease the pain after rectal band ligation?

Burcak Kayhan, Digidem Ozer, Meral Akdogan, Ersan Ozaslan, Osman Yuksel

Burcak Kayhan, Ersan Ozaslan, Osman Yuksel, Ankara Numune Training and Education Hospital, Department of Gastroenterology, Ankara 06443, Turkey

Digidem Ozer, Ankara Güven Hospital, Department of Internal Disease, Ankara 06443, Turkey

Meral Akdogan, Türkiye Yüksek İhtisas Hospital, Department of Gastroenterology, Ankara 06443, Turkey

**Author contributions:** Kayhan B and Ozer D designed research; Ozaslan E, Akdogan M and Kayhan B performed research; Yuksel O analyzed data; Kayhan B and Ozer D wrote the paper.

**Correspondence to:** Burcak Kayhan, Ankara Numune Training and Education Hospital, Department of Gastroenterology, PK: 203, Yenisehir, Ankara 06443, Turkey. [burkaygastro@hotmail.com](mailto:burkaygastro@hotmail.com)

Telephone: +90-532-5669805 Fax: +90-312-4272483

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### Abstract

**AIM:** To investigate the effect of 5-aminosalicylic acid (5-ASA) suppositories on rectal band ligation-induced pain.

**METHODS:** Sixty patients were randomized into two treatment groups.

**RESULTS:** Our results showed that there was no difference between 5-ASA suppository group and the control group for pain control.

**CONCLUSION:** 5-ASA may be an alternative treatment for hemorrhoids; however, it does not affect the rectal band ligation-induced pain.

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**Key words:** Hemorrhoid; Pain; 5-aminosalicylic acid; Rectal band ligation

**Peer reviewer:** Damian Casadesus, MD, PhD, Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

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### INTRODUCTION

Patients with hemorrhoids most often consult a physician only after their symptoms become unbearable. Hemorrhoids are frequently occurring inflammatory processes involving the hemorrhoid plexus<sup>[1]</sup>. Treatments of this inflammatory process remains a difficult problem in some cases, despite the large number of conservative methods available, such as injection sclerotherapy, rubber band ligation (RBL), cryotherapy, infrared photocoagulation, bipolar diathermy galvanic generator and laser application<sup>[2]</sup>. RBL, which is an easy and inexpensive procedure, is used to treat second-degree hemorrhoids, and surgery is often necessary for third and fourth degree hemorrhoids<sup>[3]</sup>.

Although RBL is a well-established treatment of choice for symptomatic internal hemorrhoids, fatalities following RBL have been reported<sup>[4-6]</sup>. Following this treatment some patients experience pain which may be accompanied by itching. Post-RBL pain has been reported at a frequency of 8.3% in the literature<sup>[7]</sup>. These complaints usually start on the day of treatment and may last several days.

The aim of this study was to investigate the effect of 5-aminosalicylic acid (5-ASA) suppositories (250 mg BID) on RBL-induced pain.

### MATERIALS AND METHODS

#### Patients

The study was designed as a double-blind, randomized trial of 24 mo duration. One hundred and seven patients (44 males and 63 females) volunteered to take part in the study after receiving a full explanation of the nature and purpose of the trial.

Before the start of the trial, and after three weeks of treatment, patients were assessed both clinically and rectoscopically. Endoscopic examination assessed the degree of hemorrhoids<sup>[8]</sup>, and determined whether or not anal fissure was present.

Sixty patients with second degree symptomatic hemorrhoids were included in this study. All patients' histories were obtained, and complete physical examination, digital rectal examination, and rigid rectoscopy were performed. Both groups consisted of 30 patients and none of these patients suffered from other systematic disease nor were they pregnant.

All the patients were prescribed the standard fiber diet and warm sitz baths (29°C/daily, twice). All patients underwent rectal band ligation for a single hemorrhoid cushion. One group was started on 5-ASA 500 mg BID P.R. and the control group received suppositories with placebo (glycerin). Patients were asked to score their pain on postprocedure day 1 and day 2. The scale used scored no pain as 0, mild pain as 1 and severe pain as 2.

### Statistical analysis

The Statistical Package for Social Sciences (SPSS) version 10.0 for Windows was used to analyze the data. The Pearson Chi-square, McNemar and the Student-*t* test were used whenever appropriate. Differences were considered significant if  $P < 0.05$ .

## RESULTS

There were 30 patients in each group; 14 males and 16 females in the 5-ASA group (mean age,  $31.86 \pm 10.75$  years), and 9 males and 21 females in the control group (mean age,  $32.73 \pm 9.58$  years). There were no statistically significant differences between the two groups according to sex and age.

Twenty-five patients (15 in the 5-ASA group and 10 in the control group) also presented with anal fissure. There were no statistical differences between the two groups ( $P > 0.05$ ).

All patients showed clinical and endoscopic evidence of active hemorrhoid disease and complained of pain in the perianal region. In addition, some patients had bleeding, mucus in stool, tenesmus, itching or pain in the perianal region during defecation.

Table 1 shows the differences on RBL-induced pain between groups 1 and 2. With regard to RBL-induced pain, we could not find any significant differences for either of the groups on the first ( $P = 0.390$ ) or on the second day ( $P = 0.601$ ). Although there was pain in 33% of 5-ASA group patients on the first day, that ratio increased to 90% on the second day. However, that difference was not statistically significant ( $P = 0.290$ ).

In the control group, 20% of patients did not have pain on the first day, and 93.3% had no pain on the second day. This difference was statistically not significant ( $P = 0.490$ ).

We did not find any statistical difference between two groups according to sex ( $P = 0.441$  for 5-ASA group;  $P = 0.080$  for control group) between the first day and the second day ( $P = 0.233$  for 5-ASA group;  $P = 0.523$  for control group). When we compared first day pain after RBL between fissure (+) and (-), it was statistically significant for both groups ( $P = 0.005$  for the 5-ASA group;  $P = 0.007$  for the control group). On the other hand, there were no differences among the groups on the second day ( $P = 0.189$  for the 5-ASA group;  $P = 0.103$  for the control group).

If we compare the pain scores between the two groups who had fissures (+), there were no differences ( $P = 0.665$  for the first day of treatment;  $P = 0.659$  for the second day of treatment).

Table 1 Comparison of pain in each group one and two days after RBL *n* (%)

Pain (score)	Group1 (day 1)	Group 1 (day 2)	Group 2 (day 1)	Group 2 (day 2)	<i>P</i> (day 1)	<i>P</i> (day 2)
No (0)	10 (33)	27 (90)	6 (20)	28 (93.3)	0.390	0.601
Mild (1)	13 (43)	2 (6.7)	18 (60)	2 (6.7)		
Severe (2)	7 (23)	1 (3.3)	6 (20)	0		

## DISCUSSION

Pain is the main symptom for second degree internal hemorrhoids. The exact etiopathogenesis of hemorrhoids-induced pain remains unknown. It is known that spasm of the internal sphincter is believed to be responsible for the discomfort after hemorrhoidectomy<sup>[8]</sup>. Furthermore, RBL behaves like hemorrhoidectomy presenting with similar pain. However, incarceration of smooth muscle fibers and mucosa in the transfixed vascular pedicle and epithelial denudation of the anal canal may also constitute the reason for pain<sup>[9]</sup>. A proper technique of ligation dictates that rubber bands are placed well above the dentate line. Sharp pain immediately after installment of a band denotes encroachment on the pain-sensitive area adjacent to the dentate line and necessitates removal of the band<sup>[10]</sup>. Theories for such pain include the band being placed in the receptive field of an aberrant somatic cutaneous nerve of the internal sphincter being drawn into the band, causing spasm and pain<sup>[11]</sup>. Other possible causes of pain include pressure sensation caused by edema of the hemorrhoid bundle and also, foreign body sensation of the band on the rectal mucosa.

Therefore, a number of substances with anti-inflammatory (corticosteroids), anesthetic (xylocaine) or vasoconstrictive action, are currently in use with various degrees of success, 5-ASA is a powerful anti-inflammatory agent, which has been extensively used in patients with inflammatory bowel disease as an alternative topically applied corticosteroid<sup>[12]</sup>. It reduced the intensity of all symptoms evaluated, probably *via* its anti-inflammatory activity, and decreased significantly the congestion of the hemorrhoid plexus<sup>[11]</sup>. On the other hand, there is only one study published which has investigated the reduction of pain by an agent post-RBL. It showed that hydrocortisone-cinchocaine-framycetin suppositories, which are effective for inflammation, showed no significant difference in decreasing the severity of pain<sup>[13]</sup>.

Neiger and Widaver showed that 5-ASA, given in the form of suppositories three times daily for two weeks, was very effective in eliminating the clinical symptoms in patients with hemorrhoids. Our results showed that 5-ASA, in the form of 250 mg suppositories given twice daily, is an effective drug in the symptomatic treatment of hemorrhoids irrespective of their degree of severity, without causing any significant side effects<sup>[14]</sup>.

It is known that edema is one of the results of inflammation. Despite the fact that 5-ASA is effective against inflammation, in our study it failed to decrease



the severity of pain. Our results may be interpreted in two ways: 5-ASA may not affect the pain induced by hemorrhoids, or there may not be any correlation between pain and inflammation or edema.

However, we showed that anal fissures had a potential effect in inducing RBL pain in patients with hemorrhoids. We decided that if a patient with hemorrhoids had anal fissure, the priority treatment should be applied to the anal fissure for decreasing the severity of pain symptoms.

In conclusion, 5-ASA may be an alternative treatment for hemorrhoids, but it is not effective in RBL-induced pain.

## COMMENTS

### Background

Post Rectal Bant Ligation (RBL) pain has been reported at a frequency of 8.3% in the literature.

### Research frontiers

5-aminosalicylic acid (5-ASA) decreases the inflammation of proctitis. Inflammation is one of the reasons of RBL-induced pain.

### Innovations and breakthroughs

Anal fissures have a potential effect in inducing RBL pain in hemorrhoids patients. We decided that if a hemorrhoid patient had anal fissures, the priority treatment should be applied to the anal fissure for decreasing the severity of pain symptoms. 5-ASA may be an alternative treatment for hemorrhoids.

### Applications

Although 5-ASA decreases the inflammation of proctitis, it is not effective in RBL-induced pain.

### Peer review

These results may be interpreted in two ways: 5-ASA may not affect the pain induced by hemorrhoids, or there may not be any correlation between pain and inflammation or edema.

## REFERENCES

1 Gionchetti P, Campieri M, Belluzzi A, Brignola C, Miglioli

- M, Barbara L. 5-ASA suppositories in hemorrhoidal disease. *Can J Gastroenterol* 1992; **6**: 18-20
- 2 Dennison AR, Paraskevopoulos JA, Kerrigan DD, Shorthouse AJ. New thoughts on the aetiology of haemorrhoids and the development of non-operative methods for their management. *Minerva Chir* 1996; **51**: 209-216
- 3 Smith LE. Symptomatic internal hemorrhoids. What are your options? *Postgrad Med* 1983; **73**: 323-330
- 4 Bat L, Melzer E, Koler M, Dreznick Z, Shemesh E. Complications of rubber band ligation of symptomatic internal hemorrhoids. *Dis Colon Rectum* 1993; **36**: 287-290
- 5 O'Hara VS. Fatal clostridial infection following hemorrhoidal banding. *Dis Colon Rectum* 1980; **23**: 570-571
- 6 Russell TR, Donohue JH. Hemorrhoidal banding. A warning. *Dis Colon Rectum* 1985; **28**: 291-293
- 7 Marshman D, Huber PJ Jr, Timmerman W, Simonton CT, Odom FC, Kaplan ER. Hemorrhoidal ligation. A review of efficacy. *Dis Colon Rectum* 1989; **32**: 369-371
- 8 Ho YH, Seow-Choen F, Low JY, Tan M, Leong AP. Randomized controlled trial of trimebutine (anal sphincter relaxant) for pain after haemorrhoidectomy. *Br J Surg* 1997; **84**: 377-379
- 9 Ganio E, Altomare DF, Gabrielli F, Milito G, Canuti S. Prospective randomized multicentre trial comparing stapled with open haemorrhoidectomy. *Br J Surg* 2001; **88**: 669-674
- 10 Hooker GD, Plewes EA, Rajgopal C, Taylor BM. Local injection of bupivacaine after rubber band ligation of hemorrhoids: prospective, randomized study. *Dis Colon Rectum* 1999; **42**: 174-179
- 11 Tchirkow G, Haas PA, Fox TA Jr. Injection of a local anesthetic solution into hemorrhoidal bundles following rubber band ligation. *Dis Colon Rectum* 1982; **25**: 62-63
- 12 Goligher JC. Surgery of the Anus, Rectum and Colon, 3rd ed. London: Springfield III CC Thomas, 1975: 831-832
- 13 Williams JA, Evans JC. An assessment of anesthetic-steroid suppositories: a controlled trial following rubber-band ligation of hemorrhoids. *Dis Colon Rectum* 1972; **15**: 66-68
- 14 Govosdis V, Triantafillidis JK, Cheracakis P, Barbatzas Ch, Plaitakis Z, Delis K, Merikas E, Zervakakis A. Efficacy of the topical application of 5-aminosalicylic acid (5-ASA) in the treatment of internal hemorrhoids. *Hellenic J Gastroenterol* 1996; 322-324

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RAPID COMMUNICATION

## Fragile histidine triad gene alterations are not essential for hepatocellular carcinoma development in South Korea

Chang Woo Nam, Jung Woo Shin, Neung Hwa Park

Chang Woo Nam, Department of Surgery, University of Ulsan College of Medicine, Biomedical Research Center, Ulsan University Hospital, Ulsan 682-714, South Korea  
Jung Woo Shin, Neung Hwa Park, Internal Medicine, University of Ulsan College of Medicine, Biomedical Research Center, Ulsan University Hospital, Ulsan 682-714, South Korea  
Author contributions: Park NH and Nam CW designed the research; Nam CW and Park NH performed the research; Shin JW and Park NH analyzed the data; and Nam CW and Park NH wrote the paper.

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Correspondence to: Dr. Neung Hwa Park, Department of Internal Medicine, Ulsan University Hospital, 290-3 Jeonha-dong, Dong-gu, Ulsan 682-714, South Korea. [nhpark@uuh.ulsan.kr](mailto:nhpark@uuh.ulsan.kr)  
Telephone: +82-52-2508845 Fax: +82-52-2518235

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Therefore, our study suggests that *FHIT* plays a role in relatively few HCC cases in South Korea.

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**Key words:** Fragile histidine triad; Aberrant transcripts; Microsatellite instability; Protein expression; Hepatocellular carcinoma

**Peer reviewers:** Osman C Ozdogan, Associate Professor, Department of Gastroenterology, Liver Unit, Marmara University School of Medicine, Istanbul 34662, Turkey; Dr. Michael A Zimmerman, Division of Transplant Surgery, University of Colorado Health Sciences Center, 1635 N. Ursula St, PO Box 6510, Aurora 80045, Uruguay

Nam CW, Shin JW, Park NH. Fragile histidine triad gene alterations are not essential for hepatocellular carcinoma development in South Korea. *World J Gastroenterol* 2008; 14(22): 3526-3533 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3526.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3526>

### Abstract

**AIM:** To establish the role of *FHIT* in the pathogenesis hepatocellular carcinoma (HCC).

**METHODS:** We examined genomic alterations, as well as, mRNA and protein expression patterns from the *FHIT* gene, in 48 surgically resected hepatocellular carcinoma (HCC) tissues. Additionally, *p53* mutations were analyzed.

**RESULTS:** Aberrant *FHIT* transcripts were detected in 11 of 48 surrounding non-tumor liver tissues and 27 of 48 HCC samples (22.9% vs 56.3%,  $P = 0.002$ ). No point mutations were identified within the open reading frame region of *FHIT*. Loss of heterozygosity (LOH) of the *FHIT* locus was detected in 4 of 42 informative cases for D3S1300, and 3 of 29 informative cases for D3S1313. Reduced expression of *FHIT* protein (Fhit) was observed in 8 (16.7%) of 48 HCC samples, with complete loss of Fhit in only 1 case. There were no associations with abnormal transcripts, LOH, and Fhit expression. *p53* mutations were identified in 9 of the 48 HCC cases. However, none of the cases displayed a G to T transversion at *p53* codon 249.

**CONCLUSION:** Aberrant *FHIT* transcripts were more common in HCC tissues as compared to non-cancerous liver tissues. However, Fhit expression was lost or reduced in a minor fraction of HCC tissues, while it was strongly expressed in non-cancerous liver tissues.

### INTRODUCTION

Hepatocellular carcinoma (HCC) is currently the fifth most common cancer worldwide and the fourth leading cause of cancer-related deaths. The number of new cases is estimated as more than 500 000 per year, accounting for 4% of all newly diagnosed cancers<sup>[1]</sup>. More than 80% of HCC cases occur in developing countries, especially in South-East Asia and sub-Saharan Africa, but the incidence is increasing in economically developed regions, including Japan, Western Europe, and the United States<sup>[1-2]</sup>. The overall prognosis of HCC is poor, because many patients at presentation are already in an advanced and unresectable state, and will have a median survival time of less than 6 mo<sup>[3]</sup>. High mortality may be partially attributable to the fact that the nanocapsular part of the liver lacks sensory fibers, leading to symptoms presentation only in the advanced HCC<sup>[3]</sup>. Therefore, only a small proportion of patients are eligible for liver resection, which results in a 5-year survival rate of about 40%<sup>[4]</sup>. However, even following surgical resection, recurrence rates can be as high as 50% at 2 years<sup>[4]</sup>.

Hepatocarcinogenesis is a multistep process involving different genetic alterations that ultimately lead to the malignant transformation of hepatocytes.

Numerous genetic and epigenetic alterations contribute to the activation of carcinogenic pathways in HCC<sup>[4]</sup>. Whereas most studies on the mechanisms of tumorigenesis have focused on genetic changes, various epigenetic changes have been increasingly identified in HCC<sup>[4]</sup>. The short arm of human chromosome 3 is one of the most common sites of chromosomal abnormality in malignant diseases<sup>[5-8]</sup>. One candidate is the fragile histidine triad (*FHIT*) gene, located on 3p14.2, spanning the FRA3B common fragile site<sup>[9-10]</sup>. The *FHIT* gene is composed of 10 exons encompassing 1.8 Mb genome regions, of which only exons 5 to 9 code for protein. It encodes a small mRNA of 1.1 kb, and a small protein of 16.8 kDa<sup>[9]</sup>. Studies on the functional aspects of *FHIT* have been reported that this gene is a bona fide tumor suppressor gene<sup>[11]</sup>. The *FHIT* gene and its protein have may be involved in the regulation of cell proliferative and apoptotic processes<sup>[11]</sup>. Down-regulation of *FHIT* inhibits apoptosis and *FHIT* protein (Fhit) interacts with a number of key proteins involved in cancer progression, including *p53*<sup>[11-12]</sup>. Coexpression of *FHIT* and *p53* synergistically inhibits the proliferation of various tumor cell lines. These synergistic effects may occur because of stabilization of *p53* related to Fhit-mediated downregulation of MDM2<sup>[12]</sup>. Altered transcripts and allelic loss of the *FHIT* gene are frequently identified in premalignant and malignant lesions of various tumors<sup>[13-18]</sup>. Moreover, loss or reduction of Fhit expression has been found in most tumors including HCC<sup>[10-14,19-22]</sup>.

To establish the role of *FHIT* in the pathogenesis of HCC, we examined genomic alterations, as well as, mRNA and protein expression in surgically resected HCCs and their associated non-cancerous surrounding liver tissues. We also investigated the possible associations between *FHIT* abnormalities and *p53* mutations.

## MATERIALS AND METHODS

### Patients

HCC samples and their corresponding non-cancerous liver tissues were obtained from 48 patients who had undergone surgical resection at Ulsan University Hospital in South Korea. Written informed consent was obtained from all patients participating in this study, and the study was approved by the Institutional Review Boards at Ulsan University Hospital. All cancer samples were obtained from within the tumor. Matching surrounding non-cancerous liver tissues were obtained as far as possible from the tumors. All specimens were frozen immediately after surgical resection, and stored at -70°C. HCC diagnosis was based on histologic confirmation or elevated serum AFP (> 400 ng/mL) with radiologic findings, or at least two coincident radiologic findings (contrast-enhanced dynamic computer tomography (CT), contrast-enhanced dynamic magnetic resonance imaging (MRI), and Doppler ultrasonography compatible with HCC.

Postoperative follow-up included a dynamic CT/MRI study every 3 mo, and laboratory testing of the serum

AFP level every 1 to 3 mo at our outpatient clinic. In the case of suspected HCC recurrence, further examinations, including angiography and lipiodol CT, were performed. If necessary, ultrasound-guided biopsy was conducted to confirm the diagnosis. Bone scintigraphy or chest CT was performed when clinically indicated. The follow-up period was defined as the interval from the date of surgical resection of HCC until the date diagnosis of the recurrence, or the date of death, or the end of follow-up.

### Reverse transcription polymerase chain reaction (RT-PCR) and sequencing of *FHIT* gene transcripts

Total RNA and DNA were isolated from the HCCs and corresponding non-cancerous tissues, using the TRIZOL reagent (Gibco BRL Life Technologies, Gaithersburg, MD, USA). cDNA was synthesized by reverse transcriptase from 1 µg of total RNA. One ul of the RT reaction was used to amplify the *FHIT* cDNA as described by Ohta *et al.*<sup>[9]</sup>. To amplify *FHIT* exons 3-10, a nested PCR was carried out in 10-µL final volume with 30 ng of primers (5U2-3D2 and 5U1-3D1, according to Ohta *et al.*<sup>[9]</sup>). The first round of PCR amplification was performed in 10 uL of reaction mixture comprising 1 µmol/L primers 5U2-3D2 and 5U1-3D1, 200 µmol/L each dNTP, 1 × PCR buffer, 2.5 U of ampliTaQ, and 0.5 µL of the synthesized cDNA mixture under the following reaction conditions: denaturation for 10 min at 95°C, 34 cycles of 30 s at 95°C, 30 s at 56°C, and 45 s at 72°C, and a final extension step for 10 min at 72°C. After 10-fold dilution of the amplified product 10-fold in DW, 2 µL of aliquots were subjected to a second round of PCR amplification using nested primers, 5U1 and 3D1, under the above conditions. Each nested RT-PCR assay was repeated at least twice with the original extracted RNA for confirmation. All reactions were performed at least twice and the integrity of the RNA samples verified. To confirm the presence of mRNA and rule out nonspecific amplification, 25 cycles of *β-actin* cDNA were preformed after RT, both with and without the RT enzyme. PCR products were directly sequenced using primer 5U1 and 3U1 with an automated Applied Biosystem Model 3730 DNA sequencer (Perkin-Elmer, Foster City, CA, USA). For sequence analysis of abnormal *FHIT* transcripts, the PCR products were resolved in 1.5% ethidium bromide agarose gel. Bands were excised from gels, and PCR products were purified using the QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA, USA). PCR products excised from gels were directly sequenced using the sequencing primers described by Zekri *et al.*<sup>[18]</sup>.

### Microsatellite polymorphism analysis

To investigate the allelic loss of *FHIT* gene, a PCR-based approach was performed utilizing primers that amplify two polymorphic microsatellite markers internal to and flanking the *FHIT* gene. The microsatellite marker D3S1300 is located in intron 5 of the *FHIT* gene, whereas marker D3S1313 is slightly telomeric to *FHIT*. The 10 µL PCR mixture contained 1 × Gold Buffer, 0.8 µL 25 mmol/L MgCl<sub>2</sub>, 0.8 µL 10 mmol/L

dNTP, 1  $\mu$ L 5  $\mu$ mol/L concentrations of each primer pair, 0.5  $\mu$ L 0.25 unit Amplitaq Gold, and 2  $\mu$ L of the 10 ng extracted DNA. 1  $\mu$ L of diluted PCR product was mixed with 9  $\mu$ L of loading buffer (formamide: Ro  $\times$  500 size standard, 1:39). The mixture was denatured at 95°C for 5 min, chilled on ice, and loaded on an ABI PRISM 3100 automatic sequencer. The data were analyzed using the GeneScan and Genotyper software (Perkin-Elmer/Applied Biosystems). Constitutional homozygosity was regarded as uninformative. For informative cases, allelic loss was scored as positive if the signal of one allele was lost or reduced to at least 50% in tumor DNA, compared with the corresponding normal allele. All samples were run twice to confirm the presence or absence of allelic imbalance.

### Western blotting analysis

Protein concentration was measured using the BCA protein assay (Pierce, Rockford, IL). Ten micrograms of lysate protein was separated by SDS-PAGE using a 12% polyacrylamide gel and electroblotted onto a Hybond ECL (Amersham Pharmacia Biotech). After blockage of nonspecific binding sites for 1 h with 5% nonfat milk in TPBS (PBS and 0.1% Tween 20), the membrane was incubated at room temperature for 2 h with rabbit anti-FHIT antibody (Zymed Laboratories, South San Francisco, CA, USA) at a dilution of 1:500. The membrane was washed three times with TPBS, incubated further with horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (New England Biolabs, Beverly, MA, USA) at room temperature, and then washed three times with TPBS. Membranes were developed with the ECL chemiluminescence system (Amersham Pharmacia Biotech). The intensity of Fhit expression in HCC tumors was compared with that in non-cancerous surrounding liver tissues.

### p53 mutation analysis

The primers used were oligonucleotides complementary to the sequence flanking the exon/intron junctions of exons 5-9. The sequence of the primers is as follows: exon 5, 5'-CTGACTTTCAACTCTG-3' (forward) and 5'-AGCCCTGTCGTCTCT-3' (reverse); exon 6, 5'-CTC TGATTCCTCACTG-3' (forward) and 5'-ACCCAGTTGCAAACC-3' (reverse); exon 7, 5'-TGCTTGCCACAGGTCT-3' (forward) and 5'-ACAGCAGGCCAGTGT-3' (reverse); exon 8, 5'-AGGACCTGATTTTCCTTAC-3' (forward) and 5'-TCTGAGGCATAACTGC-3' (reverse); and exon 9, 5'-TATGCCTCAGATTCAC-3' (forward) and 5'-ACTTGATAAGAGGTCC-3' (reverse). The same primers sets were used for DNA sequencing. PCR was carried out in a 20- $\mu$ L reaction contained; 50 ng DNA, 10  $\times$  PCR buffer, 200  $\mu$ mol/L each of dNTP, 5 pmol of each primer and 1 U of Taq DNA polymerase. PCR conditions were as follows: 95°C (10 min) for 1 cycle, 95°C (40 s), 63°C (40 s; for exons 4, 5, and 7-9) or 67°C (40 s; for exon 6), 72°C (40 s) for 40 cycles, and a final extension step of 72°C (10 min). PCR products were separated by electro-

phoresis and visualized with 1.5% ethidium bromide. Samples without the DNA template were included in all assays as negative controls. PCR products were purified using the QIAquick PCR purification kit (QIAGEN Inc., Valencia, CA, USA), and they were sequenced by the dideoxychain termination method with the Big Dye Terminator cycle sequencing kit (Perkin-Elmer Corporation, Foster City, CA, USA). Cycle sequencing was performed for 25 cycles of denaturation (96°C, 30 s), annealing (50°C, 15 s), and extension (60°C, 4 min), according to the Big Dye Terminator protocol. After spin column purification with Centri-Sep columns (Perkin-Elmer Corporation), samples were analyzed with the automated Applied Biosystem Model 3730 DNA sequencer (Perkin-Elmer, Foster City, CA, USA).

### Statistical analysis

All continuous variables were compared using the Mann-Whitney test, or the one-way analysis of variance (ANOVA). Categorical variables were compared using the Fisher's exact test. All data were analyzed using the statistical package SPSS (version 14.0: SPSS Inc., Chicago, IL, USA). In all cases, a 2-tailed *P* value of less than 0.05 was considered statistically significant.

## RESULTS

### Patient characteristics

The baseline characteristics of the 48 patients were shown in Table 1. The patients included 35 men and 13 women with a mean age of 50 years (range 28-71 years). HBV was the most common etiology, accounting for 38 (79%) of the 48 cases. Antibody to HCV was identified in three cases. Seven patients were negative for HBV and HCV. Alpha-fetoprotein (AFP) level ranged from 1.0 to 240 000 ng/mL with a median level of 202 ng/mL. Twelve (25%) of the 48 patients had normal AFP levels (< 11 ng/mL), whereas 20 (41.7%) patients had AFP levels of > 400 ng/mL. Preoperative diagnosis of HCC was based on histologic confirmation in 28 cases, or elevated serum AFP (> 400 ng/mL) with radiologic findings, or at least two coincident radiologic findings compatible with HCC in the remaining 20 cases. In 22 of the 48 patients, cirrhosis was observed in the surrounding non-cancerous liver tissue, and chronic hepatitis was identified in parenchyma surrounding the tumors in the remaining 26 cases. According to the Child-Pugh classification at the time of operation, 19 patients were class A (CPT 5-6), 3 were class B. Based on UICC TNM staging<sup>[23]</sup>, 5 patients were stage I, 41 stage II, and 2 stage III. Small HCC was defined as a single tumor < 5 cm, or 2-3 tumors < 3 cm without invasion of major veins larger than sub-segmental branches. These criteria are similar to those applied to define early stage HCC in the BCLC (Barcelona Clinic Liver Cancer) staging scheme<sup>[24]</sup>. Tumors exceeding these limits were regarded as advanced HCC. At initial diagnosis, 26 patients had small HCC, while 22 patients were advanced HCC. The mean tumor size was



**Table 1** Baseline characteristics of 48 patients with HCC

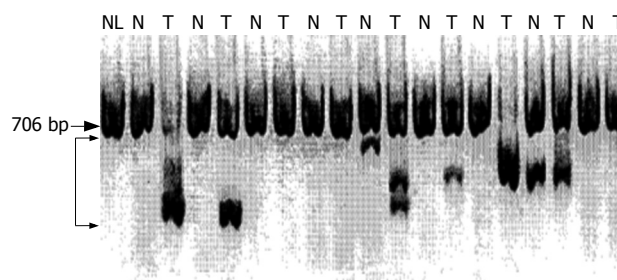
Characteristics	
Mean age (yr)	50 (20-71)
Sex (M:F)	35:13
Etiology of liver disease	
HBV	38
HCV	3
Non-HBV, Non-HCV	7
Tumor size	
≤ 3 cm	11
3-5 cm	15
> 5 cm	22
Mean (range) (cm)	5.8 ± 3.9
UICC TNM stage	
I	5
II	41
III	2
Edmonson-Steiner's grade	
I	8
II	13
III	20
IV	5
Underlying liver disease	
Cirrhosis	22
Chronic hepatitis	26
AFP (ng/mL)	
< 11	12
11-400	16
≥ 400	20

AFP: Alpha-fetoprotein.

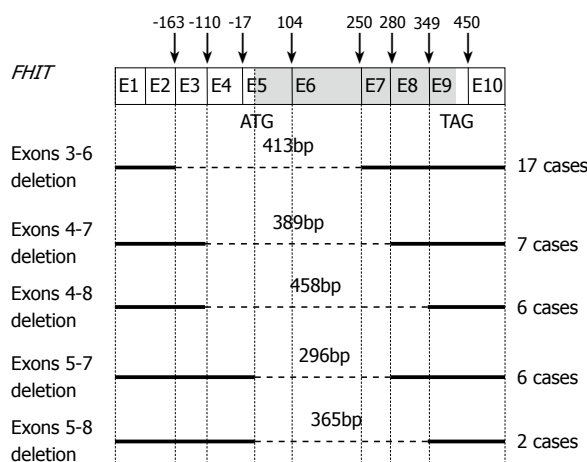
5.8 ± 3.9 cm. Among the 46 patients with solitary tumors, a diameter of 3 cm or less was observed in 11 (23.9 %), between 3 cm and 5 cm in 15 (32.6%), and greater than 5 cm in 20 (43.5%). There were no tumors with positive lymph nodes or macroscopic vascular invasion by HCC. Of these HCCs, 8 were at grade I, 13 at grade II, 20 at grade III and 5 at grade IV according to the Edmondson and Steiner's grade<sup>[25]</sup>. Microscopically, vascular and tumor capsular invasions were detected in 13 (27%) and 5 (10%) patients, respectively.

### RT-PCR and sequencing of *FHIT* transcripts

Representative results of nested RT-PCR analysis are shown in Figure 1. Abnormal-sized bands representing aberrant RT-PCR products were detected in 11 (22.9%) of 48 surrounding non-tumorous liver tissues and 27 (56.3%) of the 48 HCC samples. Thus, the number of tumors with aberrant transcripts was greater than that of non-tumor tissues with aberrant transcripts ( $P = 0.002$ ). Such abnormal-sized transcripts were seen at five different positions at 296, 365, 389, 413, and 458 bp (Figure 1). Interestingly, 13 cases displayed two or three aberrant transcripts. Sequence analysis of the aberrant transcripts revealed deletions of exons 3-6 (nt-164 to 249) in 17 cases, exons 4-7 (nt-110 to 279) in 7 cases, exons 4-8 (nt-110 to 348) in 6 cases, exons 5-7 (nt-17 to 279) in 6 cases, and exons 5-8 (nt-17 to 348) in 2 cases (Figure 2). Among 11 surrounding non-tumorous liver tissue samples with aberrant transcripts, 5 were chronic hepatitis, whereas in the remaining 6 cases, the parenchyma surrounding the tumor was cirrhosis.

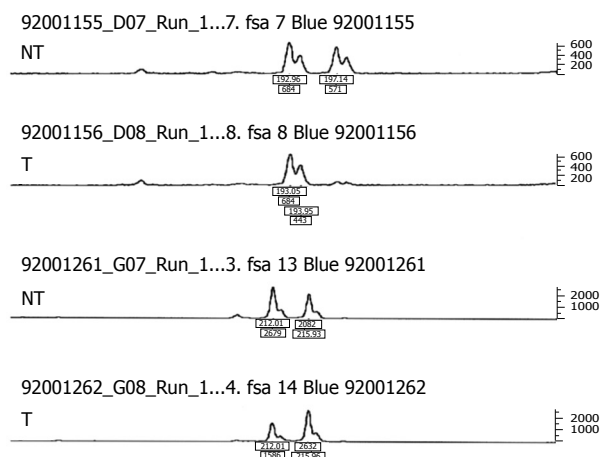


**Figure 1** Analysis of mRNA expression of *FHIT* gene in matched surrounding non-cancerous and cancerous tissues by RT-PCR. Large arrow: Normal sized *FHIT* cDNA (706 bp); small arrows: Aberrant transcripts (458 bp, 413 bp, 389 bp, 365 bp, and 296 bp); NL: Normal liver tissue; T: Tumor tissue; N: Non-tumor liver tissue.



**Figure 2** Schematic representation of aberrant *FHIT* transcripts in HCC tissues. Sequence analysis of aberrant transcripts reveals deletions of exons 3-6 (nt-164 to 249) in 17 cases, exons 4-7 (nt-110 to 279) in 7 cases, exons 4-8 (nt-110 to 348) in 6 cases, exons 5-7 (nt-17 to 279) in 6 cases, and exons 5-8 (nt-17 to 348) in 2 cases.

In total, 8 patients displayed aberrant transcripts in both tumor and surrounding non-tumor tissues, 5 of whom displayed the same abnormal patterns in the paired tumor and non-tumor samples; however, in the remaining 3 cases, the pattern was different between the paired samples. Whereas 19 cases had aberrant transcripts only in their tumor tissues, we also found 3 cases showing an aberrant transcript in their non-tumor tissues only. However, no point mutations of the *FHIT* gene were found in the region of the open reading frame. All normal-sized transcripts of 706 bp exhibited wild-type sequences. All of 21 tumor tissues without aberrant transcripts were presented normal sized transcripts. Normal-sized transcripts were also observed in 22 of the 27 HCCs with aberrant *FHIT* transcripts, but were barely present or completely absent in the remaining 5 cases. However, normal-sized bands were observed in all surrounding non-tumor tissues samples, regardless of the presence of aberrant transcripts. The presence of *FHIT* aberrations was not associated with clinical parameters, including age, sex, Child-Pugh classification, histological grade of tumor, presence of tumor capsule, AFP, tumor size, microscopic invasion, tumor recurrence, or survival ( $P > 0.05$ ).



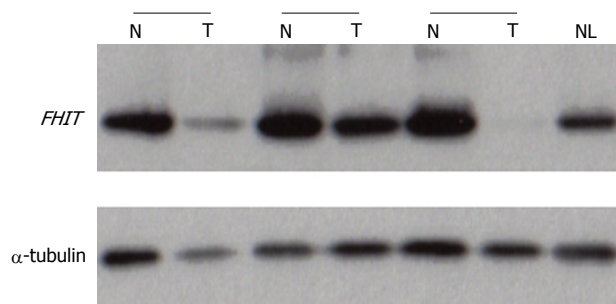
**Figure 3** LOH of *FHIT* locus. An example of LOH in tumor (T) and matched non-tumorous (NT) DNA from patients with HCC. LOH is defined as if the signal of one allele is lost or reduced to at least 50% in tumor DNA, compared to the corresponding normal allele.

### Loss of heterozygosity (LOH)

Among the 48 HCCs, 7 displayed LOH of D3S1300, D3S1313, or both markers. Specially, 4 of the 42 informative cases at D3S1300, and 3 out of the 29 cases informative at D3S1313, exhibited LOH. LOH involving both allelic markers was not observed. Examples are shown in Figure 3. In total, 6 of 30 cases with aberrant transcripts, and only 1 of 18 cases without aberrant transcripts showed LOH. Thus, the incidence of LOH appeared higher in cases with aberrant transcripts than cases without aberrant transcripts, although the data were not statistically significant ( $P = 0.23$ ). Moreover, we observed a trend towards *FHIT* aberration in tumors with LOH, although again the difference was not statistically significant ( $P = 0.23$ ). LOH of the *FHIT* gene was not associated with the clinical parameters examined.

### Western blot analysis

Representative results of Western blot analysis are shown in Figure 4. Eight (16.7%) of 48 HCCs exhibited reduced or no Fhit expression, compared with normal liver tissues. Complete loss of Fhit was only identified in one of the above 8 cases. Reduced Fhit expression was observed in 4 among the 30 samples with aberrant transcripts. The remaining 4 cases with reduced Fhit did not show aberrant transcripts. Moreover, Fhit expression was not reduced in the 5 cases where normal transcripts were absent or barely detected. Reduced Fhit expression was observed in only one patient displaying LOH at *FHIT* with no aberrant transcripts. The remaining six cases with reduced Fhit expression did not display LOH at the *FHIT* locus. We observed no association between LOH and abnormal *FHIT* transcripts, abnormal *FHIT* transcripts and abnormal Fhit expression, or, LOH and abnormal Fhit expression. Moreover, the extents of Fhit were not associated with clinical parameters, such as age, sex, Child-Pugh classification, histological grade of tumor, presence of tumor capsule, AFP, tumor size, microscopic invasion, tumor recurrence, or survival ( $P > 0.05$ ).



**Figure 4** Representative results of Fhit expression by Western blot analysis.  $\alpha$ -tubulin is used as the internal control to verify equal loading. The relative values of FHIT protein were normalized internally to  $\alpha$ -tubulin signals. NL: Normal liver tissue; N: Non-cancerous tissue; T: Tumor tissue.

**Table 2** *p53* mutation analysis in 48 patients with HCC

Mutation sites	<i>n</i>	Mutation sites	<i>n</i>
Exon 5		Exon 8	
Arg(CGC)158Leu(CTC)	1	Arg(CGT)273Cys(TGT)	1
Exon 6		Exon 7	
His(CAT)193Tyr(TAT)	1	Arg(TAC)236Cys(TGC)	1
Ser(AGT)215Arg(AGG)	1	Arg(TAC)236Asp(GAC)	1
Tyr(TAT)220Cys(TGT)	1	Met(ATG)246Leu(TTG)	1
		Met(ATG)246Val(GTG)	1

### *p53* mutation analysis

To investigate the association between *p53* mutations and genetic changes in the *FHIT* gene in the pathogenesis of HCC, direct sequencing analysis was performed. *p53* mutations were identified in tumors of 9 of the 48 patients (Table 2), but not in the surrounding liver tissues. However, none of the cases displayed G to T transversion at *p53* codon 249.

## DISCUSSION

Common fragile sites are prone to genomic alterations in cancer, and the majority of cancers exhibit alterations at fragile sites<sup>[26]</sup>. The *FHIT* gene located at one of the most common fragile sites is altered in a variety of tumor cell lines, as well as, in premalignant and malignant lesions of primary tumors, in line with its role as a tumor suppressor gene whose loss or inactivation may contribute to cancer development or malignant progression<sup>[13,15-20]</sup>. Moreover, several studies have been reported results for chromosome 3p rearrangements, decreased or absent *FHIT* mRNA expression, intragenic deletions and absence of protein expression, in HCC cell lines and primary HCC<sup>[13,15-19,21-22]</sup>.

Aberrant *FHIT* transcripts have been identified in 39%-70% of HCC cases<sup>[13,15-18]</sup>. These transcripts are generated by exon skipping, use of alternative 5' and 3' splice sites, and recognition of cryptic splice sites, resulting in insertions of intronic sequences. Consequently, the patterns of aberrant *FHIT* transcripts vary, but the majority of transcript splicing occurs within two large introns 4 and 5<sup>[15-18]</sup>. As the fusion functions coincide exactly with splice sites, aberrant transcripts may represent alternatively spliced products<sup>[9]</sup>. However, point

mutations of the *FHIT* gene are very rare<sup>[13,15-17]</sup>. In our study, all aberrant *FHIT* transcripts displayed deletion of exons 5 and 6. Additionally, no point mutations in the *FHIT* gene were identified in the region of the open reading frame. Aberrant transcripts of the *FHIT* have been identified in both HCC and surrounding non-cancerous tissues<sup>[15-18]</sup>. In our study, abnormal sized bands representing aberrant RT-PCR products were detected in 22.9% of the surrounding non-tumorous liver tissues and 56.3% of HCCs. These results suggested that aberrant *FHIT* transcripts had already accumulated even at the chronic liver diseases, in which persistent viral infection and sequential inflammation have occurred. As alterations of the *FHIT* gene in non-neoplastic tissues of smokers and ex-smokers are observed, *FHIT* alteration may be an early change during the preneoplastic phase of hepatocarcinogenesis<sup>[15-18]</sup>. Gramantieri *et al* reported that HCC cases with aberrant *FHIT* transcripts showed a significantly higher relapse rate and shorter recurrence time<sup>[14]</sup>. However, we observed no correlation between aberrant *FHIT* transcripts and clinicopathological parameters. Ohta *et al* detected full-length RT-PCR products in nearly all cases with aberrant transcripts<sup>[9]</sup>. Most of HCCs also exhibited aberrant and normal-sized transcripts<sup>[15-18]</sup>. Consistent with previous reports, our result showed that almost all cancerous and non-cancerous liver tissues exhibited normal-sized transcripts. In these cases, the normal transcripts have derived from admixed normal cells<sup>[9]</sup>. In our study, mRNA was extracted from tissue homogenates and, it was therefore not possible to assess whether or not the same cells produced normal and abnormal messengers. However, Chen *et al* reported that the normal products were observed in all tumor cell lines<sup>[17]</sup>. It has been suggested that these normal products are derived from neoplastic cells. Moreover, Schlott *et al* reported that aberrant transcripts did not differ between malignant, benign proliferating, and normal hepatocytes<sup>[16]</sup>. That is, formation of aberrant *FHIT* transcripts appears to be a common feature of benign, non-neoplastic hepatocytes. Alternative splicing definitely occurs in normal human tissues<sup>[27]</sup>. It is thus possible that the *FHIT* gene is simply located near to but is not the true target that drives a clonal selection process<sup>[17]</sup>. The *FHIT* gene, containing a common fragile region, FRA3B, is susceptible to the breakage caused by physical or chemical carcinogens. Similar effects may lead to a higher frequency of changes in the *FHIT* gene in chronically damaged liver tissues.

Several mechanisms are associated with dysfunction of the *FHIT* gene, the major being genomic deletion. These chromosomal deletions are frequently identified in HCC cell lines<sup>[13]</sup>. However, LOH at *FHIT* gene in human HCC is only occasionally detected<sup>[13,15,17]</sup>. Our study revealed that LOH of the *FHIT* gene was found in only 15% of HCCs. Although a great number of loci should be analyzed to further define any possible small deletions and rearrangements, these results so far suggest that LOH of *FHIT* gene is an uncommon in hepatocarcinogenesis. Allelic loss at *FHIT* is occasionally observed either in the presence or absence of aberrant

transcripts<sup>[15]</sup>. In our study, LOH of *FHIT* gene was observed in 6 of 30 cases with aberrant transcripts and 1 of 18 cases without aberrant transcripts. However, no significant correlations were evident between the expression of aberrant transcripts and LOH of *FHIT*. These findings imply that LOH alone does not completely suppress *FHIT* expression.

Several studies show that whereas all normal and non-cancerous liver tissues show a strong expression of Fhit, most HCC cell lines and primary HCC express reduced or no Fhit<sup>[13,19,21,22]</sup>. However, our study, reduced Fhit expression was observed in only 8 (17%) of 48 HCCs, and complete loss of Fhit in only one of these cases. Moreover, the extent of Fhit expression was not associated with aberrant transcripts or LOH presence. The data suggests that, in some cases, aberrant splice transcripts are actually transcribed. This hypothesis requires confirmation with a larger, more extended study. Inconsistent with our study, the incidence of Fhit expression was lower in HCC developed in patients chronically infected with HBV and exposed to chemical carcinogens, particularly in Qidong, China<sup>[13,19]</sup>. The exact reasons for the variation in Fhit expression are currently unclear, but may be dependent on different geographic and environmental factors. Hepatitis B virus infection is the most common etiologic factors for the development of HCC in South-East Asian regions, such as China and South Korea. Epidemiological and experimental studies disclose synergistic effects of aflatoxin B1 (AFB1) and HBV on hepatocarcinogenesis<sup>[28]</sup>. Environmental factors, including AFB1, may also contribute to the alteration of the *FHIT* gene. The concentration of AFB1 in the environment is high in China, but, low in South Korea<sup>[29]</sup>. AFB1 is strongly associated with a G to T transversion at codon 249 of the *p53* gene<sup>[28]</sup>. However, in our study, none of the cases displayed this G to T transversion. Loss or reduction of Fhit expression in HCC has been identified in association with altered *FHIT* transcripts, and LOH at the *FHIT* locus<sup>[13]</sup>. In our study, reduced Fhit expression was observed in only 4 of 30 cases with aberrant *FHIT* transcripts, and the remaining 4 cases displayed reduced Fhit expression did not present aberrant *FHIT* transcripts. Only one case with LOH at *FHIT* exhibited reduced Fhit expression. The six other cases with decreased Fhit expression did not show LOH at the *FHIT* locus. In contrast to previous studies, we observed no associations between Fhit expression and altered transcription or LOH. Loss of Fhit expression correlates with poor outcome in various cancers<sup>[14,30]</sup>. Zhao *et al* also reported that the expression of Fhit is inversely related to disease progression in HCC<sup>[19]</sup>. However, we found no correlation between Fhit expression and clinicopathological parameters. Although no interrelationship was evident between Fhit expression and LOH events, it is possible that other mutations, not investigated our study, are responsible for reduced or negative protein expression. Therefore, several genetic or epigenetic factors may potentially contribute to the loss of Fhit expression. One possibility is that inactivation

of the *FHIT* gene results from epigenetic mechanisms, such as hypermethylation of 5'-CpG islands in the promoter region<sup>[19,31]</sup>. In addition, abnormalities in post-transcriptional regulation may also abrogate expression of the *FHIT* gene. However, our results are preliminary and need to be confirmed in a larger study including more cases and an extended follow-up period.

In conclusion, our data indicate that abnormalities in *FHIT* gene transcripts occurred frequently in both cancerous and non-cancerous liver tissues. However, a normal-sized transcript without sequence abnormalities was almost always present. The *Fhit* is under-expressed only in a minor fraction of HCC tissues, while it was strongly expressed in non-cancerous liver tissues. Therefore, our study suggests that *FHIT* plays a role in relatively few HCC in lower AFB1 exposure area such as, South Korea. It is possible that such a finding was attributable to chance due to the relatively small numbers in our study. Thus, additional studies with more subjects are needed to confirm this finding.

## COMMENTS

### Background

The fragile histidine triad (*FHIT*) has been reported that this gene is a bona fide tumor suppressor gene. The *FHIT* gene and its product may be involved in the regulation of cell proliferative and apoptotic process. Down-regulation of *FHIT* inhibits apoptosis and *FHIT* has been shown to interact with a number of key proteins involved in cancer including p53

### Research frontiers

Altered transcripts and allelic loss of the *FHIT* gene are frequently found in premalignant and malignant lesions of various tumors. Moreover, loss or reduction of *FHIT* protein (*Fhit*) expression has been found in most tumors including hepatocellular carcinoma (HCC).

### Innovations and breakthroughs

Abnormalities of the *FHIT* gene transcripts occurred frequently in cancerous and non-cancerous liver tissues. However, a normal-sized transcript without sequence abnormalities was almost always present. Moreover, the *Fhit* was under-expressed only in a minor fraction of HCC tissues in lower AFB1 exposure area such as, South Korea, while it was strongly expressed in non-cancerous liver tissues. Therefore, none of the cases had a G to T transversion at p53 codon 249.

### Applications

*FHIT* behaves as a tumor suppressor gene whose loss or inactivation may contribute to HCC development or malignant progression in patients chronically infected with HBV and exposed to chemical carcinogens, particularly in areas from Qidong, China. In contrast, *FHIT* plays a role in relatively few HCC in lower AFB1 exposure area such as South Korea.

### Peer review

This is an original study that has a large big amount of work. The authors concluded that *FHIT* plays a role in relatively few HCC's in South Korea. This is an interesting study, with sound methodology.

## REFERENCES

- Bosch FX, Ribes J, Diaz M, Cleries R. Primary liver cancer: worldwide incidence and trends. *Gastroenterology* 2004; **127**: S5-S16
- Lok AS. Prevention of hepatitis B virus-related hepatocellular carcinoma. *Gastroenterology* 2004; **127**: S303-S309
- Cha C, Dematteo RP. Molecular mechanisms in hepatocellular carcinoma development. *Best Pract Res Clin Gastroenterol* 2005; **19**: 25-37
- Thomas MB, Zhu AX. Hepatocellular carcinoma: the need for progress. *J Clin Oncol* 2005; **23**: 2892-2899
- Kok K, Osinga J, Carritt B, Davis MB, van der Hout AH, van der Veen AY, Landsvater RM, de Leij LF, Berendsen HH, Postmus PE. Deletion of a DNA sequence at the chromosomal region 3p21 in all major types of lung cancer. *Nature* 1987; **330**: 578-581
- Naylor SL, Johnson BE, Minna JD, Sakaguchi AY. Loss of heterozygosity of chromosome 3p markers in small-cell lung cancer. *Nature* 1987; **329**: 451-454
- Brauch H, Johnson B, Hovis J, Yano T, Gazdar A, Pettengill OS, Graziano S, Sorenson GD, Poiesz BJ, Minna J. Molecular analysis of the short arm of chromosome 3 in small-cell and non-small-cell carcinoma of the lung. *N Engl J Med* 1987; **317**: 1109-1113
- Fujimori M, Tokino T, Hino O, Kitagawa T, Imamura T, Okamoto E, Mitsunobu M, Ishikawa T, Nakagama H, Harada H. Allelotype study of primary hepatocellular carcinoma. *Cancer Res* 1991; **51**: 89-93
- Ohta M, Inoue H, Cotticelli MG, Kastury K, Baffa R, Palazzo J, Siprashvili Z, Mori M, McCue P, Druck T, Croce CM, Huebner K. The *FHIT* gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. *Cell* 1996; **84**: 587-597
- Sozzi G, Veronese ML, Negrini M, Baffa R, Cotticelli MG, Inoue H, Tornelli S, Pilotti S, De Gregorio L, Pastorino U, Pierotti MA, Ohta M, Huebner K, Croce CM. The *FHIT* gene 3p14.2 is abnormal in lung cancer. *Cell* 1996; **85**: 17-26
- Ji L, Fang B, Yen N, Fong K, Minna JD, Roth JA. Induction of apoptosis and inhibition of tumorigenicity and tumor growth by adenovirus vector-mediated fragile histidine triad (*FHIT*) gene overexpression. *Cancer Res* 1999; **59**: 3333-3339
- Nishizaki M, Sasaki J, Fang B, Atkinson EN, Minna JD, Roth JA, Ji L. Synergistic tumor suppression by coexpression of *FHIT* and p53 coincides with *FHIT*-mediated MDM2 inactivation and p53 stabilization in human non-small cell lung cancer cells. *Cancer Res* 2004; **64**: 5745-5752
- Yuan BZ, Keck-Waggoner C, Zimonjic DB, Thorgeirsson SS, Popescu NC. Alterations of the *FHIT* gene in human hepatocellular carcinoma. *Cancer Res* 2000; **60**: 1049-1053
- Ishii H, Dumon KR, Vecchione A, Fong LY, Baffa R, Huebner K, Croce CM. Potential cancer therapy with the fragile histidine triad gene: review of the preclinical studies. *JAMA* 2001; **286**: 2441-2449
- Gramantieri L, Chieco P, Di Tomaso M, Masi L, Piscaglia F, Brilliati S, Gaiani S, Valgimigli M, Mazziotti A, Bolondi L. Aberrant fragile histidine triad gene transcripts in primary hepatocellular carcinoma and liver cirrhosis. *Clin Cancer Res* 1999; **5**: 3468-3475
- Schlott T, Ahrens K, Ruschenburg I, Reimer S, Hartmann H, Droese M. Different gene expression of MDM2, GAGE-1, -2 and *FHIT* in hepatocellular carcinoma and focal nodular hyperplasia. *Br J Cancer* 1999; **80**: 73-78
- Chen YJ, Chen PH, Chang JG. Aberrant *FHIT* transcripts in hepatocellular carcinomas. *Br J Cancer* 1998; **77**: 417-420
- Zekri AR, Bahnassy AA, Hafez M, El-Shehaby AM, Sherif GM, Khaled HM, Zakhary N. Alterations of the fragile histidine triad gene in hepatitis C virus-associated hepatocellular carcinoma. *J Gastroenterol Hepatol* 2005; **20**: 87-94
- Zhao P, Song X, Nin YY, Lu YL, Li XH. Loss of fragile histidine triad protein in human hepatocellular carcinoma. *World J Gastroenterol* 2003; **9**: 1216-1219
- Zochbauer-Muller S, Fong KM, Maitra A, Lam S, Geradts J, Ashfaq R, Virmani AK, Milchgrub S, Gazdar AF, Minna JD. 5' CpG island methylation of the *FHIT* gene is correlated with loss of gene expression in lung and breast cancer. *Cancer Res* 2001; **61**: 3581-3585
- Tannapfel A, Anhalt K, Hausermann P, Sommerer F, Benicke M, Uhlmann D, Witzigmann H, Hauss J, Wittekind C. Identification of novel proteins associated with hepatocellular carcinomas using protein microarrays. *J Pathol* 2003; **201**: 238-249



- 22 **Nan KJ**, Ruan ZP, Jing Z, Qin HX, Wang HY, Guo H, Xu R. Expression of fragile histidine triad in primary hepatocellular carcinoma and its relation with cell proliferation and apoptosis. *World J Gastroenterol* 2005; **11**: 228-231
- 23 **Greene FL**, Page DL, Fleming ID, Fritz A, Balch CM, Haller DG, Morrow M, eds. American Joint Committee on Cancer: AJCC Cancer Staging Manual. 6th ed, New York: Springer, 2002: 157-164
- 24 **Llovet JM**, Bru C, Bruix J. Prognosis of hepatocellular carcinoma: the BCLC staging classification. *Semin Liver Dis* 1999; **19**: 329-38
- 25 **Edmondson HA**, Steiner PE. Primary carcinoma of the liver: a study of 100 cases among 48,900 necropsies. *Cancer* 1954; **7**: 462-503
- 26 **Croce CM**, Sozzi G, Huebner K. Role of FHIT in human cancer. *J Clin Oncol* 1999; **17**: 1618-1624
- 27 **Boldog F**, Gemmill RM, West J, Robinson M, Robinson L, Li E, Roche J, Todd S, Waggoner B, Lundstrom R, Jacobson J, Mullokandov MR, Klinger H, Drabkin HA. Chromosome 3p14 homozygous deletions and sequence analysis of FRA3B. *Hum Mol Genet* 1997; **6**: 193-203
- 28 **Smela ME**, Currier SS, Bailey EA, Essigmann JM. The chemistry and biology of aflatoxin B(1): from mutational spectrometry to carcinogenesis. *Carcinogenesis* 2001; **22**: 535-545
- 29 **Park YM**, Kim BS, Tabor E. Precore codon 28 stop mutation in hepatitis B virus from patients with hepatocellular carcinoma. *Korean J Intern Med* 1997; **12**: 201-207
- 30 **Maruyama R**, Toyooka S, Toyooka KO, Harada K, Virmani AK, Zöchbauer-Müller S, Farinas AJ, Vakar-Lopez F, Minna JD, Sagalowsky A, Czerniak B, Gazdar AF. Aberrant promoter methylation profile of bladder cancer and its relationship to clinicopathological features. *Cancer Res* 2001; **61**: 8659-8663
- 31 **Tanaka H**, Shimada Y, Harada H, Shinoda M, Hatooka S, Imamura M, Ishizaki K. Methylation of the 5' CpG island of the FHIT gene is closely associated with transcriptional inactivation in esophageal squamous cell carcinomas. *Cancer Res* 1998; **58**: 3429-3434

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RAPID COMMUNICATION

## Relationship between T-lymphocyte cytokine levels and sero-response to hepatitis B vaccines

Vijayakumar Velu, Shanmugam Saravanan, Subhadra Nandakumar, Esaki Muthu Shankar, Appasamy Vengatesan, Suresh Sakharam Jadhav, Prasad Suryakant Kulkarni, Sadras Panchatcharam Thyagarajan

Vijayakumar Velu, Shanmugam Saravanan, Subhadra Nandakumar, Esaki Muthu Shankar, Sadras Panchatcharam Thyagarajan, Department of Medical Microbiology, Dr ALM PGIBMS, University of Madras, Chennai 600113 and National Referral Centre for Viral Hepatitis, India

Vijayakumar Velu, Vaccine Research Centre, Department of Microbiology and Immunology, Emory University, Atlanta, Georgia, 30329, United States

Sadras Panchatcharam Thyagarajan, Sri Ramachandra University, Porur, Chennai 600113, India

Saravanan Shanmugam, Esaki Muthu Shankar, Sadras Panchatcharam Thyagarajan, the YRG Centre for AIDS Research and Education, VHS campus, Taramani, Chennai 600113, India

Suresh Sakharam Jadhav, Prasad Suryakant Kulkarni, Serum Institute of India Ltd., Pune, India

Appasamy Vengatesan, Clinical Epidemiology Unit, Stanley Medical College, Chennai 600001, Tamilnadu, India

**Author contributions:** Velu V, Thyagarajan SP, Kulkarni PS, Jadhav SS designed the experiments; Velu V, Kulkarni PS organized the figures and wrote the manuscript; Velu V, Saravanan S, Nandakumar S performed the research, analyzed the data; Shankar EM and Vengatesan A performed the statistical analysis.

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**Correspondence to:** Dr. Vijayakumar Velu, Department of Medical Microbiology, University of Madras, Chennai 600113, India. [vvjai2000@yahoo.com](mailto:vvjai2000@yahoo.com)

Telephone: +91-44-22542929 Fax: +91-44-2542939

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### Abstract

**AIM:** To investigate the cellular defects by analyzing the (Th1/Th2) cytokine levels in vaccine responders and non-responders.

**METHODS:** Peripheral blood mononuclear cell (PBMC) from responders and non-responders were stimulated with or without recombinant HBsAg or PHA. Broad spectrum of cytokines viz (Th1) IFN- $\gamma$ , IL-2, TNF- $\alpha$ , IL-12 and (Th2) IL-10, IL-4 were measured after *in vitro* stimulation with recombinant HBsAg and were compared with respective antibody titers.

**RESULTS:** A significant decrease ( $P = 0.001$ ) in Th1 and Th2 cytokines namely, IL-2, INF- $\gamma$ , TNF- $\alpha$  and IL-10

in non-responders was observed. The level of IL-4 was not significant between the three groups. Furthermore, despite a strong Th1 and Th2 cytokine response, the level of IL-12 was elevated in high-responders compared to other groups ( $P = 0.001$ ) and demonstrated a positive correlation with anti-HBs titers and Th1 cytokine response.

**CONCLUSION:** Our findings suggest that unresponsiveness to recombinant hepatitis B vaccines (rHB) is multifactorial, including specific failure of antigen presentation or the lack of both T helper Th1 and Th2 response.

**Key words:** Hepatitis B vaccine; Cytokines; Humoral response; T cell response; Adult vaccines

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### INTRODUCTION

Hepatitis B is one of the world's major health problems<sup>[1]</sup>. It is estimated that more than 2 billion people are infected with hepatitis B virus (HBV) globally, and more than 400 million are chronic carriers<sup>[2]</sup>. The infection is supposed to be causally related to 1 to 2 million deaths per year worldwide<sup>[2]</sup>. Vaccination with the surface antigen of HBV (HBsAg) is considered the main strategy for effective control of the infection and viral transmission<sup>[3,4]</sup>. Recombinant hepatitis B vaccines (rHB) are recommended for Universal vaccination of neonates, as well as the high-risk healthy adult individuals<sup>[5]</sup>. Even though conventionally it is believed that the available HB vaccines induce only circulating humoral immunity, occasional reports suggest the possibility of induction of cell mediated immune response by HB vaccines.

The HB vaccine induces a protective antibody response (anti-HBs antibody  $\geq 10$  mIU/mL) in the majority of individuals after three dose regimen. However, about 10% of healthy recipients fail to generate protective levels of antibodies to the vaccine after standard immunization<sup>[6,7]</sup>. Furthermore, non-responders remain susceptible to infection with HBV<sup>[8]</sup>.

Inadequate immune response to HBsAg could be attributable to a variety of mechanisms including defect in the generation of primary HBsAg-specific T-cell<sup>[9]</sup> or B-cell repertoires<sup>[10]</sup>, expression of certain human leukocyte antigens (HLA) and haplotypes<sup>[11,12]</sup>, destruction of HBsAg-specific B-cells by antigen-specific cytotoxic T cells<sup>[13]</sup>, immunologic tolerance<sup>[14,15]</sup> and imbalance in T-helper (Th) cell function<sup>[16]</sup>.

HBsAg is a glycoprotein antigen with T-cell dependent characteristics. Induction of specific antibody to this antigen requires coordinated secretion of Th1 and Th2 cytokines leading to maturation and differentiation of the HBsAg-specific B-cell clones. Therefore, defective T-helper (Th) cell function, either Th1 or Th2, could result in failure of immune response to this antigen.

We compared three rHB vaccines; GeneVac-B<sup>®</sup> (Serum Institute of India Ltd, Pune), Engerix-B<sup>®</sup> (SmithKline Beecham Biologicals, Belgium) and Shanvac-B<sup>®</sup> (Shantha Biotechnics, India) in 400 healthy adults<sup>[17]</sup>. All three vaccines induced similar humoral immune (anti-HBs) response in the vaccines. However, a proportion of approximately 2% of the vaccines did not show adequate antibody response ( $> 10$  mIU/mL) to the vaccines. In order to better understand the non-responsiveness to these vaccines, we evaluated, for the first time in south Indian population, the broad spectrum of Th1 and Th2 cytokines levels in healthy adults vaccinated with rHB vaccines.

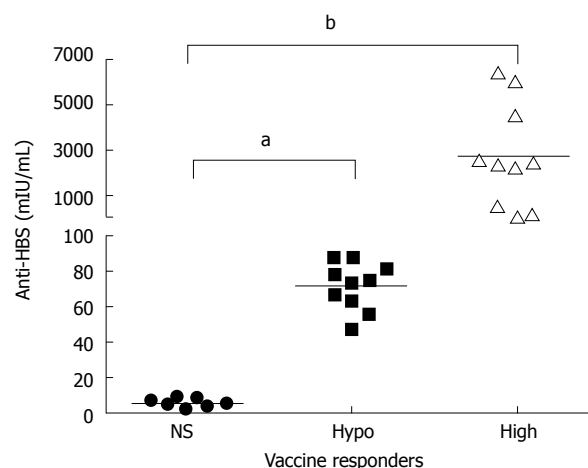
## MATERIALS AND METHODS

### Subjects

The subjects were healthy volunteers aged between 25 to 40 years. All subjects received intramuscular injections of three doses of 20  $\mu$ g of any of the three rHB vaccines with 0, 1 and 2 month regimen. Four weeks after the three dose of vaccination, serum anti-HBs levels were quantified using commercially available anti-HBs kit. Based on the anti-HBs titers, subjects were identified and recruited in the study as non-responders ( $< 10$  mIU/mL), hypo-responders (10 to 100 mIU/mL) and high-responders  $> 100$  mIU/mL (Figure 1). Four weeks following the third dose, blood samples were obtained and peripheral blood mononuclear cells (PBMCs) were separated from 27 volunteers that comprised of 7 non-responders, 10 hypo-responders and 10 high-responders to rHB vaccines for *in vitro* stimulation of the lymphocytes with HBsAg and PHA.

### Measurement of anti-HBs antibody response

Vaccines were screened for anti-HBs titers 4 wk after vaccination using the commercial Monolisa anti-HBs kit (BioRad, Belgium). Anti-HBs titers were quantified by extrapolation from a standard curve constructed using a serum sample with known concentration of antibody,



**Figure 1** Comparison of anti-HBs levels from vaccine non-responders (NS), hypo-responders (Hypo) and high-responders (High) after booster HBV vaccination. <sup>a</sup> $P < 0.05$ ; <sup>b</sup> $P = 0.001$ .

provided by the manufacturer. All these volunteers were also tested for anti-HCV and anti-HIV antibodies to rule out the possibility of immunosuppression.

### *In vitro* stimulation of PBMC

PBMC were separated from EDTA-anticoagulated venous blood by density gradient centrifugation on Ficoll-Paque (Amersham Biosciences, NJ, USA). After washing in RPMI-1640 medium (Himedia, India), PBMC were suspended in complete culture medium containing RPMI-1640 supplemented with 10% heat-inactivated fetal calf serum (FCS) (Himedia India), 2 mmol/L L-glutamine (Himedia, India), 100  $\mu$ g/mL penicillin and 100  $\mu$ g/mL Gentamicin (Gibco BRL, Scotland). PBMC were seeded at  $1 \times 10^6$  cells/mL in a 24-well sterile tissue culture plate (Nunc, USA) in the presence or absence of 5  $\mu$ g/mL of purified rHBsAg provided by Serum Institute of India, and 5  $\mu$ g/mL of PHA (Gibco BRL, Gaithersburg, MD) were used as positive controls and unstimulated cells act as a negative control. The plates were incubated for 72 h at 37°C in a humidified CO<sub>2</sub> (5%) incubator (Nuair, USA). Culture supernatants were collected and stored at -70°C until use.

### Cytokine assays

Supernatants from the PBMC proliferation assays were harvested after 72 h and cytokine levels (IFN- $\gamma$ , IL-2, TNF- $\alpha$ , IL-12, IL-10 and IL-4) were measured using commercial sandwich ELISA kits (Biosource Europe, SA) as per manufacturer's instructions. Briefly, culture supernatants distributed in 96-well plates coated with corresponding anti-cytokine antibodies were used to detect cytokine anti-cytokine complexes. The reaction was developed with TMB in 0.1 mol/L sodium acetate solutions and H<sub>2</sub>O<sub>2</sub>. Optical density was read at 450 nm. The concentration of cytokines in culture supernatants were calculated from the standard curve for each cytokine plotted on a log-log paper. The sensitivities of the assays for IFN- $\gamma$ , IL-2, TNF- $\alpha$ , IL-12, IL-10 and

**Table 1** Levels of cytokines secreted *in vitro* from PBMC of high, hypo and non-responder adults following stimulation with HBsAg

Cytokines/units	Stimulations	High-responders	Hypo-responders	Non-responders	P value	Multiple comparison <sup>2</sup>
IFN- $\gamma$ (U/mL)	HBsAg	18 $\pm$ 5.3	13.4 $\pm$ 5.6	6.8 $\pm$ 3.6	0.001 <sup>1</sup>	Ns vs Hy vs Hi
IL-2 (U/mL)	HBsAg	15.9 $\pm$ 5.5	8.4 $\pm$ 1.6	5.1 $\pm$ 1.8	0.001 <sup>1</sup>	Ns vs Hi
TNF- $\alpha$ (pg/mL)	HBsAg	201.5 $\pm$ 86.9	64.8 $\pm$ 49.0	21.4 $\pm$ 4.5	0.001 <sup>1</sup>	Ns vs Hi
IL-12 (pg/mL)	HBsAg	512.8 $\pm$ 213.6	117.6 $\pm$ 58.2	23.8 $\pm$ 7.6	0.001 <sup>1</sup>	Ns vs Hi
IL-10 (pg/mL)	HBsAg	260.8 $\pm$ 128.8	60 $\pm$ 49.7	47 $\pm$ 38.7	0.001 <sup>1</sup>	Ns vs Hi
IL-4 (pg/mL)	HBsAg	83.2 $\pm$ 72.3	62.8 $\pm$ 44.4	49.2 $\pm$ 39.9	-	Not significant

<sup>1</sup>Significant; Test of significance was One way ANOVA F-test; <sup>2</sup>Multiple comparison by Bonferroni t-test.

**Table 2** Comparison of cytokine levels secreted after *in vitro* stimulation with HBsAg and PHA

Cytokines	Stimulation	High-responders	Hypo-responders	Non-responders
IFN- $\gamma$ (U/mL)	HBsAg	18 $\pm$ 5.3	13.4 $\pm$ 5.6	6.8 $\pm$ 3.6
	PHA	21.2 $\pm$ 5.0	21.9 $\pm$ 2.8	18 $\pm$ 3.8
	P	0.19	0.001	0.001
IL-2 (U/mL)	HBsAg	15.9 $\pm$ 5.5	8.4 $\pm$ 1.6	5.1 $\pm$ 1.8
	PHA	19.8 $\pm$ 2.3	19 $\pm$ 3	16.8 $\pm$ 3.4
	P	0.06	0.001	0.001
TNF- $\alpha$ (pg/mL)	HBsAg	201.5 $\pm$ 86.9	64.8 $\pm$ 49.01	21.4 $\pm$ 4.5
	PHA	680.2 $\pm$ 138.4	626.4 $\pm$ 168.6	621.8 $\pm$ 198.7
	P	0.001	0.001	0.001
IL-12 (pg/mL)	HBsAg	512.8 $\pm$ 213.6	117.6 $\pm$ 58.2	23.8 $\pm$ 7.6
	PHA	611.3 $\pm$ 140.0	597.7 $\pm$ 141.8	491 $\pm$ 130.76
	P	0.24	0.001	0.001
IL-10 (pg/mL)	HBsAg	260.8 $\pm$ 128.8	60 $\pm$ 49.7	47 $\pm$ 38.7
	PHA	651.6 $\pm$ 132.07	606 $\pm$ 165.88	519.1 $\pm$ 152.2
	P	0.001	0.001	0.001
IL-4 (pg/mL)	HBsAg	83.2 $\pm$ 72.3	62.8 $\pm$ 44.4	49.2 $\pm$ 39.9
	PHA	610 $\pm$ 135.63	607.9 $\pm$ 163.4	569 $\pm$ 109.34
	P	0.001	0.001	0.001

Test of significance was student independent t test.

IL-4 were 0.1 U/mL, 0.1 U/mL, 3 pg/mL, 1.5 pg/mL, 1 pg/mL, 2 pg/mL, respectively.

### Statistical analysis

The data generated were analyzed using the statistical package for social sciences, (SPSS, version 13.0, Chicago, IL, USA). Anti-HBs response to all volunteers is expressed as geometric mean titers statistical difference was obtained by student t test. Differences in cytokine concentrations between the three groups of subjects were analyzed with the one-way ANOVA F test. Comparison of three groups between two different stimulations were analyzed by using multiple comparisons by Bonferroni "P" test. Correlations were calculated using Pearson's test. P values  $\leq$  0.05 were considered as significant.

## RESULTS

### Booster vaccination to HBV elicited broad spectrum of cytokines in high-responders

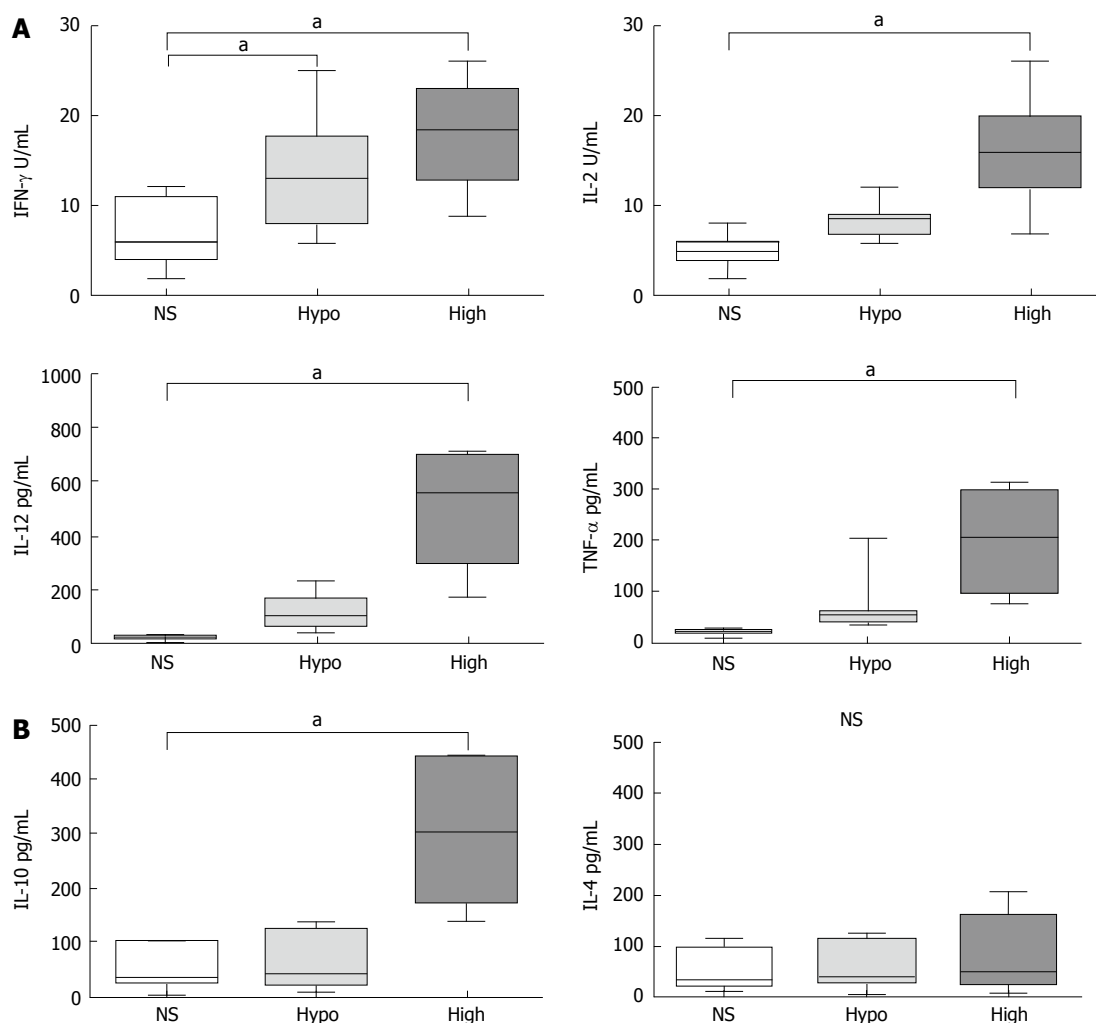
Booster HB vaccination induces a strong humoral response; however, approximately 2% of healthy adults in our study fails to induce Ab response. Based on the production of the antibody levels volunteers were classified in to non responders and responders (Figure 1).

There was a general correlation between serum anti-HBs level and the levels of Th1 and Th2 type cytokine response *in vitro*. A significant increase in the production of Th1 and Th2 cytokines was observed following stimulation of PBMC from high-responders with HBsAg except the Th2 cytokine IL-4. On the other hand, PHA induced two to three fold higher levels of all the cytokines in all the groups compared to HBsAg stimulation (Tables 1 and 2). This difference was significant for TNF- $\alpha$ , IL-10, and IL-4. However, there was no difference in the IFN- $\gamma$ , IL-2 and IL-12 levels. In addition, there was a several fold difference between the production of all cytokines between HBsAg and PHA stimulation in non-responders and hypo-responders (Table 2) which clearly demonstrate that the non-responsiveness is restricted to HBsAg specific response.

### Deficient Th1 cytokine levels in non-responders after booster immunization

The overall Th1 cytokine profile was elevated in high-responders compared with other two groups (Figure 2A). IFN- $\gamma$  production was significantly higher in high and hypo-responders compared to non-responders (P = 0.001). However, high-responders secreted higher levels of IL-2 and TNF- $\alpha$  compared with hypo-





**Figure 2** Cytokine secreted by PBMC's from high-responders, hypo-responders and non-responder adults after *in vitro* stimulation with HBsAg. **A:** Comparison of Th1 cytokines (IFN- $\gamma$ , IL-2, TNF- $\alpha$ , and IL-12) between the three groups of vaccine responders; **B:** Comparison of Th2 cytokines (IL-10 and IL-4) between the three groups of vaccine responders. \* $P < 0.05$ .

responders and non-responders (Table 1 and Figure 2A). IFN- $\gamma$  and IL-2 levels with HBsAg in high-responders were comparable with those of PHA, however, which was not observed in hypo or non-responders. A stronger positive correlation was observed between Th1 cytokine IFN- $\gamma$  ( $r^2 = 0.254$ ) and IL-2 ( $r^2 = 0.424$ ) production when compared with the anti-HBs response between the three groups of volunteers (Figure 3A). On the contrary, a weak correlation was observed with TNF- $\alpha$  ( $r^2 = 0.183$ ) production. Furthermore, the levels of IFN- $\gamma$  and IL-2 strongly correlated with the levels of IL-12 ( $r^2 = 0.52$ ) when compared between the three groups (data not shown).

#### Modulation of IL-12 production in non-responders and high-responders

There was a significant enhancement in production of IL-12 ( $P = 0.001$ ) in high responders compared with hypo-responders and non-responders (Figure 2A). Furthermore, a strong correlation ( $r^2 = 0.436$ ) was observed between the *in vivo* anti-HBs and *in vitro* IL-12 levels (Figure 3A). On the whole, our results suggest the existence of a strong association between the levels of IL-12 and Th1

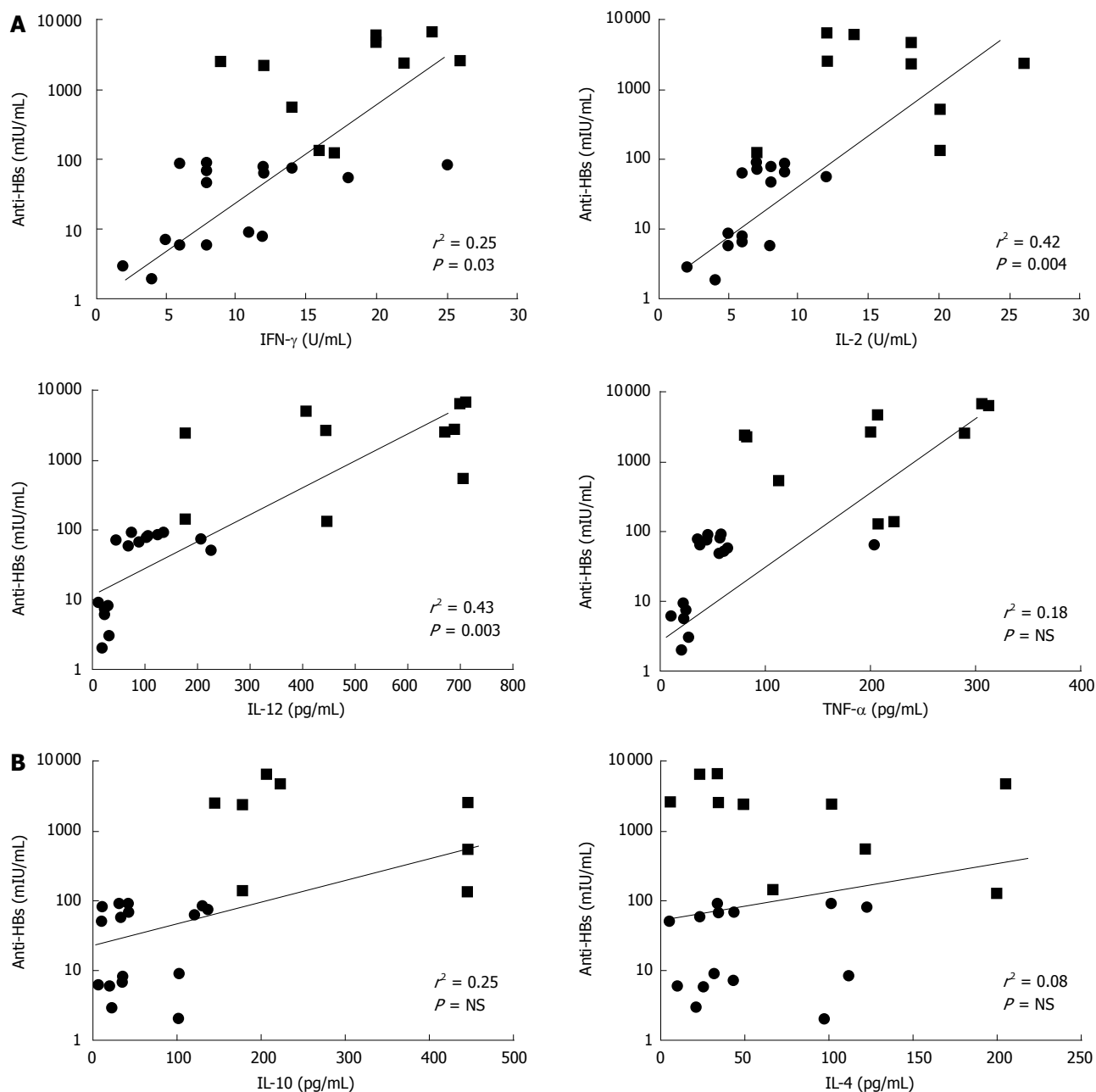
cytokines, which strongly implies that IL-12 may play a major regulatory role in the modulation of Th1 cytokine production in high-responders.

#### Elevated levels of Th2 cytokine response in high-responders after booster immunization

PBMC from high-responders produced significantly more IL-10 levels than those of hypo-responders and non-responders ( $P = 0.001$ ). The levels of IL-4 were very low and were comparable between the three groups of volunteers. However, they were significant when compared with PHA stimulations ( $P = 0.001$ ). No significant correlation was observed between anti-HBs production and IL-4 response ( $r^2 = 0.088$ ) (Figures 2B and 3B). In addition, a strong negative correlation was observed between the Th1 cytokine production and the IL-4 response (data not shown). In contrast, a weakly significant correlation was observed between IL-10 production and anti-HBs response ( $r^2 = 0.25$ ) in the volunteers (Figure 3B).

## DISCUSSION

Correlation between the humoral response *in vivo* and the



**Figure 3** Correlation between anti-HBs titers and cytokine levels secreted after *in vitro* stimulation of PBMC with HBsAg from high-responder, hypo-responder and non-responder adults. **A:** Correlation of anti-HBs antibody response and the Th1 cytokine (IFN- $\gamma$ , IL-2, IL-12 and TNF- $\alpha$ ) response to HBsAg; **B:** Correlation of anti-HBs antibody response and the Th2 cytokine (IL-10 and IL-4) response to HBsAg.

cellular response *in vitro* to HBsAg is dependent on the cytokine secretion profile of activated T lymphocytes<sup>[18]</sup>. Protective immune response to HBsAg is associated with the production of HBsAg specific neutralizing antibody<sup>[19,20]</sup>. The process of antibody production to this HBsAg is T-cell dependent and requires Th cell activation<sup>[20]</sup>. Secretion of Th2-like cytokines, such as IL-4, IL-5, IL-6, IL-10 and IL-13, is thought to be detrimental for B-cell differentiation and production of specific antibodies, whereas secretion of Th1-like cytokines, namely IL-2, IFN- $\gamma$ , TNF- $\alpha$  and transforming growth factor (TGF)- $\beta$  triggers the cell-mediated immune response leading to cure of HBV infected hepatocytes or destruction of HBV-infected cells<sup>[21-23]</sup>.

Several investigators have tried to study the pattern of

cytokine production in a variety of diseases<sup>[20,24]</sup>, including investigations in unresponsiveness to HBsAg in the recent past<sup>[26]</sup>. The results have been contradictory in analysis of *in vitro* HBsAg-induced cytokine production. These included absence of Th1 cytokine production in non-responders<sup>[9,26,27]</sup>, lack of Th2 response in both responders and non-responders<sup>[27]</sup>, absence of Th1 and Th2 cytokines in non-responders<sup>[27,28]</sup> and production of both cytokines in high-responders<sup>[28]</sup>. However, in contrast to the above findings there are also report showing no correlation between the function and cytokine production of HBsAg specific CD4 T cells<sup>[29,30]</sup>. In addition, predominant Th2 and Th1 responses have also been reported in high and low-responders, respectively<sup>[31]</sup>.

Our findings of diminished Th1 and Th2 responses

in non-responders confirmed and extended the results reported by others<sup>[25,28]</sup>. However, those studies assessed only a few cytokines, whereas in our study we looked at a broad spectrum of cytokines which represents Th1/Th2 profiles. Both types of cytokines Th1 cytokines (IL-2, IFN- $\gamma$ , TNF- $\alpha$ , IL-12) and Th2 cytokines (IL-10, IL-4) were secreted at significantly higher levels in high-responders compared with hypo-responders and non-responders. In addition the regulatory cytokine levels IL-12 were highly elevated in the high-responders compared to hypo-responders and non-responders, furthermore it is demonstrated that IL-12 induction of Th1 response is important for viral clearance in subjects suffering with chronic HB infection<sup>[24,27]</sup>.

The limitations in our study would be a small sample size and the variations in responder's cytokine profiles. However, the overall differences between the three groups of vaccines are pronounced (Table 1 and Figures 1 and 2). The significance of our results is more magnified when analyzed in the context of PHA-induced cytokine production. Despite the production of several-folds higher concentration of all cytokines in response to PHA, as compared with HBsAg, in all three groups of responders (Table 2), no significant differences were observed between the stimulations for IFN- $\gamma$ , IL-2, IL-12, IL-10, IL-4 and TNF- $\alpha$  (Table 2). These findings strongly agree and suggest the possibility of involvement of a generalized immune dysfunction in the non-responder subjects with regard to HBsAg stimulation and culture condition.

In summary, we have demonstrated diminished production of broad range of cytokines IFN- $\gamma$ , IL-2, TNF- $\alpha$ , IL-12, IL-10 and IL-4 in PBMC from healthy non-responders to HB vaccine, suggesting insufficient or lack of Th1 and Th2 responses. This could be because of a defect in either the primary HBsAg-specific T-cell repertoire<sup>[9]</sup> or antigen presentation<sup>[32]</sup>. Our study in non-responder adults strongly suggests the contribution of IL-12 cytokine levels which may lead to the dysfunction of antigen-presenting cells in unresponsiveness to the vaccine.

## COMMENTS

### Background

Hepatitis B virus (HBV) infection is one of the leading causes of morbidity and mortality world wide. Since there is no effective treatment for HBV, vaccination plays a vital role in preventing the infection. Hepatitis B vaccine induces a protective immune response in the majority of individuals. However, 4%-10% of healthy recipients fail to generate an effective antibody response against HBV after standard immunization. Inadequate immune response could be attributed to a variety of mechanisms. Here we made an attempt to study the cellular defects attributed to HB vaccine non-responders and responders.

### Research frontiers

This study was undertaken in order to analyze the cellular (cytokines) defects associated with non-responsiveness to hepatitis B (HB) vaccine in healthy individuals. Here we demonstrated that peripheral mononuclear cells (PBMC) isolated from anti-HBs seropositive subjects after booster injection were able to make both Th1/Th2 cytokines *in vitro* by stimulation with the surface antigen of HBV (HBsAg) in responses. In contrast, under the same conditions, non-responder PBMC failed to produce cytokines *in vitro*, furthermore we also demonstrated Th1 cytokine profile dominant compared to the Th2 profile and a strong positive correlation was obtained when comparing the regulatory

cytokine (IL-12) production with the strong anti-HBs response. To the best of our knowledge this is the first study which addressed the lack of Th1/Th2 cytokine profile in the south Indian population.

### Innovations and breakthroughs

Vaccination with HBsAg induces protective immunity through T-helper (Th) cell dependent production of anti-HBs antibody. Several studies have been conducted to investigate the precise role of Th1 and Th2 derived cytokines in the immune response to hepatitis B vaccine and to get further insights into the cellular basis of unresponsiveness to HBsAg. Controversial results, however, have been reported. Analyses of *in vitro* HBsAg-induced cytokine production have revealed defects in: Th1 cytokines in non-responder subjects; Th2 response in both responder and non-responder groups; Th1 and Th2 cytokines in non-responders; Different patterns of cytokine production have been observed in T-cell clones isolated from responder subjects, with either predominant Th0 or Th2 response; or Th1 and Th2 responses in high and low responders, respectively. Insufficient production of both types of cytokines in healthy non-responder individuals has been demonstrated. However, in this study we looked into the broad spectrum of cytokines and correlated them with their antibody production. Furthermore we also observed significant difference in the production of IL-12 by HBsAg in high-responders compared with non-responders. The significance is more magnified when analyzed in the context of the PHA induced IL-12 profile. Despite the production of higher concentration of IL-12 in response to PHA, as compared to HBsAg in both responders and non-responders subjects, no significant difference was observed between the groups, these results emphasize the exclusion of the possibility of involvement of generalized immune dysfunction on non-responders adults. Furthermore increase in IL-12 levels also strongly correlated with the induction of Th1 response. Taken together our data suggest that the non-responsiveness is associated with the defective production of both Th1 and Th2 cytokines.

### Applications

Our findings of significantly increased production of all cytokines in response to HBsAg as compared to control cultures without stimulation in responder vaccines, together with significantly higher secretion of these cytokines induced by PHA compared to HBsAg in non-responder, but not responder adults, may have important implications. These results suggest that in addition to the serum levels of anti-HBs antibody, the profile of cytokine secretion could also be used as an objective criteria and distinctive parameter to identify hepatitis B vaccine responder and non-responder individuals.

### Peer review

The authors compared a broad spectrum of cytokine (Th1/Th2 cytokine) response in hepatitis B vaccine non-responders, hypo-responders and high responders. They also demonstrated that lack of both Th1/Th2 cytokine profiles are associated with the non-responsiveness to hepatitis B vaccine.

## REFERENCES

- 1 Lee WM. Hepatitis B virus infection. *N Engl J Med* 1997; **337**: 1733-1745
- 2 Maddrey WC. Hepatitis B: an important public health issue. *J Med Virol* 2000; **61**: 362-366
- 3 Margolis HS. Prevention of acute and chronic liver disease through immunization: hepatitis B and beyond. *J Infect Dis* 1993; **168**: 9-14
- 4 Lemon SM, Thomas DL. Vaccines to prevent viral hepatitis. *N Engl J Med* 1997; **336**: 196-204
- 5 Hepatitis B vaccines. *Wkly Epidemiol Rec* 2004; **79**: 255-263
- 6 Shokri F, Jafarzadeh A. High seroprotection rate induced by low doses of a recombinant hepatitis B vaccine in healthy Iranian neonates. *Vaccine* 2001; **19**: 4544-4581
- 7 Bauer T, Jilg W. Hepatitis B surface antigen-specific T and B cell memory in individuals who had lost protective antibodies after hepatitis B vaccination. *Vaccine* 2006; **24**: 572-577
- 8 Boag F. Hepatitis B: heterosexual transmission and vaccination strategies. *Int J STD AIDS* 1991; **2**: 318-324
- 9 Chedid MG, Deulofeut H, Yunis DE, Lara-Marquez ML, Salazar M, Deulofeut R, Awdeh Z, Alper CA, Yunis EJ. Defect in Th1-like cells of nonresponders to hepatitis B vaccine. *Hum Immunol* 1997; **58**: 42-51
- 10 Shokrgozar MA, Shokri F. Enumeration of hepatitis B surface antigen-specific B lymphocytes in responder and non-responder normal individuals vaccinated with recombinant

- hepatitis B surface antigen. *Immunology* 2001; **104**: 75-79
- 11 **Shokrgozar MA**, Shokri F. HLA-associated antibody response to recombinant hepatitis B vaccine in healthy Iranian adults. *Ir J Med Sci* 1999; **24**: 98-103
  - 12 **Desombere I**, Willems A, Leroux-Roels G. Response to hepatitis B vaccine: multiple HLA genes are involved. *Tissue Antigens* 1998; **51**: 593-604
  - 13 **Barnaba V**, Franco A, Alberti A, Benvenuto R, Balsano F. Selective killing of hepatitis B envelope antigen-specific B cells by class I-restricted, exogenous antigen-specific T lymphocytes. *Nature* 1990; **345**: 258-260
  - 14 **del Canho R**, Grosheide PM, Schalm SW, de Vries RR, Heijntink RA. Failure of neonatal hepatitis B vaccination: the role of HBV-DNA levels in hepatitis B carrier mothers and HLA antigens in neonates. *J Hepatol* 1994; **20**: 483-486
  - 15 **Milich DR**, Jones JE, Hughes JL, Price J, Raney AK, McLachlan A. Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? *Proc Natl Acad Sci USA* 1990; **87**: 6599-6603
  - 16 **Livingston BD**, Alexander J, Crimi C, Oseroff C, Celis E, Daly K, Guidotti LG, Chisari FV, Fikes J, Chesnut RW, Sette A. Altered helper T lymphocyte function associated with chronic hepatitis B virus infection and its role in response to therapeutic vaccination in humans. *J Immunol* 1999; **162**: 3088-3095
  - 17 **Vijayakumar V**, Hari R, Parthiban R, Mehta J, Thyagarajan SP. Evaluation of immunogenicity and safety of Genevac B: A new recombinant hepatitis b vaccine in comparison with Engerix B and Shanvac B in healthy adults. *Indian J Med Microbiol* 2004; **22**: 34-38
  - 18 **Watanabe H**, Okumura M, Hirayama K, Sasazuki T. HLA-Bw54-DR4-DRw53-DQw4 haplotype controls nonresponsiveness to hepatitis-B surface antigen via CD8-positive suppressor T cells. *Tissue Antigens* 1990; **36**: 69-74
  - 19 **Mosmann TR**, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. *Immunol Today* 1996; **17**: 138-146
  - 20 **Mosmann TR**, Coffman RL. TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* 1989; **7**: 145-173
  - 21 **Romagnani S**. The Th1/Th2 paradigm. *Immunol Today* 1997; **18**: 263-266
  - 22 **O'Garra A**. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 1998; **8**: 275-283
  - 23 **Bertoletti A**, Maini MK. Protection or damage: a dual role for the virus-specific cytotoxic T lymphocyte response in hepatitis B and C infection? *Curr Opin Microbiol* 2000; **3**: 387-392
  - 24 **Ferrari C**, Penna A, Bertoletti A, Cavalli A, Missale G, Lamonaca V, Boni C, Valli A, Bertoni R, Urbani S, Scognamiglio P, Fiaccadori F. Antiviral cell-mediated immune responses during hepatitis B and hepatitis C virus infections. *Recent Results Cancer Res* 1998; **154**: 330-336
  - 25 **Kardar GA**, Jeddi-Tehrani M, Shokri F. Diminished Th1 and Th2 cytokine production in healthy adult nonresponders to recombinant hepatitis B vaccine. *Scand J Immunol* 2002; **55**: 311-314
  - 26 **Vingerhoets J**, Vanham G, Kestens L, Penne G, Leroux-Roels G, Gigase P. Deficient T-cell responses in non-responders to hepatitis B vaccination: absence of TH1 cytokine production. *Immunol Lett* 1994; **39**: 163-168
  - 27 **Vingerhoets J**, Goilav C, Vanham G, Kestens L, Muylle L, Kegels E, Van Hoof J, Piot P, Gigase P. Non-response to a recombinant pre-S2-containing hepatitis B vaccine: association with the HLA-system. *Ann Soc Belg Med Trop* 1995; **75**: 125-129
  - 28 **Bocher WO**, Herzog-Hauff S, Schlaak J, Meyer zum Buschenfeld KH, Lohr HF. Kinetics of hepatitis B surface antigen-specific immune responses in acute and chronic hepatitis B or after HBs vaccination: stimulation of the in vitro antibody response by interferon gamma. *Hepatology* 1999; **29**: 238-244
  - 29 **Larsen CE**, Xu J, Lee S, Dubey DP, Uko G, Yunis EJ, Alper CA. Complex cytokine responses to hepatitis B surface antigen and tetanus toxoid in responders, nonresponders and subjects naive to hepatitis B surface antigen. *Vaccine* 2000; **18**: 3021-3030
  - 30 **Tsutsui H**, Mizoguchi Y, Morisawa S. There is no correlation between function and lymphokine production of HBs-antigen-specific human CD4(+) -cloned T cells. *Scand J Immunol* 1991; **34**: 433-444
  - 31 **Honorati MC**, Dolzani P, Mariani E, Piacentini A, Lisignoli G, Ferrari C, Facchini A. Epitope specificity of Th0/Th2 CD4+ T-lymphocyte clones induced by vaccination with rHBsAg vaccine. *Gastroenterology* 1997; **112**: 2017-2027
  - 32 **Wataya M**, Sano T, Kamikawaji N, Tana T, Yamamoto K, Sasazuki T. Comparative analysis of HLA restriction and cytokine production in hepatitis B surface antigen-specific T cells from low- and high-antibody responders in vaccinated humans. *J Hum Genet* 2001; **46**: 197-206

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## Abdominal compartment syndrome in patients with severe acute pancreatitis in early stage

Hong Chen, Fei Li, Jia-Bang Sun, Jian-Guo Jia

Hong Chen, Fei Li, Jia-Bang Sun, Jian-Guo Jia, Department of General Surgery, Xuan Wu Hospital of Capital Medical University, Beijing 100053, China

**Author contributions:** Chen H and Li F conceived and designed the study; Chen H acquired the data; Chen H, Li F and Jia JG analyzed and interpreted the data; Chen H and Li F drafted the manuscript; Li F, Sun JB and Jia JG critically revised the manuscript for important intellectual content; Li F and Sun JB supervised and was responsible for all aspects of the study.

**Correspondence to:** Dr. Hong Chen, Department of General Surgery, Xuan Wu Hospital of Capital Medical University, Beijing 100053, China. [chenhong@medmail.com.cn](mailto:chenhong@medmail.com.cn)

Telephone: +86-10-83198899-8463 Fax: +86-10-63037023

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### Abstract

**AIM:** To study retrospectively the influence of intra-abdominal hypertension (IAH) and abdominal compartment syndrome (ACS) in patients with early acute pancreatitis (AP) (during the first week after admission) on physiological functions, and the association of the presence of IAH/ACS and outcome.

**METHODS:** Patients ( $n = 74$ ) with AP recruited in this study were divided into two groups according to intra-abdominal pressure (IAP) determined by indirect measurement using the transvesical route *via* Foley bladder catheter during the first week after admission. Patients ( $n = 44$ ) with IAP  $\geq 12$  mmHg were assigned in IAH group, and the remaining patients ( $n = 30$ ) with IAP  $< 12$  mmHg in normal IAP group. For analysis of the influence of IAH/ACS on organ function and outcome, the physiological parameters and the occurrence of organ dysfunction during intensive care unit (ICU) stay were recorded, as were the incidences of pancreatic infection and in-hospital mortality.

**RESULTS:** IAH within the first week after admission was found in 44 patients (59.46%). Although the APACHE II scores on admission and the Ranson scores within 48 h after hospitalization were elevated in IAH patients in early stage, they did not show the statistically significant differences from patients with normal IAP within a week after admission ( $16.18 \pm 3.90$  vs  $15.70 \pm 4.25$ ,  $P = 0.616$ ;  $3.70 \pm 0.93$  vs  $3.47 \pm 0.94$ ,  $P = 0.285$ , respectively). ACS in early AP was recorded in 20 patients (27.03%). During any 24-h period of

the first week after admission, the recorded mean IAP correlated significantly with the Marshall score calculated at the same time interval in IAH group ( $r = 0.635$ ,  $P < 0.001$ ). Although ACS patients had obvious amelioration in physiological variables within 24 h after decompression, the incidences of pancreatic infection, septic shock, multiple organ dysfunction syndrome (MODS) and death in the patients with ACS were significantly higher than that in other patients without ACS (pancreatic infection: 60.0% vs 7.4%,  $P < 0.001$ ; septic shock: 70.0% vs 11.1%,  $P < 0.001$ ; MODS: 90.0% vs 31.5%,  $P < 0.001$ ; mortality: 75.0% vs 3.7%,  $P < 0.001$ ).

**CONCLUSION:** IAH/ACS is a frequent finding in patients admitted to the ICU because of AP. Patients with IAP at approximately 10-12 mmHg and early signs of changes in physiologic variables should be seriously considered for urgent decompression to improve survival.

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**Key words:** Acute pancreatitis; Abdominal compartment syndrome; Intra-abdominal pressure; Intra-abdominal hypertension; Organ dysfunction

**Peer reviewer:** Ibrahim A Al Mofleh, Professor, Department of Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia

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### INTRODUCTION

Compartment syndrome is a condition in which increased pressure in a confined anatomical space adversely affects circulation and threatens the perfusion of tissues therein. The abdominal cavity can be considered as a single compartment and any change in the volume of any of its contents will elevate intra-abdominal pressure (IAP). Abdominal compartment syndrome (ACS) is the clinical syndrome resulting from a persistent increase in IAP. Intra-abdominal hypertension

(IAH) contributes to organ dysfunction and leads to the development of ACS. The resultant ACS refers to the complications caused by elevated IAP, including diaphragmatic compression with reduced pulmonary compliance, cardiovascular and renal dysfunction, and intestinal and hepatic ischemia<sup>[1]</sup>. Recently, the elevated IAP after onset of acute pancreatitis (AP) has gained growing attentions. ACS is characterized by multiple organ dysfunction syndrome (MODS), and if unrelieved, eventually results in death. This study aims to investigate the incidence and clinical outcome of ACS in patients with AP in early stage (within the first week after admission).

## MATERIALS AND METHODS

### Criteria and definition

AP was diagnosed by the criteria based on the consensus of the international symposium on AP (Atlanta definition)<sup>[2]</sup>. The presence of IAH/ACS was defined by the criteria of the World Society of ACS (WSACS)<sup>[3]</sup>. The association between IAH and organ dysfunction characterized the ACS. IAH was defined as an IAP of 12 mmHg or higher, which often rendered many organs dysfunctional. IAH was a consistently increased IAP value of  $\geq 12$  mmHg recorded by at least three standardized pressure measurements during at least 8 h. ACS was diagnosed when the IAP exceeded 20 mmHg at which IAH caused severe physiologic derangements and associated with new, attributable single organ dysfunction or MODS. Septic shock was defined according to the American-European conference consensus definitions<sup>[4]</sup>.

### Patients

The study population included 74 patients with AP admitted to the surgical intensive care unit (SICU), Xuan Wu Hospital of Capital Medical University, from May 2002 to May 2006. General inclusion criteria for AP were defined as: (1) a time interval between onset of typical abdominal symptoms and study inclusion of 72 h and less; (2) the presence of systemic inflammatory response syndrome (SIRS) manifested by two or more of the following conditions: temperature  $> 38^{\circ}\text{C}$  or  $< 36^{\circ}\text{C}$ ; heart rate (HR)  $> 90$  beats/min; respiratory rate  $> 20$  breaths/min or  $\text{PaCO}_2 < 32$  mmHg; WBC count  $> 12\,000/\text{mm}^3$  or  $< 4000/\text{mm}^3$ , or  $> 10\%$  immature(band) forms; and (3) at least 3-fold elevated serum amylase or lipase levels, or a APACHE II score  $> 8$ , or a C-reactive protein (CRP) of  $\geq 250$  mg/L.

All patients were treated according to our standard management of pancreatitis protocol and the practice guidelines in AP<sup>[5]</sup>. The patients recruited in this study were divided into two groups according to IAP determined during the first week after admission. Patients ( $n = 44$ ) with IAP  $\geq 12$  mmHg were assigned in IAH group, and the remaining patients ( $n = 30$ ) with IAP  $< 12$  mmHg in normal IAP group. Patients with IAH or ACS were treated by lowering IAP with promoting enterokinesia, and/or percutaneous abdominal decompression and drainage and/or

decompressive emergency laparotomy. A silastic covering was placed to achieve a pressure-free abdominal closure when decompressive laparotomy was performed. The definitive closure was performed within 5-7 d. In the setting of gallstone pancreatitis and evidence of common bile duct obstruction, urgent endoscopic retrograde cholangiopancreatography (ERCP) with sphincterotomy was indicated to remove the impacted stone.

### Anthropometric examination and measurement

Patients with AP admitted to SICU underwent intermittent measurement of IAP, recording of the clinical data and calculation of MODS scores by Marshall *et al*<sup>[6]</sup>. Since intravesical pressure (IVP) measurement had been described as a standard and validated technique of indirectly reflecting IAP and rapidly recognizing ACS<sup>[7]</sup>, urinary bladder pressure (UBP) was routinely measured in all AP patients in our study. In brief, the patient was placed in complete supine position with a Foley catheter. One end of a transducer was connected to the Foley catheter through 3-way stopcocks. The monitor was zeroed with the transducer at the level of the symphysis pubis. After clamping the drainage tube of the urinary catheter, the bladder was instilled with 50 mL of sterile normal saline injected through a Foley catheter under aseptic condition, the bladder was then emptied by removing the clamp off the drainage tube. IVP was measured using a pressure transducer, and a series of reading were obtained every 4 h apart and their average calculated as one standardized measurement in mmHg (1 mmHg = 1.36 cmH<sub>2</sub>O). Only end-expiratory values were used in an effort to avoid interference from respiratory excursion of the diaphragm into the intra-abdominal space. A sustained increase in IAP was detected based upon at least three consecutive standardized measurements during at least 8 h.

Hemodynamics and other physiologic parameters, including HR, mean arterial pressure (MAP), central venous pressure (CVP), urine output (UO), peak airway pressure (PAP), arterial carbon dioxide partial pressure ( $\text{PaCO}_2$ ), modified respiratory index (MRI) expressed as the arterial oxygen partial pressure ( $\text{PaO}_2$ ) to fraction of inspired oxygen ( $\text{FiO}_2$ ) ratio ( $\text{PaO}_2/\text{FiO}_2$ ), arterial pH, arterial base deficits (BE) and arterial lactic acid, were registered before and after decompression of ACS. The worst Acute Physiology and Chronic Health Evaluation (APACHE) II score and Ranson scoring system during the first 24 or 48 h of SICU stay were recorded respectively in the identification of the severity of pancreatitis. In this study, we applied a Marshall subscore of 2 or more in any of the 6 organs (respiratory, cardiovascular, coagulation, central nervous system, hepatic, and renal) to indicate organ dysfunction associated with IAH (IAP  $> 20$  mmHg), and defined the presence of ACS. MODS was defined as two or more organs having persistently 2 or more Marshall subscores for more than 24 h despite aggressive resuscitation and organ support. Total Marshall scores were computed daily and for the entire duration of the intensive care stay.

All 74 patients with AP were detected using contrast

Table 1 Etiology of attacks in 74 patients with AP

Etiology of attacks	Number of patients (%)	IAH group No. (%)	Normal IAP group No. (%)
Gallstones	46 (62.16)	26 (59.09)	20 (66.67)
Hyperlipidemia	11 (14.86)	7 (15.91)	4 (13.33)
Alcohol	8 (10.81)	5 (11.36)	3 (10.00)
Idiopathic	9 (12.16)	6 (13.64)	3 (10.00)
Total	74	44	30

Table 2 Comparison of clinical data on admission between two groups (mean  $\pm$  SD)

Clinical data on admission	IAP $\geq$ 12 mmHg ( <i>n</i> = 44)	IAP < 12 mmHg ( <i>n</i> = 30)	$\chi^2$ or <i>t</i> value	<i>P</i> value
Age (yr)	62.61 $\pm$ 11.05	63.57 $\pm$ 7.71	-0.41	0.684
Gender (male/female)	23/21	16/14	0.01	0.929
APACHE II score	16.18 $\pm$ 3.90	15.70 $\pm$ 4.25	0.50	0.616
Ranson score	3.70 $\pm$ 0.93	3.47 $\pm$ 0.94	1.08	0.285
CTSI	5.25 $\pm$ 2.27	5.63 $\pm$ 2.01	0.75	0.458

APACHE II score: Acute physiology and chronic health evaluation II score; CTSI: CT scan severity index.

enhanced computed tomography (CT) on admission. The contrast-enhanced CT scan severity index (CT-SI) developed by Balthazar and coworkers was used for evaluating severity of pancreatitis<sup>[8]</sup>. CT-SI was measured for all patients on admission in this study. The incidences of pancreatic infection, septic shock, MODS, and the in-hospital mortality were also recorded. Pancreatic infection included infected necrosis and pancreatic abscess. Necrosis formation of the pancreas was assessed by contrast-enhanced CT. Patients with pancreatic necrosis or large peripancreatic fluid collections who manifested clinical signs of sepsis with a non-improving or deteriorating clinical course despite a reasonable time of medical therapy underwent fine-needle aspiration (FNA) of the necrotic areas or fluid collections under CT or ultrasound guidance to determine the presence of bacterial contamination or pancreatic infection.

### Statistical analysis

Patients' clinical records were retrospectively reviewed. Data were analyzed using the software package SPSS version 11.5. Continuous data are presented as mean  $\pm$  SD. Categorical variables are expressed as numbers and percentages for the group from which they were derived. The comparisons of age, gender, APACHE II score and CT-SI on admission, Ranson score within 48 h after admission, incidences of pancreatic infection and MODS, and the in-hospital mortality between the two groups were analyzed using the independent sample *t* test or  $\chi^2$  test as appropriate. Comparison of clinical parameters between before decompression and after decompression of ACS was performed by paired samples *t* test. Bivariate correlations were analyzed by Pearson test between the mean IAP in any 24-h period within the first week after admission and the Marshall score at the same time interval, or, between the IAP on admission and the

APACHE II score on admission or Ranson score within 48 h after admission. Difference was defined as statistically significant if  $P < 0.05$ .  $P^*$  (corrected  $P$ )  $< 0.017$  indicated statistically significant difference in multiple comparisons of complications and outcome between patients with and without ACS in  $\chi^2$  test.

## RESULTS

### IAH group and normal intra-abdominal pressure group

Seventy-four patients (39 men and 35 women) were included in the study; the mean age was 63.00  $\pm$  9.79 years (range, 42-87 years). The mean time interval between onset of AP and admission was 34.97  $\pm$  30.16 h. IAH developed in 44 (59.46%) of 74 patients with AP. The etiologic causes of their AP are detailed in Table 1. The difference in etiologic causes between the two groups was not statistically significant,  $P = 0.803$  (Fisher two-tail exact probability test). The differences in age, gender, APACHE II score, Ranson score and CT-SI on admission were also not statistically significant between IAH group and group with normal IAP (Table 2). Neither age nor gender was correlated with IAH development. Similarly, APACHE II score and CT-SI on admission, and Ranson score within 48 h after admission, contrary to accepted belief, were not found predictive of early IAH development.

During any 24-h period of the first week after admission, the recorded mean IAP was correlated significantly with the Marshall score calculated at the same time interval in IAH group ( $r = 0.635$ ,  $P < 0.001$ ), but IAP on admission had no significant correlation with APACHE II score on admission and Ranson score within 48 h after admission ( $r = 0.127$ ,  $P = 0.248$ , and  $r = 0.145$ ,  $P = 0.263$ , respectively).

### ACS patients

ACS was recorded during the first week after admission in 20 patients (27.03%) in whom IAH (IAP  $> 20$  mmHg) was associated with organ dysfunction or failure. In 14 of these patients, IAH and organ dysfunction were present on admission, 2 patients had IAH on admission and developed organ dysfunction 24-48 h later, whereas 4 patients had respiratory failure on admission without IAH but went on to develop IAH and ACS 3-5 d after admission. Medicine and physical therapy were applied in all IAH patients to promote enterokinesia for recovery of IAH/ACS. The administration of prokinetic motility agents such as erythromycin, metoclopramide, or neostigmine appeared to hold promise in evacuating the intraluminal contents and decreasing the size of the viscera. Moreover, sedation and muscle relaxant (only for mechanical ventilated patients) were also used properly to lower IAP. Seven ACS patients had restoration of organ function with their UBP lowering less than 12 mmHg *via* non-invasive intervention mentioned above. The remaining 13 ACS patients had progressive deterioration of organ function and increases in IAP despite active persistent non-invasive decompression management for 24 h. Therefore, these



**Table 3** Changes in physiological variables after invasive decompressive procedure in patients with ACS ( $n = 13$ , mean  $\pm$  SD)

Variable	Before decompression	After decompression	$t$ value	$P$ value
MAP (mmHg)	53.77 $\pm$ 8.98	74.15 $\pm$ 6.68	7.23	< 0.001
UBP (mmHg)	36.69 $\pm$ 5.33	18.31 $\pm$ 3.25	-8.77	< 0.001
UO (mL/h)	21.76 $\pm$ 13.42	85.78 $\pm$ 18.46	13.29	< 0.001
CVP (mmHg)	15.01 $\pm$ 5.24	13.02 $\pm$ 5.01	-1.97	0.072
BE	-9.31 $\pm$ 3.09	-1.69 $\pm$ 2.59	6.67	< 0.001
PH	7.28 $\pm$ 0.10	7.36 $\pm$ 0.06	2.35	0.037
PaO <sub>2</sub> /FiO <sub>2</sub> (mmHg)	129.22 $\pm$ 41.30	229.24 $\pm$ 63.47	4.33	0.001
PAP (cmH <sub>2</sub> O)	41.23 $\pm$ 12.90	24.46 $\pm$ 5.41	-4.25	0.001
HR (bpm)	132.38 $\pm$ 13.68	111.62 $\pm$ 12.85	-5.19	< 0.001
PaCO <sub>2</sub> (mmHg)	47.61 $\pm$ 11.24	38.63 $\pm$ 5.78	-2.38	0.034
Lactate (mmol/L)	4.48 $\pm$ 0.95	2.02 $\pm$ 0.80	-8.11	< 0.001

MAP: Mean arterial pressure; UBP: Urinary bladder pressure; UO: Urine output; CVP: Central venous pressure; BE: Arterial base deficits; pH: Arterial Ph; PaO<sub>2</sub>/FiO<sub>2</sub> (MRI): Arterial oxygen partial pressure/fraction of inspired oxygen; PAP: Peak airway pressure; HR: Heart rate; PaCO<sub>2</sub>: Arterial carbon dioxide partial pressure; Lactate: Arterial lactic acid.

patients had abdominal decompression procedure very soon. The mean time interval between diagnosis of ACS and initiation of invasive decompressive procedure was  $28.38 \pm 2.29$  h (range, 26-33 h). Prior to invasive decompression, aggressive attempts should be made to correct coagulation deficits, acidosis and hypovolemia.

The invasive decompressive procedure included two ways: abdominal percutaneous decompression drainage and decompressive laparotomy with temporary closure. IAP was reduced in one of the two ways. The insertion of abdominal drains was performed in 8 patients under CT or ultrasound guidance in the presence of large intra-abdominal collections more than 800 mL. These patients had their abdomens decompressed by percutaneous placement of a large-bore hemodialysis catheter into the intra-abdominal space, with drainage of intra-abdominal fluid. Percutaneous drainage was a continual process with fluid draining for several days. Once the drainage stopped, the catheter was removed. No complication related to the procedure occurred in our study. Decompressive drainage reduced the IAP by less than 20 mmHg and then further resulted in relief of the IAH in 5 patients. Decompressive laparotomy was performed in the remaining 5 patients with ACS and 3 patients of failure in previous percutaneous abdominal decompressive drainage for the reduction in IAP. The typical open abdomen operation was performed with a temporary silastic covering obtained from the sterilized inner surface of an intravenous bag sewn to the fascia to protect the underlying abdominal contents from injury and desiccation, and to prevent excessive amounts of fluid from leaking onto surrounding bandages. Removal of silastic covering and scheduled closure of abdominal wall should be subsequently attempted as soon as possible after the acute episode resolved and the edema and/or inflammatory fluid collections reduced significantly. No complication directly related to decompressive laparotomy itself occurred in our study.

All patients with bladder pressure of more than

20 mmHg immediately benefited from the alternative of invasive decompression methods. Emergency invasive decompressive procedure resulted in statistically significant improvement in physiologic parameters, with the exception of the CVP, within 24 h after decompression (Table 3). Pre-decompression bladder pressure was significantly elevated, with a mean pressure of  $36.69 \pm 5.33$  mmHg. Abdominal compartment release resulted in a dramatic decrease of IAP to  $18.31 \pm 3.25$  mmHg ( $P < 0.001$ ). The high PAP improved significantly from  $41.23 \pm 12.90$  cmH<sub>2</sub>O pre-decompression to  $24.46 \pm 5.41$  cmH<sub>2</sub>O post-decompression ( $P = 0.001$ ). Other general physiologic condition also improved significantly with decompression, as evidenced by a significant improvement in serum pH, BE, PaO<sub>2</sub>/PaCO<sub>2</sub>, arterial lactic acid, MAP and UO. Only one parameter registered in this study, e.g. CVP, was not affected by decompression for ACS ( $P = 0.072$ ).

### Prognosis

Seventeen of 74 patients died with a mortality rate of 22.97%. It appeared that the incidences of pancreatic infection and MODS in patients with ACS in early stage were considerably higher than other patients without ACS. Subsequently, the unacceptably high mortality rate in patients with ACS during their first week after admission was much higher than that in patients without ACS in the course of their attack of AP (75% *vs* 3.33% and 4.17%) (Table 4).

Multiple comparisons of complications and outcome were made between ACS patients (IAP > 20 mmHg), IAH patients without ACS ( $12 \text{ mmHg} \leq \text{IAP} \leq 20 \text{ mmHg}$ ) and normal IAP patients (IAP < 12 mmHg). The incidences of pancreatic infection, septic shock, MODS and in-hospital mortality in patients with ACS were significantly higher than those in IAH patients without ACS and normal IAP patients, respectively (pancreatic infection:  $\chi^2 = 13.42$ ,  $P < 0.001$ ;  $\chi^2 = 16.93$ ,  $P < 0.001$ , septic shock:  $\chi^2 = 12.84$ ,  $P < 0.001$ ;  $\chi^2 = 22.12$ ,  $P < 0.001$ , MODS:  $\chi^2 = 11.01$ ,  $P = 0.001$ ;  $\chi^2 = 21.33$ ,  $P < 0.001$ , in-hospital mortality:  $\chi^2 = 23.65$ ,  $P < 0.001$ ;  $\chi^2 = 28.32$ ,  $P < 0.001$ , respectively). However, the difference in incidences of pancreatic infection, septic shock, MODS and in-hospital mortality was not statistically significant between IAH patients without ACS and normal IAP patients (pancreatic infection:  $P = 1.000$ , Fisher two-tail exact probability test; septic shock:  $\chi^2 = 0.527$ ,  $P = 0.468$ ; MODS:  $\chi^2 = 2.078$ ,  $P = 0.149$ ; in-hospital mortality:  $P = 1.000$ , Fisher two-tail exact probability test).

### DISCUSSION

The ACS is an increasingly recognized complication of both medical and surgical patients. This syndrome has been described in a wide variety of clinical scenarios and results from a persistent elevation in IAP characterized by graded organ system dysfunction. The definitions and diagnosis of IAH or ACS depend greatly on the accuracy and reproducibility of the IAP measurement technique. As a result, IAP must be measured with an



Table 4 Comparison of complications and outcome between patients with and without ACS

Complications and outcome	ACS ( <i>n</i> = 20) (IAP > 20 mmHg)	IAH ( <i>n</i> = 24) (12 mmHg ≤ IAP ≤ 20 mmHg)	Normal IAP ( <i>n</i> = 30) (IAP < 12 mmHg)	$\chi^2$ value	<i>P</i> value
Pancreatic infection (%)	12 (60.00)	2 (8.33)	2 (6.67)	23.84	< 0.001
Septic shock (%)	14 (70.00)	4 (16.67)	2 (6.67)	26.34	< 0.001
MODS (%)	18 (90.00)	10 (41.67)	7 (23.33)	21.85	< 0.001
In-hospital mortality (%)	15 (75.00)	1 (4.17)	1 (3.33)	41.93	< 0.001

MODS: Multiple organ dysfunction syndrome.

accurate, reproducible, and reliable tool. The diagnosis of ACS requires a high level of clinical suspicion combined with an increased IAP, usually obtained *via* UBP measurement. UBP measurement as an estimation of IAP is simple, reliable and widely accepted.

The bladder gold standard measurement techniques reported are not uniform<sup>[9]</sup>. The volume instilled in the bladder is important. This was shown by Fusco *et al*<sup>[7]</sup>, who compared direct laparoscopic insufflation pressure with IVP measured with different bladder volumes. They found that a bladder volume of 50 mL revealed the least bias in measuring elevated IAP. The current standard of IAP measurement in our study *via* the urinary catheter is labor intensive, and its intermittent nature could prevent timely recognition of significant changes in IAP. The continuous IAP measurement proposed by Balogh *et al*<sup>[10]</sup> can be accurately measured via the irrigation port of a three-way catheter and has good agreement with the standard intermittent IAP. Recently, a fully automated IAP measurement technique was described that it can minimize the pitfalls that may alter the accuracy and reproducibility of intermittent IAP measurements (such as volume instilled, zero reference level, air bubbles, over- or underdamping). The IAP catheter is introduced like a nasogastric tube and is equipped with an air pouch at the tip. Automated IAP measurement had good correlation with the standard IVP method<sup>[11]</sup>. Schachtrupp *et al*<sup>[12]</sup> compared different direct and indirect IAP measurement methods in a porcine model and found a very good correlation between the above-mentioned air pouch system and direct insufflator pressure. However, the air pouch system was not available for use in our study.

IAH was defined in our study as a mean IAP ≥ 12 mmHg, whereas ACS was defined as a gradually and consistently increased IAP value of > 20 mmHg associated with at least one organ dysfunction or failure that was not previously present. This study confirms that IAH and ACS are frequent occurrences in patients with AP because these conditions were observed in 59.46% and 27.03% of the studied patients, respectively.

Although ideally the diagnosis of ACS should be made based on the clinical picture and confirmed by measurements of bladder pressure or equivalent, Pickhardt and others<sup>[13]</sup> described the CT findings in four patients with confirmed ACS. They reported that the anteroposterior to transverse abdominal ratio was increased (round belly sign) in patients with ACS. The ACS patients had a ratio of 0.85 compared with 0.70 in controls. Al-Bahrani *et al*<sup>[14]</sup> concluded in their

prospective evaluation of CT features that the presence of round belly sign and bowel wall thickening with enhancement on CT images should alert clinicians to the possibility of presence of IAH and ACS, and to prompt measurement of the IAP and consideration of suitable interventions. The radiological data of CT scans that paralleled with the development of ACS in any course of the disease were deficient in our clinical information, so, we could not analyze the CT findings in ACS patients with SAP in our study.

Patients with AP are at risk for IAH/ACS because of the large volume of intra-abdominal and peripancreatic inflammatory fluid collection, capillary leakage caused by increased permeability, bowel and splanchnic edema, resuscitation fluid, and other factors. Gastrointestinal ileus or distension is a common risk factor for IAH among patients with AP. Both air and fluid within the hollow viscera can raise IAP and lead to IAH. IAH also leads to intestinal edema and visceral swelling triggering a vicious cycle. IAH impairs organ perfusion and leads to organ dysfunction. Manifestations of ACS include cardiovascular, pulmonary, renal, splanchnic and neurologic impairment. Hypoperfusion of the gastrointestinal tract was reported at IAP of 12 mmHg<sup>[15]</sup>. Oliguria and marked reduction in cardiac output have been shown to develop at an IAP greater than 20 mmHg<sup>[16,17]</sup>. The relationship among rise in IAP, greater organ dysfunction and, subsequently, higher disease mortality was well illustrated in our study. A positive significant correlation was observed between IAP and Marshall organ dysfunction score. We also observed significant improvement in Marshall score and MODS of patients with resolution of IAH. The overall mortality rate in our study of approximately 23% is comparable to the 10%-50% reported in AP patients by others<sup>[18-21]</sup>. The mortality in ACS patients in our study (75%), however, was much higher than that in patients without ACS which is not acceptable for us, although post-injury ACS has been consistently reported to have a high mortality ranging from 25%-75%. We then compared the early onset of organ dysfunction (within 7 d of admission with AP) and risk of the disease.

While non-operative medical management strategies are now recognized as playing a vital role in both the prevention and treatment of physiologic compromise and organ dysfunction due to elevated IAP, surgical decompression is commonly considered the only treatment for aggravated ACS. All patients with IAH/ACS in our study were initially managed with

noninvasive measures. In an attempt to decrease the elevated IAP, nasogastric decompression, prokinetic motility agents, bowel care, sedation, analgesia and pharmacologic paralysis were administered. Those patients who failed to improve rapidly after institution of these conservative measures underwent percutaneous abdominal decompressive drainage or operative abdominal compartment release. Although the obvious amelioration in physiological variables within 24 h after decompression have been observed in ACS patients, the clinical relevance of ACS in patients with AP in our study was illustrated, in part, by the greater probability of pancreatic infection, MODS and mortality. For this reason, a high index of suspicion and low threshold for decompressive procedure appear appropriate in patients with AP. Patients at risk for ACS warrant close monitoring and we recommend prompt abdominal decompression following documentation of increased IAP in the setting of physiologic compromise, despite in the absence of organ dysfunction/failure.

However, there is no clear consensus on the critical level of IAP at which decompression is necessary. The critical level of IAP requiring decompression thus has not been established for AP patients. Evidence of significant organ dysfunction has been demonstrated at an IAP of 10 mmHg<sup>[22]</sup>. It partially explains why the outcome of ACS patients in our study is poor even though invasive decompression appeared to be effective in reducing IAP and potentially ameliorating IAH-induced physiologic compromise. One of the most important determinants of mortality is the time interval between occurrence of IAH and sustained reduction in IAP of < 12 mmHg by decompression. As shown above, hypoperfusion and acidosis start occurring at pressures from 10 to 12 mmHg, an IAP near 10 mmHg is thus gaining acceptance as a cut-off value in our institution.

Some papers have demonstrated that a persistent splanchnic hypoperfusion may induce irreversible damage in organ function and death<sup>[23-25]</sup>. We speculate that a global mechanism of ischemia and reperfusion may explain these findings. Increased IAP resulted in a decrease of mucosal blood flow to 63% of baseline despite maintaining normal mean arterial blood pressure<sup>[17]</sup>. In addition, elevated IAP could significantly reduce bowel tissue oxygenation due to bowel ischemia<sup>[26]</sup>. In the 1990s, several authors observed a positive correlation between bacterial translocation and IAP in animal models, even when IAP was raised for less than 1 h. This result was caused by increased gut permeability induced by splanchnic ischemia with and without reperfusion<sup>[27,28]</sup>. The mechanism by which the necrotic pancreas becomes infected is unclear, but experimental and clinical data suggest that the gastrointestinal tract is the likely source of organisms, since intestinal colonization by pathogens often precedes pancreatic infection<sup>[29-33]</sup>. The gut clearly plays a major role in the development of MODS. IAH has been shown to be associated with increased bacterial translocation to pancreas and probability of pancreatic infection followed by MODS and death. This increase may be more pronounced when the rise in IAP is followed

by splanchnic ischemia/reperfusion after decompression because of ACS. Moreover, ACS decompression showed to provoke and amplify proinflammatory cytokine release that served as a second insult for the induction of severe organ dysfunction in the two-hit model of MODS<sup>[34]</sup>. Time interval effect of decompression is consistent with the "vulnerable window" of inflammatory mediators cascade priming. As a result, abdominal decompression of established ACS probably causes a fulminant reperfusion syndrome. The mean time interval between diagnosis of ACS and initiation of invasive decompressive procedure in our study was  $28.38 \pm 2.29$  h. The relatively long time for persistence of ACS before invasive decompression might be enough to induce the higher pre-decompression IAP ( $36.69 \pm 5.33$  mmHg) and occurrence of splanchnic ischemia/reperfusion, and soon thereafter, to trigger the bacteria residing within the gastrointestinal lumen to cross the intact intestine into pancreas<sup>[35]</sup>. The sequential effects might mainly contribute to pancreatic infection, MODS and the higher mortality of ACS patients in our study. We therefore advocate performing invasive decompression for an acute IAP of 20-25 mmHg rather than 30-40 mmHg. We also should keep firmly in mind that the earlier treatment is instituted, the more likely a progression to irreversible damage is prevented. The key to managing IAH and ACS is the early recognition of the harmful effects. It is better to prevent ACS than to allow it to occur, and manage the sequelae. However, the timing, indications and threshold value for surgical decompression are controversial with very few large trials available to give firm guidance. Decompression must be strongly considered if the IAP continues to rise or if clinical deterioration occurs. We agree with the concept that IAH is a part of a continuum leading to ACS; therefore, early detection and treatment are preferable to treating the overt clinical manifestations of ACS.

The widely disparate patient populations who may develop IAH/ACS make a standardized therapeutic approach to this syndrome difficult. Thus a single threshold value of IAP cannot be globally applied to the decision making of all patients. No one management strategy can be uniformly applied to every patient with IAH/ACS. Several fundamental management concepts, however, remain appropriate among all patients with AP. While initial conservative measures are implemented and the patient nonetheless proceeds to develop IAPs by greater than 20 mmHg, invasive abdominal decompression should be performed immediately, particularly in the presence of general trend of signs towards overt ACS, including a tendency towards high airway pressures or oliguria refractory to aggressive resuscitation. In AP patients, decompression can be accomplished either by percutaneous decompression with a large-bore catheter inserted or by formal laparotomy. Failure of catheter decompression invariably leads to formal laparotomy. With an increased awareness of the signs of ACS, early conservative treatment of IAH and rapid abdominal decompression when the trend towards the syndrome manifests, clinicians can expect a lower mortality in severe AP patients. In our experience with 74 patients with AP,

the following clinical features may predispose ACS in early stage of the disease: gastrointestinal ileus or distension, a large volume of intra-abdominal and peripancreatic inflammatory fluid collection, massive fluid resuscitation and oliguria.

Indeed, the data presented from our retrospective study raise more questions than answers. Further prospective multi-center studies involving a large number of AP patients are necessary to identify subgroups that might benefit from a therapeutic intervention. The outcome of ACS remains very poor in present study. This result suggests that efforts at prevention may be as fruitful as the efforts directed at early recognition and decompression. Further efforts should focus on prevention of the syndrome. For better prevention of ACS, further studies are essential to identify the independent risk factors for ACS in AP patients and build prediction models for the syndrome to identify high-risk patients with early signs and symptoms of ACS, so as to permit prevention or timely modified treatment before organ failure occurs.

In summary, we conclude that ACS is one of the most important causes of significant morbidity and mortality in AP patients. Early detection and rapid treatment of IAH via abdominal decompression should be essential to preventing the subsequent development of pressure induced organ dysfunction in AP patients.

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## COMMENTS

### Background

Intra-abdominal hypertension (IAH) contributes to organ failure in patients with abdominal trauma and sepsis and leads to the development of abdominal compartment syndrome (ACS). ACS has also been described in patients with severe acute pancreatitis (SAP). Significant visceral edema associated with massive fluid resuscitation, paralytic ileus and formation of pancreatic ascites in patients with SAP can lead to ACS which may cause the early development of multiple organ dysfunction syndrome (MODS), especially in the early stages of the disease. Despite recent advances in the management of patients, SAP remains a disease with an unpredictable clinical course and significant morbidity and mortality. Pancreatic infection and the presence of organ dysfunction still remain the most fearing complications and are increasingly recognized as important risk factors for mortality in patients with SAP. The development of ACS in patients with SAP seems to be associated with increased mortality. The clinical impact of IAH/ACS on pancreatic infection and organ failure in SAP patients is needed to investigate.

### Research frontiers

Only recently has the important role of ACS been recognized as a contributing factor for the multiple organ failure commonly seen in SAP. Percutaneous drainage of ascites and surgical decompression are the preferred methods of treatment for ACS. However, some studies, including this study, show that although decompression has a significant effect in lowering IAP, mortality still remains high in SAP patients with ACS. The poor response might be due to an intervention performed too late. Although the 2004 international ACS conference consensus definitions committee has proposed the evidence-based treatment guidelines to assist clinicians in the management of IAH and ACS, there is no clear consensus on the critical level of IAP at which decompression

is necessary. The critical level of IAP requiring decompression thus has not been established for AP patients. Because of the close correlation between ACS and death in SAP patients as suggested by this study, there is a great need for well-designed, prospective clinical trials to clarify the questions and issues that remain unanswered with respect to IAH and ACS.

### Innovations and breakthroughs

The notion that patients with SAP may develop ACS, which necessitates emergency abdominal decompression, has been ignored by some current literatures. The currently prevailing paradigm calls for non-operative management of SAP as long as there is no evidence of infection. The authors of this study proposed that there is a subset of patients who may necessitate an emergency abdominal decompression or laparotomy in the absence of infection in order to decompress a clinically significant ACS, which is associated with the acute pancreatitis (AP).

### Applications

The present study implicated that the timing of surgical decompression may play a crucial role in improving poor survival rates of SAP patients with ACS. Increased awareness of the syndrome of IAH/ACS in AP and transvesical measurement of intra-abdominal pressure (IAP) will reveal its prevalence and significance. This experience with the 74 patients may reinforce the enthusiasm for prevention of ACS and decompression in SAP patients with IAH. This study also gave proof of necessity for establishing a critical IAP level at which abdominal decompression or laparotomy should be performed for SAP patients.

### Terminology

IAP is the steady-state pressure concealed within the abdominal cavity. IAH is a sustained or repeated pathological elevation in IAP  $\geq 12$  mmHg. ACS is a sustained IAP  $> 20$  mmHg associated with new organ dysfunction/failure.

### Peer review

Although the IAP was described by Etienne-Jules Marey in 1863 and ACS described in details by Korn *et al* in 1984 and more information is appearing recently in the literature, it is still important to gather more knowledge about ACS complicating severe pancreatitis. This article constitutes a fairly good number of patients with ACS.

## REFERENCES

- 1 Cullen DJ, Coyle JP, Teplick R, Long MC. Cardiovascular, pulmonary, and renal effects of massively increased intra-abdominal pressure in critically ill patients. *Crit Care Med* 1989; **17**: 118-121
- 2 Bradley EL 3rd. A clinically based classification system for acute pancreatitis. Summary of the International Symposium on Acute Pancreatitis, Atlanta, Ga, September 11 through 13, 1992. *Arch Surg* 1993; **128**: 586-590
- 3 Malbrain ML, Cheatham ML, Kirkpatrick A, Sugrue M, Parr M, De Waele J, Balogh Z, Leppaniemi A, Olvera C, Ivatury R, D'Amours S, Wendon J, Hillman K, Johansson K, Kolkman K, Wilmer A. Results from the International Conference of Experts on Intra-abdominal Hypertension and Abdominal Compartment Syndrome. I. Definitions. *Intensive Care Med* 2006; **32**: 1722-1732
- 4 Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, Schein RM, Sibbald WJ. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. *Chest* 1992; **101**: 1644-1655
- 5 Banks PA, Freeman ML. Practice guidelines in acute pancreatitis. *Am J Gastroenterol* 2006; **101**: 2379-2400
- 6 Marshall JC, Cook DJ, Christou NV, Bernard GR, Sprung CL, Sibbald WJ. Multiple organ dysfunction score: a reliable descriptor of a complex clinical outcome. *Crit Care Med* 1995; **23**: 1638-1652
- 7 Fusco MA, Martin RS, Chang MC. Estimation of intra-abdominal pressure by bladder pressure measurement: validity and methodology. *J Trauma* 2001; **50**: 297-302
- 8 Balthazar EJ, Robinson DL, Megibow AJ, Ranson JH. Acute pancreatitis: value of CT in establishing prognosis. *Radiology* 1990; **174**: 331-336
- 9 Malbrain ML. Different techniques to measure intra-abdominal pressure (IAP): time for a critical re-appraisal.

*Intensive Care Med* 2004; **30**: 357-371

- 10 **Balogh Z**, Jones F, D'Amours S, Parr M, Sugrue M. Continuous intra-abdominal pressure measurement technique. *Am J Surg* 2004; **188**: 679-684
- 11 **Malbrain ML**, De Laet I, Viaene D, Schoonheydt K, Dits H. In vitro validation of a novel method for continuous intra-abdominal pressure monitoring. *Intensive Care Med* 2008; **34**: 740-745
- 12 **Schachtrupp A**, Tons C, Fackeldey V, Hoer J, Reinges M, Schumpelick V. Evaluation of two novel methods for the direct and continuous measurement of the intra-abdominal pressure in a porcine model. *Intensive Care Med* 2003; **29**: 1605-1608
- 13 **Pickhardt PJ**, Shimony JS, Heiken JP, Buchman TG, Fisher AJ. The abdominal compartment syndrome: CT findings. *AJR Am J Roentgenol* 1999; **173**: 575-579
- 14 **Al-Bahrani AZ**, Abid GH, Sahgal E, O'shea S, Lee S, Ammori BJ. A prospective evaluation of CT features predictive of intra-abdominal hypertension and abdominal compartment syndrome in critically ill surgical patients. *Clin Radiol* 2007; **62**: 676-682
- 15 **Schwarte LA**, Scheeren TW, Lorenz C, De Bruyne F, Fournell A. Moderate increase in intraabdominal pressure attenuates gastric mucosal oxygen saturation in patients undergoing laparoscopy. *Anesthesiology* 2004; **100**: 1081-1087
- 16 **Diebel L**, Saxe J, Dulchavsky S. Effect of intra-abdominal pressure on abdominal wall blood flow. *Am Surg* 1992; **58**: 573-575; discussion 575-576
- 17 **Diebel LN**, Dulchavsky SA, Brown WJ. Splanchnic ischemia and bacterial translocation in the abdominal compartment syndrome. *J Trauma* 1997; **43**: 852-855
- 18 **Tao HQ**, Zhang JX, Zou SC. Clinical characteristics and management of patients with early acute severe pancreatitis: experience from a medical center in China. *World J Gastroenterol* 2004; **10**: 919-921
- 19 **Al-Bahrani AZ**, Ammori BJ. Clinical laboratory assessment of acute pancreatitis. *Clin Chim Acta* 2005; **362**: 26-48
- 20 **Mayerle J**, Hlouschek V, Lerch MM. Current management of acute pancreatitis. *Nat Clin Pract Gastroenterol Hepatol* 2005; **2**: 473-483
- 21 **Pitchumoni CS**, Patel NM, Shah P. Factors influencing mortality in acute pancreatitis: can we alter them? *J Clin Gastroenterol* 2005; **39**: 798-814
- 22 **Sugrue M**, Buist MD, Hourihan F, Deane S, Bauman A, Hillman K. Prospective study of intra-abdominal hypertension and renal function after laparotomy. *Br J Surg* 1995; **82**: 235-238
- 23 **Malbrain ML**. Is it wise not to think about intraabdominal hypertension in the ICU? *Curr Opin Crit Care* 2004; **10**: 132-145
- 24 **Malbrain ML**, Deeren D, De Potter TJ. Intra-abdominal hypertension in the critically ill: it is time to pay attention. *Curr Opin Crit Care* 2005; **11**: 156-171
- 25 **Cheatham ML**, Malbrain ML, Kirkpatrick A, Sugrue M, Parr M, De Waele J, Balogh Z, Leppaniemi A, Olvera C, Ivatury R, D'Amours S, Wendon J, Hillman K, Wilmer A. Results from the International Conference of Experts on Intra-abdominal Hypertension and Abdominal Compartment Syndrome. II. Recommendations. *Intensive Care Med* 2007; **33**: 951-962
- 26 **Bongard F**, Pianim N, Dubecz S, Klein SR. Adverse consequences of increased intra-abdominal pressure on bowel tissue oxygen. *J Trauma* 1995; **39**: 519-524; discussion 524-525
- 27 **Jakob SM**. Clinical review: splanchnic ischaemia. *Crit Care* 2002; **6**: 306-312
- 28 **Khanna A**, Rossman JE, Fung HL, Caty MG. Intestinal and hemodynamic impairment following mesenteric ischemia/reperfusion. *J Surg Res* 2001; **99**: 114-119
- 29 **Tarpila E**, Nystrom PO, Franzen L, Ihse I. Bacterial translocation during acute pancreatitis in rats. *Eur J Surg* 1993; **159**: 109-113
- 30 **Runkel NS**, Moody FG, Smith GS, Rodriguez LF, LaRocco MT, Miller TA. The role of the gut in the development of sepsis in acute pancreatitis. *J Surg Res* 1991; **51**: 18-23
- 31 **Medich DS**, Lee TK, Melhem MF, Rowe MI, Schraut WH, Lee KK. Pathogenesis of pancreatic sepsis. *Am J Surg* 1993; **165**: 46-50; discussion 51-52
- 32 **McNaught CE**, Woodcock NP, Mitchell CJ, Rowley G, Johnstone D, MacFie J. Gastric colonisation, intestinal permeability and septic morbidity in acute pancreatitis. *Pancreatol* 2002; **2**: 463-468
- 33 **Luiten EJ**, Hop WC, Endtz HP, Bruining HA. Prognostic importance of gram-negative intestinal colonization preceding pancreatic infection in severe acute pancreatitis. Results of a controlled clinical trial of selective decontamination. *Intensive Care Med* 1998; **24**: 438-445
- 34 **Rezende-Neto JB**, Moore EE, Melo de Andrade MV, Teixeira MM, Lisboa FA, Arantes RM, de Souza DG, da Cunha-Melo JR. Systemic inflammatory response secondary to abdominal compartment syndrome: stage for multiple organ failure. *J Trauma* 2002; **53**: 1121-1128
- 35 **Steinberg SM**. Bacterial translocation: what it is and what it is not. *Am J Surg* 2003; **186**: 301-305

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## Clinical features of adverse reactions associated with telbivudine

Xue-Song Zhang, Rui Jin, Shi-Bin Zhang, Ming-Ling Tao

Xue-Song Zhang, Rui Jin, Shi-Bin Zhang, Ming-Ling Tao, Department of GI and Hepatology, Beijing Youan Hospital affiliated to Capital Medical University, Beijing 100069, China  
Author contributions: Jin R designed the research; Zhang SB, Tao ML and Zhang XS performed the research; Zhang SB and Tao ML collected and analyzed the data; and Zhang SB wrote the paper.

Correspondence to: Rui Jin, Professor, Department of GI and Hepatology, Beijing Youan Hospital Affiliated to Capital Medical University, Beijing 100069, China. [jinrui@public.bta.net.cn](mailto:jinrui@public.bta.net.cn)

Telephone: +86-10-83997117 Fax: +86-10-63395319

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and Liver Research Unit, Guadalajara University Hospital, University of Alcalá, Guadalajara 19002, Spain; Vincent Lai, Derby NHS Foundation Trust, Derby DE22 3NE, United Kingdom

Zhang XS, Jin R, Zhang SB, Tao ML. Clinical features of adverse reactions associated with telbivudine. *World J Gastroenterol* 2008; 14(22): 3549-3553 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3549.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3549>

### Abstract

**AIM:** To analyze the clinical features and risk factors of adverse reactions associated with telbivudine.

**METHODS:** Clinical data were collected from cases that presented with serious adverse reactions to telbivudine. We analyzed general information and medicine status, clinical features, results of examination, and misdiagnosis.

**RESULTS:** Out of 105 patients who were treated with telbivudine for hepatitis B in an outpatient department from January, 2007 to January, 2008, five presented with serious adverse drug reactions. Most of these five patients had used other nucleoside analogues in the past. Four were treated with a combination of telbivudine and interferon or another nucleoside analogue, while the other received an increased dose of telbivudine. The main adverse reactions were myalgia and general weakness. This was accompanied by cardiac arrhythmia in one patient, and nervous symptoms in three. Serum creatine kinase was elevated. The rate of misdiagnosis was high.

**CONCLUSION:** The adverse reactions were related to telbivudine, but the biological mechanism of the reactions is not yet clear. Combination therapy with interferon or another nucleoside analogue and a high dose may increase the risk of adverse reactions.

### INTRODUCTION

Telbivudine is a new synthetic nucleoside analogue<sup>[1]</sup>. Since it came on the market in October, 2006, it has been a new option for clinicians in treating chronic hepatitis B, because it significantly suppresses hepatitis B virus (HBV) replication. In our recent clinical practice, however, adverse reactions associated with telbivudine have been increasing. To understand this better, we retrospectively analyzed the clinical records of patients taking telbivudine. We hope that the result will provide clinic references for the future safe use of telbivudine.

### MATERIALS AND METHODS

#### Subjects

Of 105 patients who were treated with telbivudine for hepatitis B at an outpatient department from January, 2007 to January, 2008, five presented with serious adverse reactions.

#### Methods

A retrospective method was employed to analyze the medical records of the five patients, including: general information, medicine history, telbivudine treatment, dosage, combined medication, time of occurrence and clinical features of adverse reactions, possible misdiagnosis, as well as results of laboratory tests, such as routine blood analysis, myozyme, liver function, and kidney function.

### RESULTS

#### General information and medication status

All patients were male with an age range of 25-45 years, and a mean of 34 years. Four patients were infected with

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**Key words:** Adverse drug reaction; Hepatitis B; Mitochondria; Nucleoside analogue; Telbivudine

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Table 1 General information and medication status

Case	Age (yr)	Hepatitis history (yr)	Current diagnosis	Medication history	Telbivudine		Drug and time combined
					Dosage	Time (mo)	
1	45	10	Hepatocirrhosis	DLAM, ECV	600 mg <i>bid</i>	2	-
2	35	6	Hepatitis B	LAM, ADV	600 mg <i>qd</i>	5	ADV for 5 mo
3	37	37	Hepatitis B	LAM, ADV	600 mg <i>qd</i>	7	Interferon for 3 mo
4	30	1	Hepatitis B	-	600 mg <i>qd</i>	1	-
5	25	2	Hepatitis B	ADV	600 mg <i>qd</i>	7	Interferon for 3 mo
						9	Interferon for 3 mo

LAM: Lamivudine; ADV: Adefovir; ECV: Entecavir.

Table 2 Clinical symptoms of telbivudine-related adverse reactions

Case	Occurred time (mo)	Injured location	Soreness		Weakness		Numbness		Neuralgia		Cardiac arrhythmia	
			Take	Stop	Take	Stop	Take	Stop	Take	Stop	Take	Stop
1	1.5	Shoulder	+		++		-		-		-	
	2	Limb	+	-	+	-	-	-	-	-	-	-
2	5	Limb	++	+	++	+	++	+	++	+	-	-
3	0.5	Limb	++	-	++	+	++	+	-	-	++	+
4	2	Limb	+	-	+	-	++	+	-	-	-	-
5	1	Buttock	++	+	++	+	++	+	-	-	-	-

++: Very serious; +: Serious; -: Not serious.

Table 3 Blood tests results

Case	Muscle enzymes		Liver function			Blood counts			Kidney function		
	CK (IU/L)	LDH (IU/L)	ALT (IU/L)	AST (IU/L)	GGT (IU/L)	WBC ( $\times 10^9/L$ )	HGB (g/L)	PLT ( $\times 10^9/L$ )	BUN (mmol/L)	CR ( $\mu\text{mol/L}$ )	
1	438.7	182.9	97.3	216.4	115.8	4.28	144	39	3.91	101	
2	900	-	32.1	31.3	24.8	2.7	130	118	2.53	107	
3	191	117	32	24	26	7.19	153	212	8.38	100	
4	311	253	50	47	117	3.6	147	86	4.60	45	
5	400.2	424.8	92.9	68.3	21.6	3.4	133	154	-	-	

HBV after birth and one acquired the infection directly during pregnancy. One patient had been diagnosed with liver cirrhosis, and the other four with chronic hepatitis B. Four were given other nucleoside analogues before telbivudine. The duration of treatment with telbivudine varied from 1 to 9 mo. In case 1, whose dosage was changed from telbivudine 600 mg twice daily alone in the first 2 mo, to 600 mg once daily in combination with adefovir for 5 mo because of the incidence of myopathy. Cases 2, 4 and 5 were treated with a combination of telbivudine and interferon (Table 1).

### Clinical features of adverse reactions

Occurrence of adverse reactions varied from 0.5 to 5 mo after treatment. Myalgia was most commonly observed, mainly of the limb skeletal muscles, companied by general weakness. There were four cases with nervous damage which included symptoms of numbness, while one case had neuralgia. One case presented with cardiac arrhythmia. After telbivudine treatment was discontinued, myalgia was reduced to a varying extent, but cardiac and nervous system symptoms persisted for a long time (Table 2).

### Laboratory tests

Serum creatine kinase (CK) was elevated. There was

no direct correlation between CK level and severity of fatigue. Liver function was not impaired. All blood cell counts were normal except that platelets were decreased in case 1 due to hypersplenism. Neither bone marrow inhibition nor kidney toxicity was observed (Table 3).

### Initial diagnosis, misdiagnosis and confirmed diagnosis

Misdiagnosis commonly took place because clinicians failed to recognize the adverse reactions. As a result, the patients had to endure further examinations and incurred the associated extra medical expenses (Table 4).

## DISCUSSION

To date, there have been no special reports about telbivudine-related adverse reactions in the literature. In this investigation, the following suggested that the adverse events reported were related to treatment with telbivudine: (1) patients did not present with obvious adverse reactions to interferon or other nucleoside analogues in the past; (2) adverse reactions occurred after taking telbivudine and were reduced or resolved after discontinuing treatment; and (3) the ratio for the occurrence of adverse reactions was approximately 1:20 (5:105), which was high.

Table 4 Diagnosis process

Case	Confirmed time	Misdiagnosis	Examinations	Medication
1	1 d	Arthritis	No	Paste for arthritis
	2 d	Myocarditis	Cardiac enzymes	-
2	3 mo	Neuritis, arthritis	Lumber spinal MRI, laboratory examination	Vitamin B
3	0.5 mo	Neuritis, myocarditis	Myozyme, holter, ECG, ultrasoundcardiography	Mecobalamin, propafenone
4	1 d	-	-	-
5	2 mo	Neuritis	-	Vitamin B

### Possible mechanisms of telbivudine-associated adverse reactions

Telbivudine, as a new synthetic analogue of thymidine, is phosphorylated by host cellular kinases to telbivudine-5'-triphosphate, which has a half-life of 14 h *in vivo*. Telbivudine-5'-triphosphate is then incorporated into HBV DNA by HBV polymerase, through competition with thymidine-5'-triphosphate, the natural substrate. Once inserted, telbivudine-5'-triphosphate causes DNA chain termination, thereby inhibiting HBV replication<sup>[2-4]</sup>.

Some studies<sup>[5-8]</sup> have indicated that telbivudine treatment has considerable clinical efficacy, was and is well tolerated at all doses (25, 50, 100, 200, 400 and 800 mg/d), with no dose-related or treatment-related clinical or laboratory adverse events. However, other research has shown<sup>[9]</sup> that 3/680 patients treated with telbivudine presented with myopathy. However, our study is different.

At present, the biological mechanism of telbivudine-related adverse reactions is not yet clear. Given that the adverse reactions involve multiple organs, including muscle, nerves and the heart, we suggest that the mechanism is associated with cell energy metabolism. Mitochondria are involved in the production of energy. They contain many important proteins, enzymes and carriers that participate in energy transduction. Deficiency in any of these leads to a poor substrate supply for oxidative phosphorylation, and eventually to inadequate manufacture of the energy molecule ATP<sup>[10]</sup>, and this causes mitochondrial disease. Organs that are highly dependent on ATP, such as the nervous system, skeletal muscle, myocardium, retina and pancreas are the most vulnerable to mitochondrial dysfunction. Symptoms of pathological conditions often take place in related areas. Clinically, skeletal muscle is a frequent target and symptoms include fatigability, weakness, as well as myalgia in 50% of cases, and CK level is not elevated significantly<sup>[11]</sup>. ATP deficiency in the heart may result in cardiac arrhythmia, dilated cardiomyopathy, or unexplainable cardiomyopathy; while deficiency in the nervous system can lead to peripheral neuropathy. It has been suggested that muscle biopsy and chromosome examination are beneficial for the diagnosis of mitochondria disease<sup>[12-14]</sup>. There is no mature experience as to the treatment at present<sup>[15-18]</sup>.

The possible links between telbivudine and mitochondrial disease are as follows. (1) Mitochondrial toxicity<sup>[19-22]</sup> is a serious adverse reaction associated with nucleoside analogues, which can inhibit mtDNA polymerase- $\gamma$ , then interfere with energy transduction

in mitochondria. Some nucleoside analogues such as adenine arabinoside and acyclovir can cause mitochondrial toxicity<sup>[23]</sup>. (2) As a competitive substrate of natural thymidine, telbivudine is phosphorylated by host mitochondrial thymidine kinases to telbivudine-5'-triphosphate, and thymidine kinases are extensively exhausted. As a result, the normal energy transduction process is disturbed. (3) In the process of phosphorylation, high levels of phosphates are captured, which leads directly to exhaustion of ATP, which results in poor energy supply.

Although the telbivudine-related adverse reactions could not be definitely diagnosed as mitochondrial disease without a muscle biopsy and DNA study, the symptoms were identical to those of mitochondrial disease. This reminds us to pay close attention to it along with telbivudine treatment in future practice.

### Risk factors related to telbivudine treatment

In case 1, symptoms of myalgia intensified following treatment with 600 mg telbivudine twice daily, while the symptoms became milder at lower doses. This suggests that myalgia is dose-dependent. Other studies<sup>[24-27]</sup> have shown that telbivudine plasma concentration is correlated with dosage when in the range 200-600 mg/d. As a result of the long half-life of this drug, a dose of 600 mg twice daily may lead to drug accumulation. Therefore, the risk may increase with higher doses.

### Synergistic effect of drug combinations

Among the patients with adverse events mentioned above, three were given combination treatment with telbivudine and interferon. Although the therapeutic mechanisms of the two drugs are different<sup>[28-30]</sup>, they share a common feature in causing myalgia as an adverse reaction<sup>[31]</sup>. Synergistic effects can happen when two drugs are used at the same time. It is known that most nucleoside analogues are metabolized in the kidney. According to the literature<sup>[1]</sup>, plasma concentration of drugs may be elevated, if it is combined with other drugs secreted through kidney proximal tubular or altered of kidney proximal tubular function. Overall, combination therapy consisting of two or more nucleoside analogues may increase their plasma concentration, lead to a higher risk of adverse events.

Although there was no direct proof of telbivudine inducing mitochondrial disease in our study, we demonstrated that the adverse reactions were associated with telbivudine. This emphasizes that we ought to exercise caution when using telbivudine to treat hepatitis

B. First of all, practitioners need to be aware of possible adverse reactions, and inform the patient before prescription. Secondly, if telbivudine is prescribed, practitioners need to pay close attention to relevant symptoms and physical signs. Lastly, once the symptoms have been observed, an immediate medical response should be initiated. Diagnosis should be made as soon as possible, and drug therapy discontinued if necessary. Only in this way can telbivudine be used safely and effectively in clinical practice.

## COMMENTS

### Background

Telbivudine is a new synthetic nucleoside analogue. Since it came on the market in October, 2006, it has been a new option for clinicians to treat chronic hepatitis B, because it significantly suppresses replication of hepatitis B virus (HBV). There has been no special report about telbivudine-related adverse reactions to date, except in clinical trials.

### Research frontiers

According to the results of clinical trials, myopathy, one of the telbivudine-associated adverse reactions, was found in 3/680 patients.

### Innovations and breakthroughs

In the present study, the occurrence ratio of telbivudine-related myopathy was approximately 1:20 (5:105), which is different significantly different from the above result.

### Applications

The study reminds us of the importance of adverse reactions when using telbivudine to treat hepatitis B. Once the symptoms are observed, doctors can diagnose the condition and initiate an immediate medical response as soon as possible, so as to relieve pain.

### Terminology

Nucleoside analogues are synthetic nucleosides, which are phosphorylated in host cells, then incorporated into virus DNA instead of the natural nucleoside. Once inserted, they cause virus DNA chain termination, and therefore, inhibit virus replication. Nucleoside analogues can inhibit mtDNA polymerase- $\gamma$ , and then interfere with energy transduction in mitochondria.

### Peer review

The interest of the study is in the description of telbivudine-related adverse reactions during clinical practice, which may be different from those reported during clinical trials characterized by strict patient selection.

## REFERENCES

- Bryant ML, Bridges EG, Placidi L, Faraj A, Loi AG, Pierra C, Dukhan D, Gosselin G, Imbach JL, Hernandez B, Juodawlkis A, Tennant B, Korba B, Cote P, Marion P, Cretton-Scott E, Schinazi RF, Sommadossi JP. Antiviral L-nucleosides specific for hepatitis B virus infection. *Antimicrob Agents Chemother* 2001; **45**: 229-235
- Liang YL, Huang CX. New drug for Chronic Hepatitis B-telbivudine (review). *Zhongnan Yaoxue* 2007; **5**: 285-287
- Mondelli M. [New treatment options in chronic hepatitis B]. *Infez Med* 2008; **16**: 5-14
- Borgia G, Gentile I. Treating chronic hepatitis B: today and tomorrow. *Curr Med Chem* 2006; **13**: 2839-2855
- Hou J, Yin YK, Xu D, Tan D, Niu J, Zhou X, Wang Y, Zhu L, He Y, Ren H, Wan M, Chen C, Wu S, Chen Y, Xu J, Wang Q, Wei L, Chao G, Constance BF, Harb G, Brown NA, Jia J. Telbivudine versus lamivudine in Chinese patients with chronic hepatitis B: Results at 1 year of a randomized, double-blind trial. *Hepatology* 2008; **47**: 447-454
- Lai CL, Gane E, Liaw YF, Hsu CW, Thongsawat S, Wang Y, Chen Y, Heathcote EJ, Rasenack J, Bzowej N, Naoumov NV, Di Bisceglie AM, Zeuzem S, Moon YM, Goodman Z, Chao G, Constance BF, Brown NA. Telbivudine versus lamivudine in patients with chronic hepatitis B. *N Engl J Med* 2007; **357**: 2576-2588
- Lai CL, Leung N, Teo EK, Tong M, Wong F, Hann HW, Han S, Poyndar T, Myers M, Chao G, Lloyd D, Brown NA. A 1-year trial of telbivudine, lamivudine, and the combination in patients with hepatitis B e antigen-positive chronic hepatitis B. *Gastroenterology* 2005; **129**: 528-536
- Chan HL, Heathcote EJ, Marcellin P, Lai CL, Cho M, Moon YM, Chao YC, Myers RP, Minuk GY, Jeffers L, Sievert W, Bzowej N, Harb G, Kaiser R, Qiao XJ, Brown NA. Treatment of hepatitis B e antigen positive chronic hepatitis with telbivudine or adefovir: a randomized trial. *Ann Intern Med* 2007; **147**: 745-754
- Matthews SJ. Telbivudine for the management of chronic hepatitis B virus infection. *Clin Ther* 2007; **29**: 2635-2653
- Zhang HF. Mitochondria disease (lecture). *Ningxia Yixue Zazhi* 2003; **9**: 574-575
- Jackson MJ, Schaefer JA, Johnson MA, Morris AA, Turnbull DM, Bindoff LA. Presentation and clinical investigation of mitochondrial respiratory chain disease. A study of 51 patients. *Brain* 1995; **118** ( Pt 2): 339-357
- Servidei S, Zeviani M, Manfredi G, Ricci E, Silvestri G, Bertini E, Gellera C, Di Mauro S, Di Donato S, Tonali P. Dominantly inherited mitochondrial myopathy with multiple deletions of mitochondrial DNA: clinical, morphologic, and biochemical studies. *Neurology* 1991; **41**: 1053-1059
- Hammans SR, Sweeney MG, Brockington M, Lennox GG, Lawton NF, Kennedy CR, Morgan-Hughes JA, Harding AE. The mitochondrial DNA transfer RNA(Lys)A-->G(8344) mutation and the syndrome of myoclonic epilepsy with ragged red fibres (MERRF). Relationship of clinical phenotype to proportion of mutant mitochondrial DNA. *Brain* 1993; **116** ( Pt 3): 617-632
- Huoponen K, Vilkkii J, Aula P, Nikoskelainen EK, Savontaus ML. A new mtDNA mutation associated with Leber hereditary optic neuroretinopathy. *Am J Hum Genet* 1991; **48**: 1147-1153
- Sherratt EJ, Thomas AW, Alcolado JC. Mitochondrial DNA defects: a widening clinical spectrum of disorders. *Clin Sci (Lond)* 1997; **92**: 225-235
- Wang YJ. The development of the therapy in mitochondria disease. *Guowai Yixue Shenjingbingxue Shenjing Waikexue Shouce* 1996; **23**: 191-193
- Chen QT, Wu LJ, Wu QZ, Yuan Y, Jia Z, Zhang QR, Gao H, Shi ZH. Primary mitochondrial myopathy and encephalomyopathy (A report of 53 cases). *Zhongguo Shenjing Jingshen Jibing Zazhi* 1994; **20**: 16-18
- Fu ZX, Zhao D. The analysis of 25 cases of mitochondria disease. *Henan Yike Daxue Xuebao* 2000; **35**: 451
- Deng AP. Efficacy and safety of entecavir in treatment of Chronic Hepatitis B. *Zhongguo Xinyao Yu Linchuang Zazhi* 2005; **4**: 326-329
- Ray AS, Feng JY, Murakami E, Chu CK, Schinazi RF, Anderson KS. Interaction of 2'-deoxyguanosine triphosphate analogue inhibitors of HIV reverse transcriptase with human mitochondrial DNA polymerase gamma. *Antivir Chem Chemother* 2007; **18**: 25-33
- Feng JY, Murakami E, Zorca SM, Johnson AA, Johnson KA, Schinazi RF, Furman PA, Anderson KS. Relationship between antiviral activity and host toxicity: comparison of the incorporation efficiencies of 2',3'-dideoxy-5-fluoro-3'-thiacytidine-triphosphate analogs by human immunodeficiency virus type 1 reverse transcriptase and human mitochondrial DNA polymerase. *Antimicrob Agents Chemother* 2004; **48**: 1300-1306
- Johnson AA, Ray AS, Hanes J, Suo Z, Colacino JM, Anderson KS, Johnson KA. Toxicity of antiviral nucleoside analogs and the human mitochondrial DNA polymerase. *J Biol Chem* 2001; **276**: 40847-40857
- Gu CH, Luo KX. Hepatitis B Basic Biology and Clinical



- Science. 2th ed. In: Anti-virus therapy: Nucleoside analogues, Peking: People's Medical Publishing House, 2000: 396-400
- 24 **Jiang J**, Hu P, Wang HY, Shen K, Brown NA, Zhou XJ. [Study on the pharmacokinetic profile of telbivudine] *Zhonghua Ganzangbing Zazhi* 2006; **14**: 811-813
- 25 **Zhou XJ**, Fielman BA, Lloyd DM, Chao GC, Brown NA. Pharmacokinetics of telbivudine in healthy subjects and absence of drug interaction with lamivudine or adefovir dipivoxil. *Antimicrob Agents Chemother* 2006; **50**: 2309-2315
- 26 **Zhou XJ**, Lloyd DM, Chao GC, Brown NA. Absence of food effect on the pharmacokinetics of telbivudine following oral administration in healthy subjects. *J Clin Pharmacol* 2006; **46**: 275-281
- 27 **Zhou XJ**, Lim SG, Lloyd DM, Chao GC, Brown NA, Lai CL. Pharmacokinetics of telbivudine following oral administration of escalating single and multiple doses in patients with chronic hepatitis B virus infection: pharmacodynamic implications. *Antimicrob Agents Chemother* 2006; **50**: 874-879
- 28 **Zerbini A**, Pilli M, Boni C, Fisicaro P, Penna A, Di Vincenzo P, Giuberti T, Orlandini A, Raffa G, Pollicino T, Raimondo G, Ferrari C, Missale G. The characteristics of the cell-mediated immune response identify different profiles of occult hepatitis B virus infection. *Gastroenterology* 2008; **134**: 1470-1481
- 29 **Ferreira MS**, Borges AS. [Advances in the treatment of hepatitis B] *Rev Soc Bras Med Trop* 2007; **40**: 451-462
- 30 **Marcellin P**, Lada O, Asselah T. Treatment of chronic hepatitis B with the combination of pegylated interferon with lamivudine. *Hepatol Res* 2007; **37**: S55-S61
- 31 **Merup M**, Aberg W, Löfvenberg E, Svensson E, Engman K, Paul C, Gardulf A. Symptoms, symptom distress and health-related quality of life in patients with polycythaemia vera or essential thrombocythaemia during treatment with interferon-alpha. *Acta Oncol* 2002; **41**: 50-55

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RAPID COMMUNICATION

## Preparation of chitosan-polyaspartic acid-5-fluorouracil nanoparticles and its anti-carcinoma effect on tumor growth in nude mice

Dan-Ying Zhang, Xi-Zhong Shen, Ji-Yao Wang, Ling Dong, Yong-Li Zheng, Li-Li Wu

Dan-Ying Zhang, Xi-Zhong Shen, Ji-Yao Wang, Ling Dong, Li-Li Wu, Department of Gastroenterology, Zhongshan Hospital, Shanghai Medical College, Fudan University, Shanghai 200032, China

Yong-Li Zheng, Department of Macromolecular Science, Key Laboratory of Molecular Engineering of Polymers of Chinese Ministry of Education, Fudan University, 220 Handan Road, Shanghai 200433, China

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**Author contributions:** Zhang DY and Dong L contributed equally to this work; Zhang DY, Shen XZ and Dong L designed the research; Zhang DY, Dong L and Zheng YL performed the research; Zheng YL provided new reagents/analytic tools; Zhang DY, Dong L and Wu LL analyzed data; and Zhang DY and Dong L wrote the paper.

**Correspondence to:** Ling Dong, Department of Gastroenterology, Zhongshan Hospital, Shanghai Medical College, Fudan University, Shanghai 200032, China. [dltalk@tom.com](mailto:dltalk@tom.com)

Telephone: +86-21-54231990

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formation and increase of total bilirubin, and alanine aminotransferase in the 5-Fu group, but no change in those of the other three groups. There was no change in white blood cell count and creatinine among the four groups. Pathological section of liver and nephridial tissues showed that the damage in the 5-Fu group was more severe than that in the CTS-Pasp-5Fu group. 5-Fu and CTS-Pasp-5Fu groups could both down-regulate the Bcl-2 expression and up-regulate the Bax expression to different extent, and the accommodate effect of CTS-Pasp-5Fu was more obvious than 5-Fu.

**CONCLUSION:** The tumor inhibition rate of CTS-Pasp-5Fu nanoparticles is much higher than that of 5-Fu alone.

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**Key words:** 5-Fluorouracil; Chitosan; Polyaspartic acid; Nanoparticles; Gastric carcinoma

**Peer reviewer:** Yuan Yuan, Professor, Cancer Institute of China Medical University, 155 North Nanjing Street, Heping District, Shenyang 110001, Liaoning Province, China

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### Abstract

**AIM:** To prepare chitosan-polyaspartic acid-5-fluorouracil (CTS-Pasp-5Fu) nanoparticles and investigate its anti-carcinoma effect and toxicity.

**METHODS:** CTS-Pasp-5Fu nanoparticles were synthesized by ionic gelatification. Male BABL/c nude mice were injected with SGC-7901 gastric carcinoma cell line mass to establish a human gastric carcinoma model. They were randomly allocated into 4 groups: CTS-Pasp-5Fu (containing 5-Fu 1.25 mg/kg), 5-Fu (1.25 mg/kg), CTS-Pasp and normal saline groups. Tumor weight was measured and assay of colony forming unit-granulocyte and macrophage (CFU-GM) was performed. The structural change of cells and tissues was observed and the Bax and Bcl-2 genes were detected.

**RESULTS:** Compared with normal saline, the inhibition rates of tumor growth for the CTS-Pasp, 5-Fu and CTS-Pasp-5Fu groups were 5.58%, 58.69% and 70.82%, respectively. The tumor inhibition rates for the CTS-Pasp, 5-Fu and CTS-Pasp-5Fu groups were 5.09%, 65.3% and 72.79%, respectively. There was a significant decrease in the number of CFU-GM

### INTRODUCTION

As is well known, gastric carcinoma is one of the most familiar gastrointestinal malignant tumors. 5-fluorouracil (5-Fu) is universally used as an antineoplastic agent in gastrointestinal cancer<sup>[1]</sup>; however, it has a short plasma half-life period *in vivo* (only 5-10 min). This requires us to make continuous infusion (CI) schedules of 5-Fu in order to maintain an effective concentration *in vivo*<sup>[2-3]</sup>. The novel oral fluoropyrimidine appears to offer comparable efficacy while providing a more convenient schedule<sup>[4]</sup>. Chitosan (CTS) is the second most abundant polysaccharide and a cationic polyelectrolyte present in nature. CTS has shown a favorable biocompatibility<sup>[5-6]</sup>

as well as the ability to increase membrane permeability both *in vitro*<sup>[7,8]</sup> and *in vivo*<sup>[9]</sup>, which are degraded by lysozyme in serum. CTS has received more attention in the pharmaceutical field for a wide range of drug-delivery applications<sup>[10-14]</sup>. Polyaspartic acid is a kind of newly biodegradable, innocuous and friendly environmental bio-organic polymer, recognized as a green material, and widely applied in the areas such as agriculture, medicine, commodity, water treatment, *etc.* The synthesis and application of polyaspartic acid have been studied in many companies. However, little work focused on the complex of chitosan and polyaspartic acid. Our previous study showed that chitosan can encapsulate appreciable quantities of polyaspartic acid (Pasp) into stable nanoparticles, and the method of ion gelatification for preparing chitosan-polyaspartic acid-5-fluorouracil (CTS-Pasp-5Fu) nanoparticles is stable, simple and well biocompatible. Compared with 5-Fu, the Cmax of its nanoparticles is decreased, the AUC was increased and the T1/2 is prolonged obviously. The CTS-Pasp-5Fu nanoparticles are released controllably and could overcome some disadvantages of 5-Fu<sup>[15]</sup>. The major goal of the present work was to prepare CTS-Pasp-5Fu nanoparticles and investigate its anti-carcinogenic effect and toxicity.

## MATERIALS AND METHODS

### Materials

Chitosan with a deacetylation degree (DD) of 95.3% and the molecular weight (Mw) of 6-270 kDa was purchased from Kabo Biochemical Company (Shanghai, China). Polyaspartic acid was prepared by the Department of Macromolecular Science, Key Laboratory of Molecular Engineering of Polymers of Chinese Ministry of Education, Fudan University (Shanghai) as previously described<sup>[16]</sup>. The average molecular weight of PAsp was 5.0 kDa. 5-fluorouracil was purchased from Donghai Pharmaceutical Company (Shanghai, China). The human gastric cancer cell line, SGC-7901, was serially subcultivated by the Department of Laboratory of Gastroenterology, Zhongshan Hospital. Nude male BALB/c mice (aged 35-42 d and weighing 20-23 g) and Kunming male mice (weight 5-20 g) were obtained from the Department of Laboratory Animals, Fudan University. Methyl cellulose M450 was purchased from China Medicine (group) Shanghai Chemical Reagent Corporation. Iscove's modified Dulbecco's medium was purchased from GIBCO Company (UK). Fetal bovine serum was purchased from Hangzhou Sijiqing Biological Engineering Materials Co, Ltd (Hangzhou, China). Antisubstance R-0023 was purchased from Changdao Biotechnology Ltd (Shanghai). All other chemicals were of analytical grade and used without further purification.

### Preparation of CTS-Pasp-5Fu nanoparticles

Chitosan nanoparticles were prepared as reported by Calvo *et al.*<sup>[17]</sup> (1997) based on the ionic gelation of CS with TPP anions. Briefly, chitosan was dissolved in dilute acetic acid solution (10 g/L). The concentration

of acetic acid in aqueous solution was the same as that of chitosan. Quantum satis 5-Fu was dissolved in this solution at room temperature. Afterwards, under magnetic stirring, the mixture solution of 5-Fu and CS was dropped into the Pasp solution at a rate of one drop/sec. Then, opalescent suspension was formed. The obtained suspension was filtered with a paper filter for use. Glutaraldehyde crosslinking nanoparticles were dropped in to drug-loaded CTS-Pasp suspension under magnetic stirring. This mixture was further stirred for three hours at room temperature. CTS-Pasp-5-Fu nanoparticles were separated from the aqueous suspension medium by ultra-centrifugation at 35000 r/min for 30 min at 25°C, washed by dilute acetic acid (pH 5.0) solution and separated by three times ultracentrifugation. The sample was re-dispersed.

Dynamic light scattering (DLS) (Malvern, Autoszer 4700) was used to measure the hydrodynamic diameter and size distribution<sup>[18]</sup>. DLS measurement was done with a wave length of 532 nm at 25°C with an angle detection of 90°.

The morphology and dried TEM-assessed size measurement of the CTS-Pasp-5Fu nanoparticles were examined under transmission electron microscope (TEM; Hitachi, H-600). The sample was dried at room temperature and examined using a TEM without staining.

The encapsulation efficiency and loading capacity of nanoparticles were determined by separation of nanoparticles from the aqueous medium containing non-associated 5-Fu by ultracentrifugation at 35000 r/min for 30 min at 14°C. The amount of free 5-Fu in the supernatant was measured by HPLC. HPLC was performed using a LC-4A HPLC system equipped with a LC-4A pump, and a SPD-10A UV detector (Shimadzu, Kyoto, Japan). The detective wavelength was set at 270 nm. HPLC analysis of samples was performed using a Science C18 column (4.6 × 250 nm, 5 µm, Japan) preceded by a C18 guard column (GL Science, Japan). The column temperature was maintained at 30°C. The mobile phase was a mixture of methanol/3.6% acetic acid. The flow rate was 1.0 mL/min. The 5-Fu encapsulation efficiency (EE) and the 5-Fu loading capacity (LC) of the nanoparticles were calculated as follows:

$$EE = \frac{\text{The amount of 5-Fu in the nanoparticles}}{\text{Total amount of 5-Fu}} \times 100\%$$

$$LC = \frac{\text{The amount of 5-Fu in the nanoparticles}}{\text{Total amount of nanoparticles weight}} \times 100\%$$

All measurements were performed in triplicate.

### In vitro drug release from nanoparticles

*In vitro* 5-Fu release profiles of chitosan-Pasp-5-Fu nanoparticles were determined as follows. The CTS-Pasp-5-Fu nanoparticles were separated from the aqueous suspension medium through ultra-centrifugation. CTS-Pasp-5-Fu nanoparticles and 5-Fu were re-dispersed in 4.0 mL of phosphate buffer saline (PBS), respectively, and placed into a dialysis membrane bag with a molecular weight cut-off of 10 kDa, tied and placed into 40.0 mL

PBS medium. The entire system was kept at 37°C with continuous magnetic stirring. At 15 min, 1, 4, 8, 24, 48, 96, 144 or 192 h, 3 mL release medium was removed at each time point and 3 mL fresh medium PBS solution was added into the system. The amount of 5-Fu in the release medium was evaluated by HPLC. All measurements were performed in triplicate.

#### ***In vivo release from nanoparticles***

Kunming male mice (weight 20-25 g) were randomly divided into two groups. Each group was administrated with 5-Fu and CTS-Pasp nanoparticles. The plasma concentrations of 5-Fu were evaluated by HPLC after 15 min, 1, 2, 4, 6, 8, 12, 16, 24 and 48 h to compare their concentration curves<sup>[9]</sup>.

#### ***Establishment of human gastric carcinoma model and tumor inhibition experiment***

Nude BALB/c male mice (aged 35-42 d and weighing 20-23 g) were inoculated subcutaneously near the nape with the transplanted human SGC-7901 gastric carcinoma cell line ( $1 \times 10^7$  cells per mouse). Two weeks later, the exuberantly proliferating tumor tissues were cut into 1.5 mm thick pieces and inoculated subcutaneously near nape of the 32 nude male BALB/c mice (aged 33-42 d and weighing 20-23 g) under aseptic conditions. The diameter of the tumor tissue transplanted into each nude mouse was measured with a slide caliper rule.

When the tumors grew to 100-300 mm<sup>3</sup>, the animals were randomly allocated into 4 groups with 8 mice in each group: CTS-Pasp-5Fu (containing 5-Fu 1.25 g/L), 5-Fu (1.25 g/L), chitosan-polyaspartic acid, and normal saline groups. Tumor weight was measured and the diameter measurement method was used to observe the dynamic changes in the antitumor response. Twenty-five mg/kg CTS-Pasp-5Fu and 0.025 g/kg 5-Fu were given<sup>[19]</sup> each time whereas the control group was given an equal amount of normal saline or CTS-Pasp by gastric perfusion. The formula for calculating the tumor volume (TV) is:  $TV = 1/2 \times a \times b^2$ , where a and b represent the length and width<sup>[20]</sup>.

The inhibition rate of tumor growth (IR)<sup>[20]</sup> was calculated based on the results of the measurements. IR was calculated using the following formula:  $IR = (Tc - Tt)/Tc \times 100\%$ , where Tc represents control group and Tt represents treatment group.

The tumor was weighed when the experiment was over and the tumor inhibition rates (TIR) were calculated according to the following formula:  $TIR = (1 - \text{tumor weight of treatment group} / \text{tumor weight of control group}) \times 100\%$ .

The white blood cell (WBC) count, total bilirubin (TB), alanine aminotransferase (ALT) and creatinine in the four groups were detected after 2 wk.

#### ***Assay of colony forming unit-granulocyte and macrophage (CFU-GM)***

The nude mice were killed under aseptic conditions. The mice were sterilized thoroughly with 70% alcohol and the pelt was peeled off to fully expose the hip joint. Sterile

sharp dissecting scissors were used to cut the knee joint and the femur near hip joint. The tissues were removed and bones were placed in DMEM culture solution. The ends of the bones were trimmed, a 1 mL sterile syringe needle was inserted into marrow shaft at the end of the femur and the marrow was flushed with Iscove's MDM with 2% FBS in a sterile tube. Flushing was repeated and the number of nucleated cells were counted. The bone marrow cell suspension was done at a concentration of  $2 \times 10^5$ /mL and 0.3 mL of those cells was added into 3 mL MethoCult<sup>TM</sup> medium (prepared by Department of Gastroenterology, Zhongshan Hospital, Fudan University). The tubes were thoroughly mixed and let stand for 2-3 min to allow bubbles to rise to the top. MethoCult<sup>TM</sup> of 1.1 mL was dispensed into each 35 mm dish. Cultures were placed in a water-jacket at 37°C, 5% CO<sub>2</sub>,  $\geq 95\%$  humidity for 7 d. The growth of bone marrow cell colonies ( $\geq 30$  nucleated cells constituted one colony) was observed<sup>[21]</sup>.

#### ***Pathophysiology***

The tumors, liver and nephridial tissues of the four groups were collected, fixed with 10% formalin, stained with hematoxylin and eosin, paraffin embedded and sectioned. The structural change of cells and tissues was observed. Four-grade histological denominator was used: +++, total necrosis; ++, necrosis areas exceed half of total areas; +, necrosis areas less than half of the total areas; -, no necrosis<sup>[22]</sup>.

#### ***Immunohistochemical analysis***

Tumor tissues from the four groups were paraffin embedded and shaken. The sections were deparaffinized and doused with PBS 5 minutes after quenching the endogenous peroxidase with 3% hydrogen dioxide. They were repaired twice with citric acid fluid for 15 min, added with the first antistain (Bcl-2, 1:100; Bax, 1:100) and stayed overnight at 4°C. The second antistain with HRP was incubated for 30 min at 37°C and stained with a diaminobenzidine (DAB) developer. The sections were bleached with alcohol, cleared with methyl benzene, mounted with neutral balsam and photographed (ZEISS microscope, Germany). The ratio of positive expression area to the total image area was calculated.

#### ***Statistical analysis***

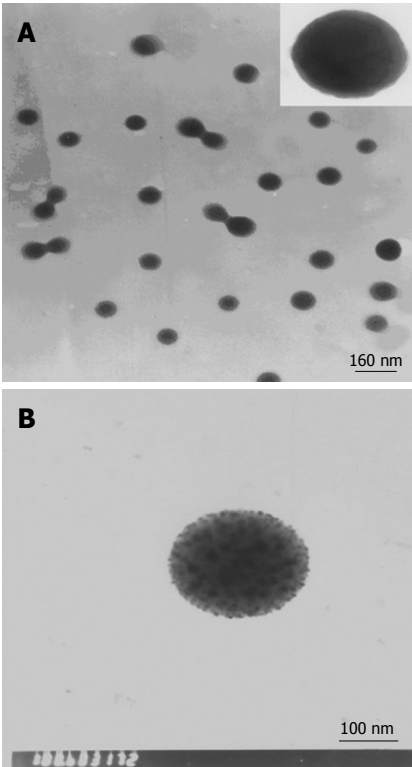
The SPSS software package (SAS Institute Inc, North Carolina, USA) was used for analysis of variances.  $P < 0.05$  was considered statistically different.

## **RESULTS**

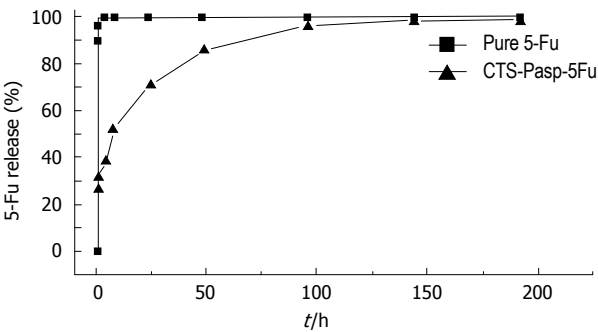
#### ***Morphology, encapsulation efficiency and loading capacity***

The mean hydrated size of particles and distribution of CTS-Pasp-5Fu nanoparticles were determined by DLS. The results indicated that the mean size and distribution of the samples were 206 nm and 0.14, respectively. The encapsulation efficiency (EE) of CTS-Pasp-5Fu was 40.2%, and the loading capacity was 34.9%. Figure 1





**Figure 1** TEM photography of CTS-Pasp-5Fu nanoparticles showing. It shows the morphological characteristics of CTS-Pasp-5Fu nanoparticles synthesized by ion gelatinification. **A:** The nanoparticles are in regular spherical shape and well distributed; **B:** The diameter of the particles is between 150-250 nm.

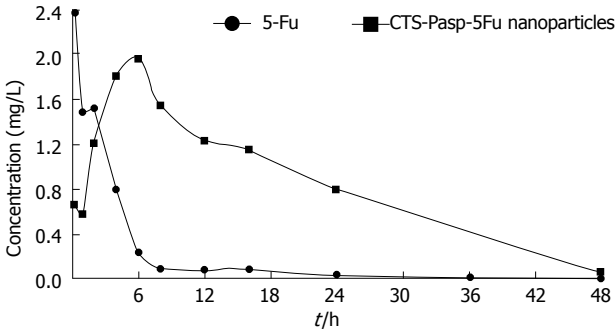


**Figure 2** Typical *in vitro* release profile of pure 5-Fu (■) and CTS-Pasp-5Fu (▲) in PBS. All pure 5-Fu escaped rapidly from the dialysis bag within the first 1 h. CTS-Pasp-5Fu nanoparticles released 5-Fu gradually up to 100% in 100 h.

shows the morphological characteristics of CTS-Pasp-5Fu nanoparticles which were synthesized by ion gelatinization. The nanoparticles were spherical in shape and well distributed. The diameter of the particles ranged from 150 to 250 nm. 5-Fu was dispersed in nanoparticles by electrostatic interaction with particulate form.

**In vitro release**

Figure 2 shows the release profile of 5-Fu and CTS-Pasp-5Fu. All the pure 5-Fu released rapidly from the dialysis bag within the first 1 h. CTS-Pasp-5Fu nanoparticles released 5-Fu gradually up to 100% in 100 h. Compared with the drug release of pure 5-Fu with 5-Fu loading CTS-



**Figure 3** The concentration-time curves of 5-Fu group and CTS-Pasp-5Fu nanoparticles group (48 h). The maximal concentration (C<sub>max</sub>) of 5-Fu group occurred within 15 min and decreased rapidly, and that of CTS-Pasp-5Fu nanoparticles group occurred 6 h after the administration and the effective concentration lasted about 14 h.

**Table 1** The mean weight in four groups (g)

Groups	Before treatment	After treatment (d)			
		3	7	10	14
NS	20.96 ± 0.82	22.35 ± 1.17	23.41 ± 1.53	24.28 ± 1.72	24.11 ± 1.73
5-Fu	22.16 ± 2.83	22.20 ± 2.86	22.58 ± 2.80	20.86 ± 3.16	18.34 ± 3.69
CTS-Pasp	20.80 ± 1.19	22.96 ± 1.34	24.45 ± 1.26	24.14 ± 1.07	24.52 ± 1.75
CTS-Pasp-5Fu	22.01 ± 2.78	23.31 ± 2.39	25.15 ± 2.15	25.46 ± 2.08	25.53 ± 1.80

Pasp nanoparticles, CTS-Pasp-5Fu nanoparticles might be used to provide a continuous release. We considered that 5-Fu embedded into the nanoparticles might be bound to PAsp by ionic reaction resulting in slow 5-Fu diffusion. 5-Fu released slowly and incompletely.

**In vivo release**

Figure 3 shows the concentration-time curves for the 5-Fu and CTS-Pasp-5Fu groups. The maximal concentration (C<sub>max</sub>) in the 5-Fu group occurred within 15 min and decreased rapidly, while that in the CTS-Pasp-5Fu group occurred 6 h after the administration and the effective concentration lasted about 14 h. The plasma C<sub>max</sub> in the CTS-Pasp-5Fu group was lower than the 5-Fu group.

**Anti-carcinoma effect of CTS-Pasp-5Fu nanoparticles on tumor growth in nude mice**

Before the treatment, the weight and tumor volume in four groups were not significantly different ( $P > 0.05$ ). After the treatment, the mice in the 5-Fu group lost weight gradually (Table 1) and became hypokinetic and were in a poor general state. The other three groups were in a good general state. At the end of the experiment, 6 of 8 nude mice survived in the 5-Fu group and no mouse died in the other three groups. The inhibition rate of tumor growth (IR) for the 5-Fu and CTS-Pasp-5Fu groups was significantly higher than that for the NS group (58.69%,  $P = 0.004$ ; 70.82%,  $P = 0.00015$ ; Table 2). The IR for the CTS-Pasp-5Fu group was also enhanced but was not significantly different from that for the 5-Fu group ( $P > 0.05$ ,  $P = 0.206$ ; Table 2). There was no difference in the IR between CTS-Pasp and NS groups

Table 2 The mean tumor volume (cm<sup>3</sup>) and inhibition rate of tumor growth (IR) in four groups

Groups	Before treatment	After treatment					
		3 d	7 d	IR	10 d	IR	14 d
NS	0.11 ± 0.03	0.19 ± 0.05	0.33 ± 0.10		0.62 ± 0.28		0.80 ± 0.23
5-Fu	0.12 ± 0.03	0.12 ± 0.04	0.24 ± 0.11	27.76%	0.31 ± 0.19	50.27%	0.33 ± 0.20
CTS-Pasp	0.12 ± 0.03	0.19 ± 0.04	0.32 ± 0.10	2.80%	0.54 ± 0.10	13.49%	0.76 ± 0.26
CTS-Pasp-5Fu	0.10 ± 0.01	0.11 ± 0.04	0.13 ± 0.05	60.76%	0.19 ± 0.05	68.99%	0.23 ± 0.07

Table 3 Tumor weight (g) and tumor inhibition rate (TIR) at the end of experiment

Groups	Mean tumor weight (g)	TIR (%)
NS	1.47 ± 0.18	-
5-Fu group	0.51 ± 0.11	65.30
CTS-Pasp group	1.40 ± 0.13	5.09
CTS-Pasp-5Fu group	0.40 ± 0.13	72.79

Table 4 WBC, TB, ALT, creatinine and cell colony culture in four groups

Groups	WBC (10 <sup>9</sup> /L)	TB (μmol/L)	ALT (U/L)	Creatinine (μmol/L)	CFU-GM (unit/plate)
NS	1.48 ± 0.84	1.24 ± 0.19	67.44 ± 19.33	27.83 ± 6.95	247 ± 18.06
5-Fu	1.42 ± 0.50	15.08 ± 4.32	145.67 ± 42.52	22.98 ± 8.33	120 ± 8.25
CTS-Pasp	1.50 ± 0.46	1.29 ± 0.67	65.43 ± 5.95	24.37 ± 4.89	241 ± 6.26
CTS-Pasp-5Fu	1.54 ± 0.23	1.26 ± 0.49	58.84 ± 4.36	22.18 ± 6.18	239.6 ± 7.40

Table 5 Histological change in four groups

Groups	Tumor number	Score of histological tumor necrosis			
		-	+	++	+++
NS	8	3	5	0	0
CTS-Pasp	8	2	6	0	0
5-Fu	6	1	4	1	0
CTS-Pasp-5Fu	8	0	4	3	1

( $P > 0.05$ ,  $P = 0.607$ ). The tumor size in the NS group was bigger than that in the 5-Fu and CTS-Pasp-5Fu groups (all  $P < 0.001$ , Table 3), and the tumor weight of CTS-Pasp and NS groups was not different ( $P > 0.05$ ). Tumor inhibition rate (TIR) for the CTS-Pasp-5Fu and 5Fu groups was significantly higher than that for the NS group (72.79% and 65.3%; Table 3). TIR for the CTS-Pasp-5Fu group was higher than that for the 5-Fu group, but the difference was not significant (Table 3, Figure 4).

Figure 4 shows all the tumors of the four groups at the end of the experiment. There were only 6 tumors left in the 5-Fu group. The tumors of those of NS and CTS-Pasp groups were obviously bigger than the other two groups. The size of tumors in the 5-Fu and CTS-Pasp-5Fu groups was not significantly different, but TIR for the CTS-Pasp-5Fu group was higher than that for the 5-Fu group (72.79% *vs* 65.3%, Table 3).

### Side effects

The white blood cell count, total bilirubin and alanine aminotransferase (ALT) in the NS, CTS-Pasp and CTS-

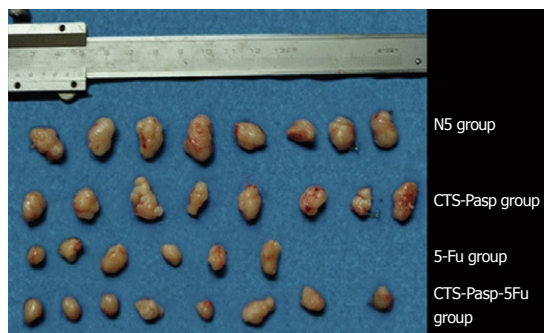


Figure 4 Tumors in four groups.

Pasp-5Fu groups were not significantly different ( $P > 0.05$ ). Those in the 5-Fu group were higher than those in the other three groups (Table 4). There was no difference in creatinine among the four groups ( $P > 0.05$ ).

The bone marrow inhibition effect in the 5-Fu group was significantly different from that in the other three groups ( $P < 0.001$ , Table 4, Figure 5).

Nucleated cells  $\geq 30$  constituted one bone marrow cell colony<sup>[16]</sup> in the four groups (Figure 5). The growth of bone marrow cell colonies in the NS, CTS-Pasp and CTS-Pasp-5Fu groups was vigorous, while that in the 5-Fu group was sparse. CTS-Pasp-5Fu did not suppress the growth of bone marrow cells.

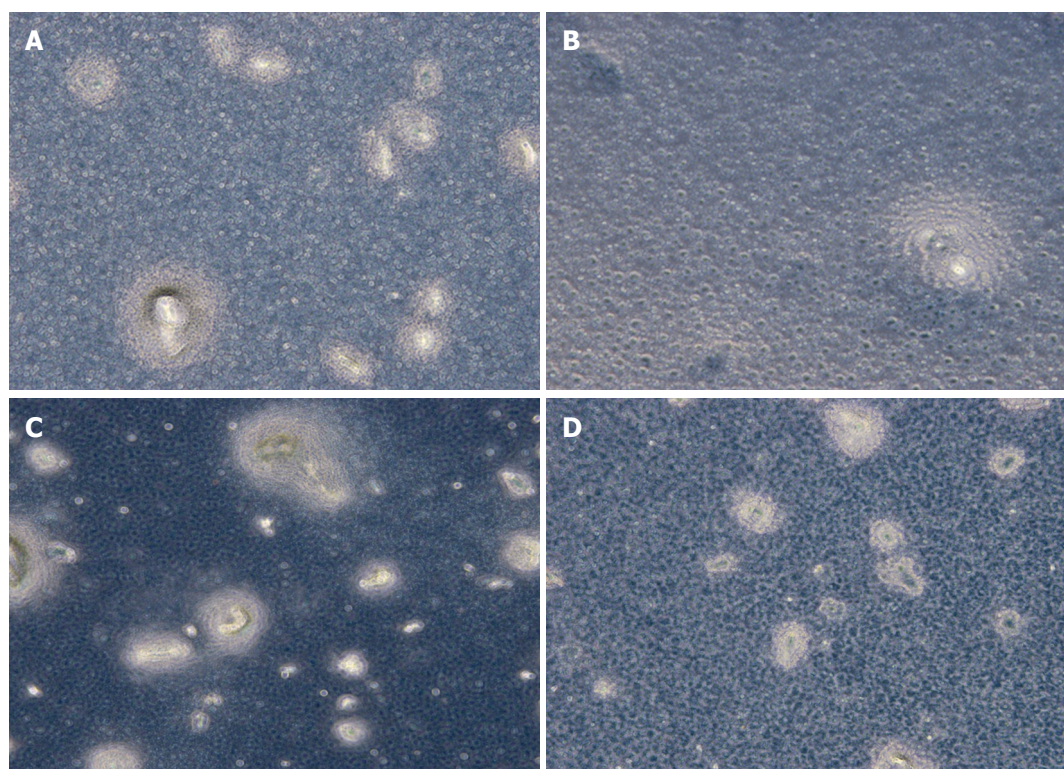
### Histological change after treatment

Table 5 shows the histological change in the four groups after treatment. The tumor cells in the NS and CTS-Pasp groups aligned regularly with slight inflammatory cell infiltration. The tumor cells in the CTS-Pasp-5Fu group were swollen with degeneration, necrosis and inflammatory cell infiltration which was less severe than in 5Fu group. The damage liver in the 5-Fu group was more severe than that in the CTS-Pasp-5Fu group.

### Immunohistochemistry

5-Fu and CTS-Pasp-5Fu down-regulate the Bcl-2 expression and up-regulation of the Bax expression were observed in the 5-Fu and CTS-Pasp-5Fu groups, but not in the CTS-Pasp group and NS group. The accommodate effect of CTS-Pasp-5Fu was more obvious than that of 5-Fu (Table 6, Figures 5 and 6).

Figure 6 shows the expression of the apoptotic proteins Bcl-2 and Bax in the four groups. The masculine expression of Bax in the CTS-Pasp-5Fu was more obvious than that in the 5-Fu group. CTS-Pasp-5Fu and 5Fu could up-regulate Bax expression. The



**Figure 5** Bone marrow cell colonies ( $\geq 30$  nucleated cells constituted one colony) in four groups ( $\times 100$ ). The growth of bone marrow cell colonies in the normal saline group (A), CTS-Pasp group (D) and CTS-Pasp-5Fu group (C) was vigorous, while the cell colonies in the 5-Fu (B) group was sparse. It demonstrated the inhibition effects of 5-Fu used alone. The marrow cell count indicated that CTS-Pasp-5Fu could not suppress the growth of bone marrow cells.

**Table 6** Expression of Bcl-2, Bax in four groups ( $n = 8$ )

Groups	Bcl-2	Bax
NS groups	$39.14 \pm 5.84$	$18.94 \pm 9.92$
CTS-Pasp	$38.62 \pm 6.53$	$18.26 \pm 7.65$
5-Fu	$31.74 \pm 5.49^a$	$33.24 \pm 4.89^a$
CTS-Pasp-5Fu	$22.74 \pm 4.55^{b,c}$	$37.22 \pm 9.21^d$

<sup>a</sup> $P < 0.05$  vs NS; <sup>c</sup> $P < 0.05$  vs 5-Fu; <sup>b</sup> $P < 0.001$  vs NS; <sup>d</sup> $P < 0.001$  vs NS group.

masculine expression of Bcl-2 in the CTS-Pasp-5Fu group was obviously lower than that in the other three groups. The difference in the expression of Bax and Bcl-2 between NS group and CTS-Pasp group was not significant (Figure 6).

## DISCUSSION

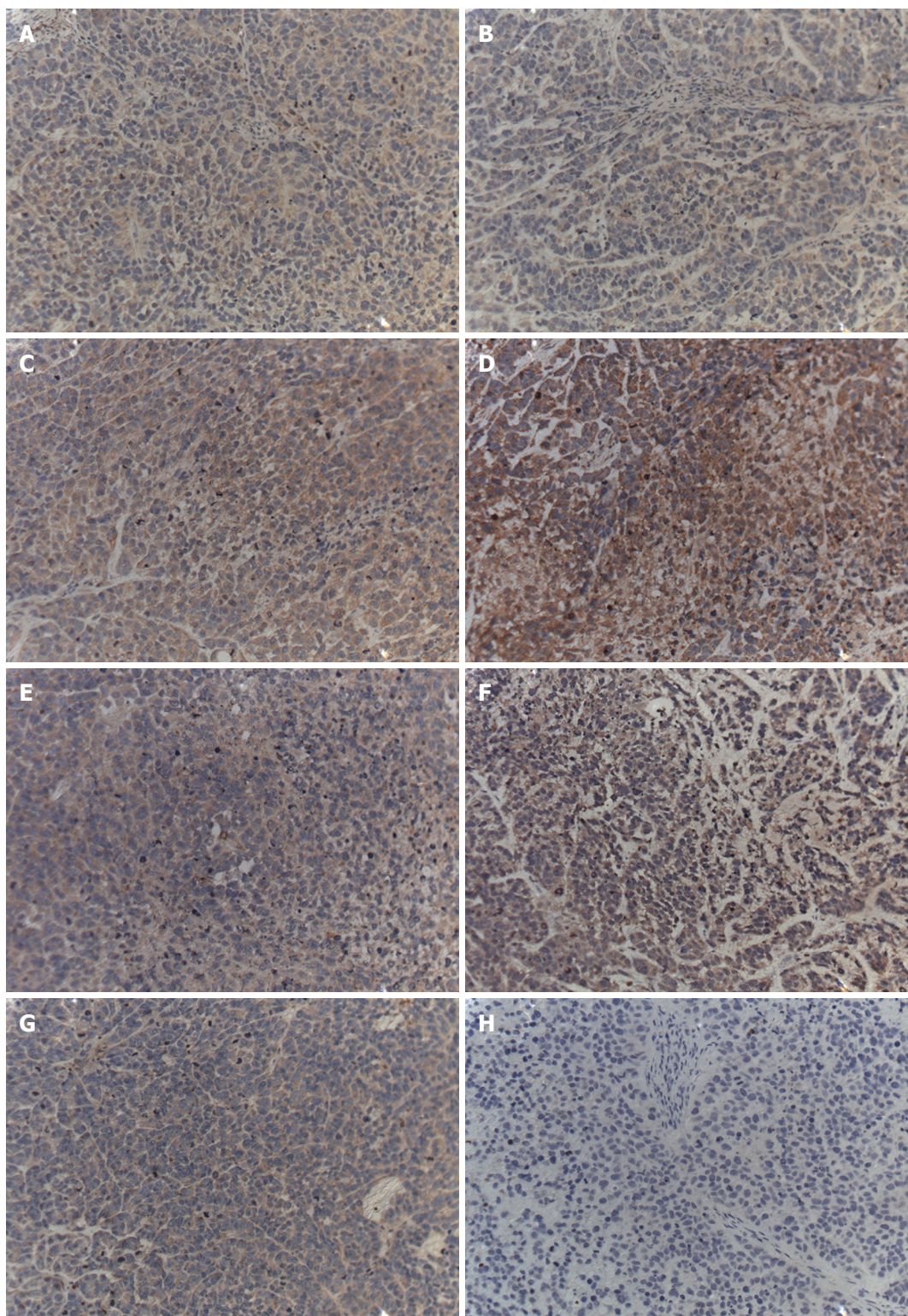
5-Fu is a thymidylate synthetase (TS) inhibitor that undermines the stability of DNA molecular structure<sup>[23]</sup>, thus it is among the top priority of consideration in chemotherapy for gastrointestinal tumors. 5-Fu is also disadvantageous in terms of irregular absorption, treatment dose, toxicity and severe side effects with high doses which result in complications by long-term intravenous administration. Consequently, the research and development of highly effective oral fluorouracil preparation or fluorouracil analogs with mild side effects has been a major topic for many medical scientists.

Biologically degradable drug-loaded nanoparticles are a novel carrier for targeted drug delivery. Its advantages include greater magnitude of drug load, controlled release, longer biological half-life, less administration time, and fewer side effects. Nanoparticles have all the

advantages of liposomes including the size property. Some investigators have also observed that the number of nanoparticles which cross the intestinal epithelium is greater than that of the microspheres ( $> 1 \mu\text{m}$ )<sup>[24]</sup>. Recently, polymer nanoparticles have been widely studied as a carrier for drug delivery. They are expected to be adsorbed in an intact form in the gastrointestinal tract after oral administration<sup>[25]</sup>. Chitosan (CTS), a poly [(1 $\rightarrow$ 4)- $\beta$ -linked 2-amino-2-deoxy-D-glucose], is prepared from chitin by N-deacetylation. It is a biologically compatible and degradable material with almost no side effects, and is receiving worldwide interest for its industrial uses as antimicrobials, biomedical materials, cosmetics, food additives, separators, sewage disposal, agricultural materials, *etc.* Nanoparticles prepared with CTS are characterized by delayed release, controlled release and targeted delivery with a higher bioavailability and fewer side effects. We have developed nanocarriers made of CS which have shown a great capacity in drug controlled release<sup>[26,27]</sup>.

Polyaspartic acid is another favorable drug carrier<sup>[28]</sup>. Polyaspartic acid or its salts are a kind of newly innocuous, biodegradable bio-organic polymer. It has been widely applied in many areas such as agriculture, medicine, commodity, water treatment, petroleum, *etc.*<sup>[29,30]</sup>. The synthesis and application of polyaspartic acid have been studied in many companies over the past years. The medical realm of polyaspartic acid has gradually caught more attention of people. Our previous studies showed that CTS-Pasp-5Fu nanoparticles prepared by ionic gelation *in vitro* and *in vivo* can delay release of 5-Fu with longer effective concentration time, and that it may render solution to the 5-Fu problems of a short half-life and more side effects brought by a higher serum concentration soon after administration.





**Figure 6** Immunohistochemical atlas of Bcl-2 and Bax expression in four groups ( $\times 200$ ). Bax expression is shown in normal saline group (A), CTS-Pasp group (B), 5-Fu group (C) and CTS-Pasp-5Fu group (D). Bcl-2 expression is shown in normal saline group (E), CTS-Pasp group (F), 5-Fu group (G) and CTS-Pasp-5Fu group (H). The masculine express of Bax in was CTS-Pasp-5Fu group was more obvious than that in the 5-Fu group. CTS-Pasp-5Fu and 5-Fu could up-regulate Bax expression compared with NS. The masculine expression of Bcl-2 in the CTS-Pasp-5Fu group was obviously lower than that in the other three groups. Both CTS-Pasp-5Fu and 5-Fu could both down-regulate the Bcl-2 expression.

We observed in our experiment that chitosan-polyaspartic acid-5fluorouracil (CTS-Pasp-5Fu) nanoparticles could slowly release 5-Fu compared to 5-Fu. The  $C_{max}$  of CTS-Pasp-5Fu nanoparticles was decreased, and the AUC was increased. The CTS-Pasp-5Fu nanoparticles were controllably released.

It is observed in the transplant nude mouse model of human gastric cancer that the inhibition rate of tumor growth (IR) and tumor inhibition rate (TIR) for the CTS-Pasp-5Fu group were significantly higher than

those for the 5-Fu group (70.82%, 58.69%, 72.79%, 65.3%), and oncopathology documented prominent degeneration and necrosis of tumor cells in the CTS-Pasp-5Fu group compare with the other three groups. These results demonstrated that the anti-tumor effects of CTS-Pasp-5Fu were better than those of 5-Fu at the same dose, and this preparation method enhanced the pharmacodynamics of the drugs.

Nude mice in the 5-Fu group seemed to be obviously emaciated, in which 2 died at the end of the study. The



total serum bilirubin and ALT in the 5-Fu group were higher than those in the other three groups, and CFU-GM values were much lower. However, biochemical examination and CFU-GM values were not significantly different between CTS-Pasp-5Fu, CTS-Pasp and NS groups, indicating that single use of 5-Fu brings about liver toxicity and bone marrow suppression. Nevertheless, there was no significant difference in liver or kidney functions and CSF-GM values of the bone marrow between CTS-Pasp-5Fu and NS groups, suggesting that chitosan-Pasp nanoparticles coated with 5-Fu bring about less bone marrow suppressing effect, and can be used as a safe and effective drug carrier.

The Bcl-2 gene family is an important regulatory factor group for apoptosis, including Bcl-2, Bcl-xl, *etc.* The ratio of Bcl-2/Bax is an important index affecting apoptosis<sup>[31]</sup>. Bcl-2 inhibits cell apoptosis by inhibiting the release of cytochrome c while Bax promotes cell apoptosis by promoting the release of cytochrome c and activates the key enzyme caspase in the process of apoptosis. Activated caspase causes tumor apoptosis by its effect on substrate<sup>[32]</sup>. The previous investigation *in vitro* showed that 5-Fu can up-regulate Bax protein expression in some tumor cells<sup>[33]</sup>. Our immunohistochemical detection of the apoptosis-related genes among four tumor groups showed that 5-Fu and CTS-Pasp-5Fu could down-regulate the Bcl-2 expression, and up-regulate Bax expression. Since the regulation effect of CTS-Pasp-5Fu nanoparticles is more powerful, it can enhance the inhibitory effect compared with 5-Fu alone, and induce the apoptosis of the gastric carcinoma.

In conclusion, in this study we successfully synthesized CTS-PASP nanoparticles coated with 5-Fu, which demonstrated significant anti-tumor effects in the transplanted nude mouse model of human gastric cancer, with less bone marrow suppression. Therefore, it may be used as a safe and effective novel anti-tumor preparation with profound prospects in clinical application.

## COMMENTS

### Background

5-Fluorouracil (5-Fu) is often used as an anti-tumor agent in the treatment of gastrointestinal cancer. However, it often causes inconvenience of patients because of its short plasma half-life and requires continuous infusion (CI) schedules. So it is important to investigate and develop new drugs which can reduce the side effects of 5-Fu.

### Research frontiers

Chitosan (CTS) has received more attention recently in the pharmaceutical field for a wide range of drug-delivery applications. Polyaspartic acid is a kind of a new biodegradable, innocuous and friendly environmental bio-organic polymer, recognized as a green material, and widely used in the areas such as agriculture, medicine, commodity, water treatment, *etc.*

### Innovations and breakthroughs

In this study, the authors successfully synthesized chitosan-polyaspartic acid-5-fluorouracil (CTS-Pasp-5Fu) nanoparticles and investigated their anti-carcinoma effect and toxicity to overcome the disadvantage of 5-Fu in gastric carcinoma mouse model. The results show that the tumor inhibition rate of CTS-Pasp-5Fu nanoparticles was much higher than that of 5-Fu alone, and the inhibition on bone marrow was alleviated efficiently. Thus, it may be used as a safe and effective novel anti-tumor preparation with profound prospects in clinical application.

### Applications

Based on the results of this study, it is supposed that CTS-Pasp-5Fu nano-

particles may substitute 5-Fu in the future treatment of gastrointestinal cancer.

## Peer review

This study focused on the preparation of CTS-Pasp-5Fu nanoparticles and investigation of its anti-carcinoma effect and toxicity to overcome the disadvantage of 5-Fu in gastric carcinoma mouse model. The data demonstrate that CTS-Pasp-5Fu appears to offer comparable efficacy while providing a more convenient schedule and showed insight into the novel oral fluoropyrimidine.

## REFERENCES

- 1 Grem JL. 5-Fluorouracil: forty-plus and still ticking. A review of its preclinical and clinical development. *Invest New Drugs* 2000; **18**: 299-313
- 2 Llorca Ferrandiz C, Esquerdo Galiana G, Cervera Grau JM, Briceo Garcia HC, Calduch Broseta JV, Del Pino Cuadrado J. [5-Fluorouracil-induced small bowel toxicity in a patient with colorectal cancer] *Clin Transl Oncol* 2005; **7**: 356-357
- 3 Saif MW. Capecitabine versus continuous-infusion 5-fluorouracil for colorectal cancer: a retrospective efficacy and safety comparison. *Clin Colorectal Cancer* 2005; **5**: 89-100
- 4 Diasio RB, Johnson MR. The role of pharmacogenetics and pharmacogenomics in cancer chemotherapy with 5-fluorouracil. *Pharmacology* 2000; **61**: 199-203
- 5 Ogawa K, Yui T, Okuyama K. Three D structures of chitosan. *Int J Biol Macromol* 2004; **34**: 1-8
- 6 Prabakaran M, Mano JF. Chitosan-based particles as controlled drug delivery systems. *Drug Deliv* 2005; **12**: 41-57
- 7 Aspden TJ, Mason JDT, Jones NS. Chitosan as a nasal delivery system: the effect of chitosan solutions on in vitro and in vivo mucociliary transport rates in human turbinates and volunteers. *J Pharm Sci* 1997; **86**: 509-513
- 8 Chornet E, Dumitriu S. Inclusion and release of proteins from polysaccharide-based polyion complexes. *Adv Drug Deliv Rev* 1998; **31**: 223-246
- 9 Takeuchi H, Yamamoto H, Niwa T, Hino T, Kawashima Y. Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes. *Pharm Res* 1996; **13**: 896-901
- 10 Park SB, You JO, Park HY, Haam SJ, Kim WS. A novel pH-sensitive membrane from chitosan-TEOS IPN; preparation and its drug permeation characteristics. *Biomaterials* 2001; **22**: 323-330
- 11 Mi FL, Wu YB, Shyu SS, Schoung JY, Huang YB, Tsai YH, Hao JY. Control of wound infections using a bilayer chitosan wound dressing with sustainable antibiotic delivery. *J Biomed Mater Res* 2002; **59**: 438-449
- 12 Shahidi F, Abuzaytoon R. Chitin, chitosan, and co-products: chemistry, production, applications, and health effects. *Adv Food Nutr Res* 2005; **49**: 93-135
- 13 Tang ES, Huang M, Lim LY. Ultrasonication of chitosan and chitosan nanoparticles. *Int J Pharm* 2003; **265**: 103-114
- 14 Janes KA, Fresneau MP, Marazuela A, Fabra A, Alonso MJ. Chitosan nanoparticles as delivery systems for doxorubicin. *J Control Release* 2001; **73**: 255-267
- 15 Wu LL, Zheng YL, Shen XZ, Fu SK, Dong L. Pharmacokinetics of Chitosan-Polyaspartic acid-5Fluorouracil Nanoparticles in vitro. *Fudan Univ J Med Sci* 2006; **33**(6): 757-760
- 16 Nakato T, Kusuno A, Kakuchi T. Synthesis of poly (succinimide) by bulk polycondensation of L-aspartic acid with an acid catalyst. *Polym Sci Pol Chem* 2000; **38**: 117-122
- 17 Calvo P, Remunan-Lopez C, Vila-Jato JL, Alonso MJ. Novel hydrophilic chitosan-polyethylene oxide nanoparticles as protein carriers. *Journal of Applied Polymer Science* 1997; **63**: 125-132
- 18 Chu B, Wang Z, Yu J. Dynamic light scattering study of internal motions of polymer coils in dilute solution. *Macromolecules* 1991; **24**: 6832-6838
- 19 Moehler M, Teufel A, Galle PR. New chemotherapeutic strategies in colorectal cancer. *Recent Results Cancer Res* 2005; **165**: 250-259
- 20 Okuno S, Harada M, Yano T, Yano S, Kiuchi S, Tsuda N, Sakamura Y, Imai J, Kawaguchi T, Tsujihara K. Complete re-

- gression of xenografted human carcinomas by camptothecin analog carboxymethyl dextran conjugate (T-0128). *Cancer Research* 2000; **60**: 2988-2995
- 21 **Kuwata T**, Wang IM, Tamura T, Ponnampertuma RM, Levine R, Holmes KL, Morse HC, De Luca LM, Ozato K. Vitamin A deficiency in mice causes a systemic expansion of myeloid cells. *Blood* 2000; **95**: 3349-3356
- 22 **Jenkins WT**, Evans SM, Koch CJ. Hypoxia and necrosis in rat 9L glioma and Morris 7777 hepatoma tumors: comparative measurements using EF5 binding and the Eppendorf needle electrode. *Int J Radiat Oncol Biol Phys* 2000; **46**: 1005-1017
- 23 **Chu E**, Drake JC, Koeller DM, Zinn S, Jamis-Dow CA, Yeh GC, Allegra CJ. Induction of thymidylate synthase associated with multidrug resistance in human breast and colon cancer cell lines. *Mol Pharmacol* 1991; **39**: 136-143
- 24 **Desai MP**, Labhasetwar V, Amidon GL, Levy RJ. Gastrointestinal uptake of biodegradable microparticles: effect of particle size. *Pharm Res* 1996; **13**: 1838-1845
- 25 **Jung T**, Kamm W, Breitenbach A, Kaiserling E, Xiao JX, Kissel T. Biodegradable nanoparticles for oral delivery of peptides: is there a role for polymers to affect mucosal uptake? *Eur J Pharm Biopharm* 2000; **50**: 147-160
- 26 **Zheng Y**, Wu Y, Yang W, Wang C, Fu S, Shen X. Preparation, characterization, and drug release in vitro of chitosan-glycyrrhetic acid nanoparticles. *J Pharm Sci* 2006; **95**: 181-191
- 27 **Wu Y**, Yang WL, Wang CC, Hu JH, Fu SK. Chitosan nanoparticles as a novel delivery system for ammonium glycyrrhizinate. *Int J Pharm* 2005; **295**: 235-245
- 28 **Tauro JR**, Gemeinhart RA. Development of amine-containing polymeric particles. *J Biomater Sci Polym Ed* 2005; **16**: 1233-1244
- 29 **Ehtezazi T**, Govender T, Stolnik S. Hydrogen bonding and electrostatic interaction contributions to the interaction of a cationic drug with polyaspartic acid. *Pharm Res* 2000; **17**: 871-878
- 30 **Jiang HL**, Zhu KJ. Comparison of poly(aspartic acid) hydrogel and poly(aspartic acid)/gelatin complex for entrapment and pH-sensitive release of protein drugs. *J Appl Polymer Sci* 2006; **9**: 2320-2329
- 31 **Mirjolef JF**, Barberi-Heyob M, Didelot C, Peyrat JP, Abecassis J, Millon R, Merlin JL. Bcl-2/Bax protein ratio predicts 5-fluorouracil sensitivity independently of p53 status. *Br J Cancer* 2000; **83**: 1380-1386
- 32 **Sawa H**, Kobayashi T, Mukai K, Zhang W, Shiku H. Bax overexpression enhances cytochrome c release from mitochondria and sensitizes KATOIII gastric cancer cells to chemotherapeutic agent-induced apoptosis. *Int J Oncol* 2000; **16**: 745-749
- 33 **Kawakami K**, Tsukuda M, Mizuno H, Nishimura G, Ishii A, Hamajima K. Alteration of the Bcl-2/Bax status of head and neck cancer cell lines by chemotherapeutic agents. *Anticancer Res* 1999; **19**: 3927-3932

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## Imaging findings and transcatheter arterial chemoembolization of hepatic malignancy with right atrial embolus in 46 patients

Hong-Yan Cheng, Xiao-Yan Wang, Guo-Li Zhao, Dong Chen

Hong-Yan Cheng, Xiao-Yan Wang, Guo-Li Zhao, Dong Chen, Department of Radiology, Eastern Hepatobiliary Surgery Hospital, 225 Changhai Road, Shanghai 200438, China

**Author contributions:** Cheng HY designed the research, Cheng HY and Wang XY wrote the paper, Cheng HY, Zhao GL and Chen D performed the research and analyzed the data.

**Correspondence to:** Hong-Yan Cheng, MD, Department of Radiology, Eastern hepatobiliary Surgery Hospital, 225 Changhai Road, Shanghai 200438, China. [chengys9304@yahoo.com.cn](mailto:chengys9304@yahoo.com.cn)

Telephone: +86-21-25070861 Fax: +86-21-25070861

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**Peer reviewers:** Aydin Karabacakoglu, MD, Department of Radiology, Denizli Government Hospital, Denizli, Turkey; Akihito Tsubota, MD, Institute of Clinical Medicine and Research, Jikei University School of Medicine, Kashiwa, Chiba, Japan; Hiroshi Yoshida, MD, First Department of Surgery, Nippon Medical School, Tokyo, Japan; Dario Conte, Professor, GI Unit-IRCCS Osp. Maggiore, Milano, Italy

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### Abstract

**AIM:** To analyze the imaging findings of hepatic malignancy with right atrial (RA) embolus.

**METHODS:** Forty-six patients with an embolus in the RA were diagnosed, including 44 patients with hepatocellular carcinoma (HCC), 1 patient with cholangiocellular carcinoma and 1 patient with hepatic carcinoma metastasis. The diagnosis was confirmed by clinical examination, serum  $\alpha$ -fetoprotein and imaging. Seventeen patients underwent transcatheter arterial chemoembolization (TACE).

**RESULTS:** On enhancement computer tomography (CT) or magnetic resonance (MR) imaging, a nodular filling defect in the RA could be easily found, with a slight enhancement in the arterial phase. The coronal images of CT or MR showed the extent of lesion. Lipiodol entered the embolus after TACE, hence reducing the speed of embolus growth. There was a survival benefit for patients receiving anticancer treatment.

**CONCLUSION:** Patients with HCC, showing a filling defect of the inferior vena cava (IVC), hepatic vein (HV) and RA on images, can be diagnosed with RA embolus. Encroachment of the RA is very rare in patients with hepatic malignancies. Furthermore, a prolongation of survival time is found in those patients who underwent TACE.

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**Key words:** Carcinoma; Liver; Neoplasm; Metastasis; Embolus; Right atrium; Computed tomography; X-Ray; Magnetic resonance

### INTRODUCTION

Malignant tumors of various organs and tissues, originating from all over the body, may disseminate to the right atrium (RA) and form a nodular embolus. Current literature reports that malignant tumors, such as hepatocellular carcinoma (HCC), pancreatic carcinoma, adrenal carcinoma, testicular cancer, lymphoma, leiomyosarcoma, hysterocarcinoma, nasopharyngeal cancer, esophageal cancer, Ewing's sarcoma and Wilm's tumor can encroach on the RA<sup>[1-3]</sup>.

HCC is the most prevalent of the liver tumors, and the portal vein (PV), hepatic vein (HV), and the inferior vena cava (IVC) are often affected. Although it is rare, tumors may also intrude on the RA. This study describes the imaging findings in 46 patients with malignant hepatic tumors that formed a tumor embolus in the RA.

### MATERIALS AND METHODS

#### Patients

From August, 2001 to May, 2007, 46 patients with hepatic malignancy and RA embolus were enrolled (41 men and 5 women, aged 23-75 years; average 52.5 years). The patients were diagnosed by computed tomography (CT) and magnetic resonance (MR), laboratory values (i.e.,  $\alpha$ -fetoprotein) and clinical examination. The malignant hepatic tumors were designated as HCC ( $n = 44$ ), intrahepatic cholangiocellular carcinoma ( $n = 1$ ) and lung and liver metastasis from post-operation retroperitoneal

sarcoma ( $n = 1$ ). All these patients had encroachment of the IVC and the RA.

Two of the 46 patients underwent surgery while 17 patients had transcatheter arterial chemoembolization (TACE). TACE was performed 4 times in 4 patients, twice in 10 patients and once in 3 patients. The remaining 27 patients had serious liver function damage. These patients were not treated with anti-tumor drugs, but were given liver protective medication until discharged.

### **Imaging protocols**

CT was performed using multi-slice spiral CT (Lightspeed QX/i, GE) scanning with high-quality scanning mode, thickness 5 mm, 120 kV, 270-300 mA. The contrast medium was 100 mL Ioversol Injection 350 (Mallinckrodt, Canada), and the velocity was 3 mL/s.

The MR image (Signa Excite Twin Speed 1.5T, GE) was preformed with a T2-weighted image using RFSE+FS, TR: 5000-10000 ms, TE: 80-130 ms, ETL: 16-23; the T1-weighted image using FSPGR, TR: 175 ms, TE: 4.2 ms; and the 2D Fiesta coronal image using TR: 4.2 ms, TE: 1.5 ms. The contrast medium was 30 mL gadopentetic acid dimeglumine salt injection (Schering, Germany), and the velocity was 4 mL/s. Dynamic CT and MR images were produced with an arterial phase of 25 s, a portal phase of 60 s, and a delayed phase of 3 min.

Plate digital subtraction angiography (DSA, INNOVA 4100, GE) with 40 mL of contrast medium, and a velocity of 6 mL/s were used in TACE treatment. Angiography was initially performed on the abdominal cavity and superior mesenteric artery to determine the variant hepatic arterial supply, the blood supply type, and size, number and position of tumors in all patients. Then, the celiac axis was selected. A 5 Fr Yashiro angiographic catheter (Terumo) was advanced over a 0.035 inch guide wire (Terumo) into the desired hepatic artery branch, depending on the tumor location. Because the optimal agents or doses for TACE have not been determined,<sup>[4]</sup> the dose of anticancer drug and lipiodol (Lipiodol Ultra Fluide, Guerbet, France) was determined based on the size, number, and blood supply of the tumor, and also on the basis of practical infusion procedures during the operation<sup>[5]</sup>. Anticancer drugs used were epirubicin hydrochloride (20-40 mg), hydroxycamptothecin (OPT, 10 mg) and ultra-liquefied lipiodol (10-30 mL). Before infusion, epirubicin hydrochloride and lipiodol were fully mixed together to form an emulsion. At the end, some gel foam sponge powder was infused to decrease blood flow. Lidocaine (5 mL) was also given intra-arterially after chemoembolization for pain control.

The relationship between RA embolus and hepatic cancer was observed to determine the development pathway.

### **Follow-up of life span and growth velocity of the embolus**

Patients were monitored from the moment of discovery of the RA embolus to an occurrence of hard end-point (i.e. patients' death) or to the end of the study period

(May 30, 2007). The growth velocity of the embolus was monitored in 10 cases. In these cases the RA embolus was discovered during follow-up and the patients had no RA embolus at first examination. The relationship between the type of intrahepatic tumor and RA embolus was observed.

## **RESULTS**

### ***Intra-hepatic malignancy and external hepatic metastasis***

The types of intra-hepatic malignancy found were: huge-mass type in 37 cases (80.4%), multinodular type in 8 cases (17.4%) and single nodular type in 1 case. The location of the tumor was the right hepatic lobe in 39 cases, the left hepatic lobe in 5 cases, and between the right and left lobe in 2 cases. There were 4 cases of lung metastasis and 5 cases of abdominal lymph node metastasis.

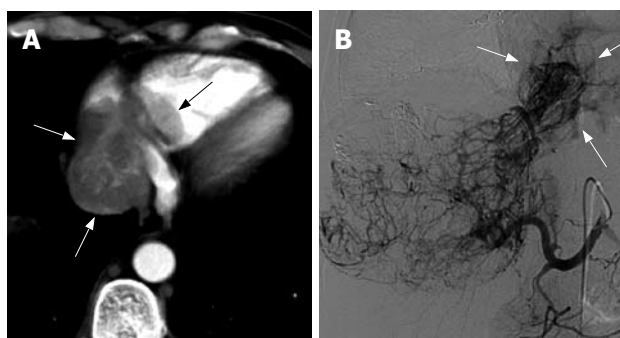
### ***Imaging findings of RA embolus***

On imaging, the RA embolus was commonly a regular, slight irregular round, orbicular-ovate, or lobulated type. The maximum size was 6.1 cm × 12 cm, almost filling the entire RA. The minimum size was 1.5 cm × 2.1 cm.

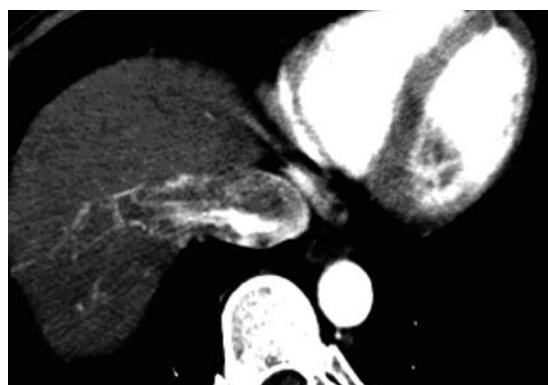
A plain CT scan showed low-density or iso-density, which corresponded to a normal cordis density. The CT value was 27-52 HU, without an obvious density difference and sometimes hardly differentiated. There was a low density line around the embolus edge. On the arterial phase scan, the contrast medium filled the cordis intracavity and the embolus showed a well-defined filling defect. On this arterial phase scan, the embolus had a slight inhomogenous "stick"-like enhancement (Figure 1A), while the tumor encroaching the IVC could be seen as a "stick"-like enhancement arterial vessel inside the IVC (Figure 2). The CT value of the embolus increased by about 30 HU following enhancement. On the portal phase scan, the contrast medium in the cordis intracavity was partially discharged, and the density of the embolus was slightly increased or decreased. The edge of the embolus was comparatively blurred. On the delay phase scan, the density of the embolus decreased, but the edge was clear; hence there was a small increase in embolus size when compared to the arterial phase scan.

The embolus showed a nodular high or iso-signal in the T1-weighted image (Figure 3A). A higher signal was found in the T2-weighted image (Figure 3B) because in this image, larger vessels, the atrium and the ventricles, have a flowing void effect. There was a low signal in the lumina and cavity of the greater vessels and cordis, while the thrombus signal was an indicator of intrahepatic HCC with a higher signal. In the arterial phase image, the thrombus was shown as a low signal filling defect with a slightly "stick"-like enhancement (Figure 3C). The portal and delay phase images showed low nodular signals. In the coronal image, the relationship between the intrahepatic tumor, IVC, and RA embolus could be seen very clearly (Figure 3D and Figure 4B).

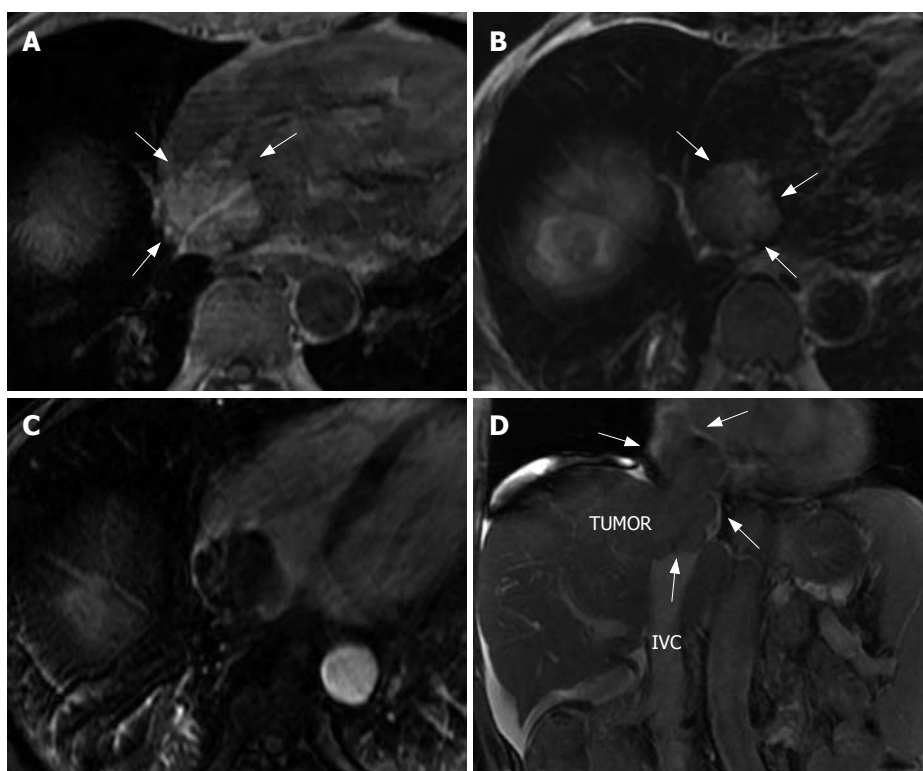




**Figure 1** A 52-year-old man with a big massive HCC located in the right lobe, the tumor encroaches the PV and IVC and intrudes into the RA. The embolus increased 5 cm within 3 mo. **A:** Arterial phase of CT scan shows a well-defined lobulated filling defect (6.7 cm x 7.5 cm) in the RA (arrow) and an irregular "stick"-like enhancement; **B:** Angiographic image in the second time TACE shows that the tumor is a hypervascular lesion and the artery enters the RA by passing the IVC, and is a "grating"-like type (arrow).



**Figure 2** A 42-year-old man with a massive HCC located in the right lobe, the tumor encroaches the HV and IVC and intrudes into the RA. Arterial phase of CT scan shows a "stick"-like enhanced arterial vessel entering the entrance of the RA.



**Figure 3** A 69-year-old man with a multinodular HCC located in the right lobe, 7 mo after the 6th TACE, embolus in the RA increased to 4 cm (**A-C** are the same patient). **A:** T1-weighted image shows a higher signal nodular (TR: 195 ms/TE: 4.2 ms, ST: 8.0 mm) in RA (arrow); **B:** T2-weighted image shows a higher signal nodular of HCC in the right diaphragmatic dome, the same well-defined higher signal nodular in the RA and a low signal around the core-cavity (TR: 7058.8 ms/TE: 89.2 ms, ST: 8.0 mm); **C:** The arterial phase image shows an embolus with low signal filling defect and a "stick"-like enhancement (TR: 190 ms/TE: 1.9 ms, ST: 8.0 mm); **D:** The coronal image shows the tumor entering into the RA via the widened IVC, the IVC lumen was almost completely filled (arrow) (TR: 3.7 ms/TE: 1.6 ms, ST: 7.0 mm).

Angiography images showed that the tumor vessel entered the RA and was a grating-like type (Figure 1B). After TACE with lipiodol perfusion, the lipiodol was retained in the HCC and the embolus and there were higher density group masses in the intra-liver area and the RA (Figure 4C). This suggested that TACE could be used successfully to treat an embolus of the HCC.

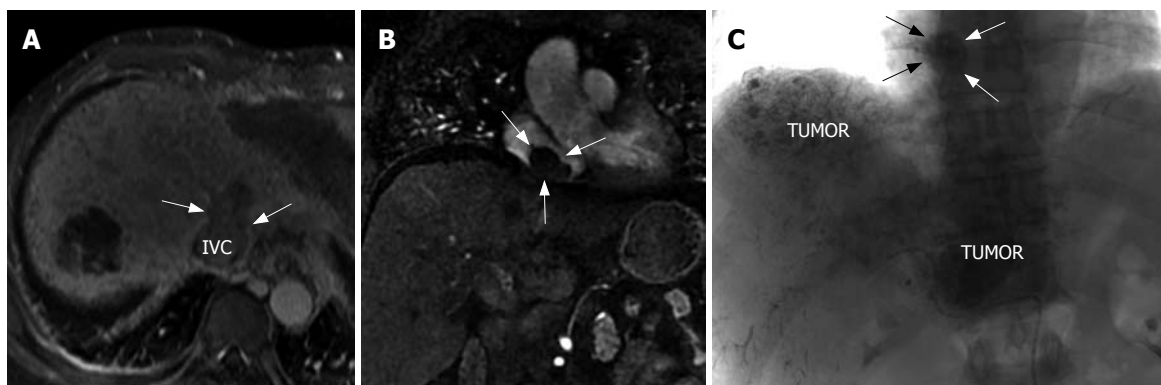
Invasion of the PV, HV, and IVC was seen in 38 cases, 11 cases, and all cases, respectively. The range of invasion of IVC varied in size in different patients and the longest invasion size was 8.3 cm. The coronal images from CT and MR showed the relationship between the intra-liver tumor, IVC and the RA embolus best (Figures 3D, 4A and B). Vein invasion was discovered by the presence of a filling defect in the vena cava. In a portal phase cross section

image there was no contrast medium in the center of the IVC cava with embolus and there was clear enhancement around the embolus, which showed up as a "ring" sign (Figure 5).

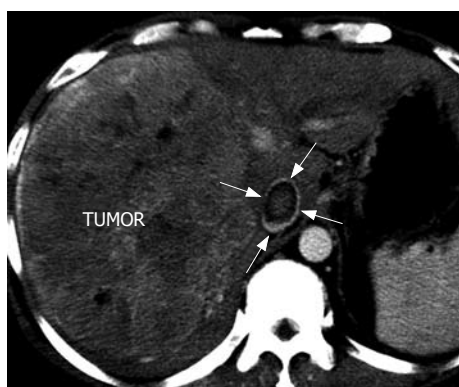
There were two routes of HCC invasion into the RA. One directly invaded into the IVC, then upward into the RA and downward into IVC. The second one was invasion into the HV, and then into the IVC and RA. The first route was more common.

#### Growth velocity of RA embolus

Thirty-six of the 46 patients had an RA embolus when imaging examination was performed, and the embolus was discovered during follow-up in 10 cases. The growth velocity of the embolus in these 10 cases was slow



**Figure 4** A 49-year-old man with multinodular recurrence after HCC operation, with the embolus of RA discovered at the same time. The patient underwent TACE twice. **A:** The portal phase T1-weighted image shows a low signal embolus entering the IVC from middle HV (arrow) (TR: 220 ms/TE: 18ms, ST: 8.0 mm); **B:** The coronal image shows a well-defined "bottle-gourd" -shape filling defect in RA (arrow) (TR: 3.3 ms/TE: 1.5 ms/TI: 7.0 ms, ST: 3.0 mm); **C:** The image after perfused Lipiodol shows a nodular and lump tumor of the right and left lobe filled with Lipiodol, which was also retained in the embolus of RA (arrow).



**Figure 5** A 46-year-old man with a large mass of HCC in the right lobe. The intra-liver tumor entered the RA by IVC. Cross sectional CT scan in portal phase shows a well-defined filling defect in IVC and a "ring"-like sign (arrow).

growth of 3 cm in 6 mo, and quick growth of 4 cm in 1 mo. The embolus may remain in steady state for a long time after TACE treatment.

### Survival time

Twenty-seven patients without anticancer treatment died after 6 mo. The shortest time to death was 1 mo, and longest time to death was 8 mo (average 3.5 mo). In 17 patients who received TACE treatment the longest survival time was 19 mo, and the shortest survival time was 4 mo (average 8.4 mo). The survival time of 2 patients who underwent surgery were 5 and 7 mo, respectively.

## DISCUSSION

About 70% patients with HCC have HV and PV invasion, but encroachment into the RA is very rare. According to the literature<sup>[3,6-10]</sup>, less than 50 cases have been reported. A combination of literature and our hospital clinical data indicates HCC encroachment into the RA may not be an overt symptom. However, when the embolus obstructs the tricuspid orifice and coronary sinus opening, it may result in a severe hemodynamic disorder. This can include venous engorgement of both

upper extremities and the chest, hydropneumothorax, pleural effusion, and flustered or even dyspneic respiration. An electrocardiogram will show right bundle branch block. When the IVC is obstructed, engorgement of the veins of the lower extremities will appear, leading quickly to Budd-Chiari syndrome<sup>[10,11]</sup>, and in this situation the prognosis of patients is very bad. Even when the HCC and embolus are surgically resected the patient may quickly die due to the severity of the pathogenetic condition or cardiac failure<sup>[12-15]</sup>.

The formation of the RA embolus had a growth process. Firstly, the tumor perforated, involving the wall of the HV and/or IVC, then the tumor extended into the lumen of the vein and grew there. The tumor within the lumen can grow upward and/or downward, and if it grows upward it may get into the RA and continue slow growth there. On the basis of overview data from 10 patients, the quickest velocity of embolus growth was 4 cm in 1 mo, and the slowest velocity of growth was 3 cm in 6 mo (average growth of 3.7 cm in 3.2 mo). Slow growth of the embolus was due to receiving TACE. TACE treatment was effective and controlled hepatic malignancy and tumor embolus.

Using the plain CT scan, the density difference between the embolus and cardiac tissue was not obvious, but sometimes there was a low density single band appearance between them. When this appearance is found, we should distinguish between normal interventricular septum, interatrial septum and tumor in the cordis as the location of each was not the same.

In the arterial phase scan, the malignant embolus was mildly enhanced. The heart chambers were filled with a high concentration of contrast medium whereas the embolus showed a filling defect so that there was a very significant contrast and a clear density difference. The arterial phase scan produced an image showing the embolus. In the portal phase scan, the embolus continued to display slight enhancement. In the delay phase, most of the contrast medium in the heart chambers was discharged, but the density of the embolus was still lower than that of cardiac tissue. In this phase, the size of the embolus was increased

compared with the arterial phase. This was because the high density contrast medium submerged part of the edge of the embolus in the arterial phase.

CT and MR images can display and distinguish between a tumor, thrombi, and a malignant embolus<sup>[16]</sup>. The malignant embolus was round, orbicular-ovate or cauliflower in shape. The embolus was part of the arterial blood supply. On angiography, a lot of blood vessels and signs of “stria” are seen with a hypervascular tumor, but there is no enhancement or staining for hypovascular tumors. The thrombus was not enhanced when it has lower density and there was no intrahepatic malignant tumor. Cardiac tumors can be divided into 2 types: primary tumor and metastasis. The most common is the primary myxoma; 93.5% of them are located in the left atrium and 6% are located in the RA. A myxoma can activate and the stem can cohere on the atrial septum. But when the malignant embolus was solid and was not transfigured, the stem cohered on the IVC.

MR images showed the embolus better than CT as MR showed it from different direction of view.

The PV, IVC and HV may be encroached by HCC, and show a filling defect of the venous lumen. Most patients with HCC had PV encroachment. IVC encroachment was relatively rare. The tumor encroached from the IVC to the RA and formed an embolus. When the tumor grew in the IVC the image showed a prismatic filling defect. The vessel wall was not destroyed unless the tumor encroached directly upon the wall. There was a gap between the embolus and vessel wall and the contrast medium flowed through the gap. This gap was shown as a high density ring-shape with low density in the center on the arterial phase scan, like a “ring”. Tumors in the right hepatic lobe usual encroach upon the hepatic vein and directly affect the IVC, and tumors in the left lobe firstly encroach upon the left hepatic vein and middle hepatic vein, get into IVC, then re-enter the RA.

Patients who could not take TACE generally had PV embolus, pulmonary metastasis, retroperitoneal lymph node metastasis, medium dose or high dose ascites, and a grade C Child-Pugh hepatic function classification. Patients could take TACE treatment if they had class A or B hepatic function and if they could tolerate TACE treatment. The tumor could be controlled for a certain time and to a certain degree after TACE treatment, and the survival time of patients could be prolonged<sup>[7,17-19]</sup>.

CT and MR images can exhibit and distinguish between tumor, thrombi and malignant embolus<sup>[16]</sup>. The malignant embolus was round, orbicular-ovate or cauliflower shape. The embolus was arterial blood supply, on the angiography, it may show a lot of blood vessel and “stria” sign for hypervascular tumor, but for hypovascular tumor it showed no enhancement and stain. The thrombus was no enhancement with more low density and no intrahepatic malignant tumor.

and form a nodular embolus. Hepatocellular carcinoma (HCC) is the most prevalent of the liver tumors, and the portal vein (PV), hepatic vein (HV) and the inferior vena cava (IVC) are often affected. Although rare, the RA may show signs of tumor intrusion.

### Research frontiers

Nodular filling defects in the RA could be easily found on enhancement computer tomography (CT) or magnetic resonance (MR) imaging. The coronal images of CT or MR show the extent of the lesion. Lipiodol can enter the embolus after transcatheter arterial chemoembolization (TACE) and reduce the speed of embolus growth.

### Innovations and breakthroughs

Lipiodol can enter the embolus after TACE and reduce the speed of embolus growth. There was a survival benefit associated with anticancer treatment.

### Applications

CT and MR images can exhibit and distinguish between the tumor, thrombi and a malignant embolus. MR images showed the embolus better than CT because MR can show it from a different direction of view.

### Terminology

TACE: One of most commonly used technologies for liver cancer therapy. The chemotherapeutic agents and embolic agents (and/or particles) were delivered via selective catheter placement to the tumor(s) feeding vessels in an attempt to achieve cytoreduction by enabling more focused drug delivery or deposition of a higher concentration of drug within the liver cancer.

### Peer review

This study reported imaging characteristics of an RA tumor embolus in patients with advanced HCC. It arouses interest for readers and provides an important clue for diagnosing and treating patients with tumor emboli.

## REFERENCES

- 1 **Cheng HY**, Xu AM, Chen D, Xu W, Jia YC. Multi-slice helical CT findings of tumor thrombus in the right atrium from hepatocellular carcinoma. *Zhonghua Fangshexue Zazhi* 2003; **37**: 989-991
- 2 **Nokamura K**, Kohmoto T, Kawada M, Shimizu S, Kuroko Y, Kawabata T, Sano S. [Left atrial thrombus in an 80-year-old woman scheduled to have an operation for uterus cancer; report of a case] *Kyobu Geka* 2005; **58**: 838-840
- 3 **Miyazawa M**, Torii T, Asano H, Yamada M, Toshimitsu Y, Shinozuka N, Koyama I. Does a surgery for hepatocellular carcinoma with tumor thrombus highly occupying in the right atrium have significance? A case report and review of the literature. *Hepatogastroenterology* 2005; **52**: 212-216
- 4 **Reidy DL**, Schwartz JD. Therapy for unresectable hepatocellular carcinoma: review of the randomized clinical trials-I: hepatic arterial embolization and embolization-based therapies in unresectable hepatocellular carcinoma. *Anticancer Drugs* 2004; **15**: 427-437
- 5 **Cheng HY**, Xu AM, Chen D, Jia YC. [Adjustment of lipiodol dose according to tumor blood supply during the transcatheter arterial chemoembolization for large hepatic carcinoma by multidetector helical CT] *Zhonghua Zhongliu Zazhi* 2003; **25**: 186-189
- 6 **Vauthey JN**, Lauwers GY, Esnaola NF, Do KA, Belghiti J, Mirza N, Curley SA, Ellis LM, Regimbeau JM, Rashid A, Cleary KR, Nagorney DM. Simplified staging for hepatocellular carcinoma. *J Clin Oncol* 2002; **20**: 1527-1536
- 7 **Wallace MJ**. Transatrial stent placement for treatment of inferior vena cava obstruction secondary to extension of intracardiac tumor thrombus from hepatocellular carcinoma. *J Vasc Interv Radiol* 2003; **14**: 1339-1343
- 8 **Wilson K**, Guardino J, Shapira O. Pulmonary tumor embolism as a presenting feature of cavoatrial hepatocellular carcinoma. *Chest* 2001; **119**: 657-658
- 9 **Yogita S**, Tashiro S, Harada M, Kitagawa T, Kato I. Hepatocellular carcinoma with extension into the right atrium: report of a successful liver resection by hepatic vascular exclusion using cardiopulmonary bypass. *J Med Invest* 2000; **47**: 155-160
- 10 **Wu CC**, Hsieh S, Ho WM, Tang JS, Liu TJ, P'eng FK.

## COMMENTS

### Background

Malignant tumors of various organs may disseminate to the right atrium (RA)

- Surgical treatment for recurrent hepatocellular carcinoma with tumor thrombi in right atrium: using cardiopulmonary bypass and deep hypothermic circulatory arrest. *J Surg Oncol* 2000; **74**: 227-231
- 11 **Saisse J**, Hardwigsen J, Castellani P, Caus T, Le Treut YP. Budd-Chiari syndrome secondary to intracardiac extension of hepatocellular carcinoma. Two cases treated by radical resection. *Hepatogastroenterology* 2001; **48**: 836-839
- 12 **Okuda K**. Natural history of hepatocellular carcinoma including fibrolamellar and hepato-cholangiocarcinoma variants. *J Gastroenterol Hepatol* 2002; **17**: 401-405
- 13 **Nonami T**, Nakao A, Harada A, Kaneko T, Kurokawa T, Takagi H. Hepatic resection for hepatocellular carcinoma with a tumor thrombus extending to inferior vena cava. *Hepatogastroenterology* 1997; **44**: 798-802
- 14 **Zaczek M**, Franczyk M, Mikulski A. [Right atrial and right ventricular thrombus in a patient with hepatic carcinoma - a case report] *Kardiologia Pol* 2003; **59**: 321-324
- 15 **Valera JM**, Merino R, Palavecino P, Sepulveda L, Smok G, Fernandez M, Brahm J. [Hepatocellular carcinoma with cardiovascular invasion. Report of five cases] *Rev Med Chil* 2004; **132**: 1517-1522
- 16 **Krombach GA**, Spuentrup E, Buecker A, Mahnken AH, Katoh M, Temur Y, Higgins CB, Gunther RW. [Heart tumors: magnetic resonance imaging and multislice spiral CT] *Rofo* 2005; **177**: 1205-1218
- 17 **Izaki K**, Matsumoto S, Konishi J, Higashino T, Tsurusaki M, Fukuda T, Akasaka Y, Mori T, Sugimoto K, Fujii M, Sugimura K, Yoshikawa T, Hirota S, Hayashi Y. [Temporary placement of inferior vena cava filter prior to transcatheter arterial embolization (TAE) for hepatocellular carcinoma with IVC tumor thrombus--prevention of pulmonary tumor emboli after TAE] *Gan To Kagaku Ryoho* 2001; **28**: 1708-1711
- 18 **Chang JY**, Ka WS, Chao TY, Liu TW, Chuang TR, Chen LT. Hepatocellular carcinoma with intra-atrial tumor thrombi. A report of three cases responsive to thalidomide treatment and literature review. *Oncology* 2004; **67**: 320-326
- 19 **Yogita S**, Tashiro S, Harada M, Kitagawa T, Kato I. Hepatocellular carcinoma with extension into the right atrium: report of a successful liver resection by hepatic vascular exclusion using cardiopulmonary bypass. *J Med Invest* 2000; **47**: 155-160

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## Preventive effect of *Qianggan-Rongxian* Decoction on rat liver fibrosis

Chun-Hui Li, Li-Hui Pan, Zong-Wei Yang, Chun-Yu Li, Wen-Xie Xu

Chun-Hui Li, Zong-Wei Yang, Department of Pathology, Affiliated Hospital of Chengde Medical College, Chengde 067000, Hebei Province, China

Li-Hui Pan, Chun-Yu Li, Chengde Medical College, Chengde 067000, Hebei Province, China

Wen-Xie Xu, Department of Physiology, College of Medicine, Shanghai Jiaotong University, Shanghai 200030, China

**Author contributions:** Li CH designed the research; Pan LH, Yang ZW, Li CY and Xu WX performed the research; Li CY and Xu WX provided the analytic tools; Li CH wrote the paper. Supported by the Research Program of Department of Science and Technology of Hebei Province, No. 200721047

**Correspondence to:** Chun-Hui Li, Department of Pathology, Affiliated Hospital of Chengde Medical College, Chengde 067000, Hebei Province, China. [chli612@yahoo.com.cn](mailto:chli612@yahoo.com.cn)

Telephone: +86-314-2279447 Fax: +86-314-2270251

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### Abstract

**AIM:** To study the preventive effects of *Qianggan-Rongxian* Decoction on liver fibrosis induced by dimethylnitrosamine (DMN) in rats.

**METHODS:** Male Wistar rats were randomly divided into hepatic fibrosis model group, control group and 3 treatment groups (12 rats in each group). Except for the normal control group, all the rats received 1% DMN (10  $\mu$ L/kg body weight, i.p), 3 times a week for 4 wk. The rats in the 3 treatment groups including a high-dose DMN group (10 mL/kg), a medium-dose DMN group (7 mL/kg), and a low-dose DMN group (4 mL/kg) were daily gavaged with *Qianggan-Rongxian* Decoction, and the rats in the model and normal control groups were given saline vehicle. Enzyme-linked immunosorbent assay (ELISA) was used to determine the changes in serum hyaluronic acid (HA), laminin (LN), and type IV collagen levels. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using routine laboratory methods. Pathologic changes, particularly fibrosis, were examined by hematoxylin and eosin (HE) and Sirius red staining. Hepatic stellate cells (HSC) were examined by transmission electron microscopy.

**RESULTS:** Compared with the model control group, the serum levels of HA, LN, type IV collagen, ALT and AST were decreased markedly in the other groups after treatment with *Qianggan-Rongxian* Decoction, especially in the medium-dose DMN group ( $P < 0.05$ ).

Moreover, the area-density percentage of collagen fibrosis was lower in the *Qianggan-Rongxian* Decoction treatment groups than in the model group, and a more significant drop was observed in the medium-dose DMN group ( $P < 0.05$ ).

**CONCLUSION:** *Qianggan-Rongxian* Decoction can inhibit hepatic fibrosis due to chronic liver injury, delay the development of cirrhosis, and notably ameliorate liver function. It may be used as a safe and effective therapeutic drug for patients with fibrosis.

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**Key words:** Liver fibrosis; *Qianggan-Rongxian* Decoction; Prevention; Rat model; Dimethylnitrosamine

**Peer reviewers:** James Neuberger, Professor, Liver Unit, Queen Elizabeth Hospital, Birmingham B15 2TH, United Kingdom; Dr. Yukihiro Shimizu, Kyoto Katsura Hospital, 17 Yamada-Hirao, Nishikyo, Kyoto 615-8256, Japan

Li CH, Pan LH, Yang ZW, Li CY, Xu WX. Preventive effect of *Qianggan-Rongxian* Decoction on rat liver fibrosis. *World J Gastroenterol* 2008; 14(22): 3569-3573 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3569.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3569>

### INTRODUCTION

In China, the incidence of liver cirrhosis is still high<sup>[1]</sup>. Hepatic cirrhosis results from fibrosis<sup>[2-4]</sup>. Many factors can lead to chronic liver disease and hepatic fibrosis<sup>[5-9]</sup>. Hepatic fibrosis is associated with a number of morphological and biochemical changes leading to structural and metabolic abnormalities in the liver. Hepatic stellate cells (HSC) play a major role in various types of liver fibrosis through initial myofibroblast transformation. Although new therapeutic approaches have recently been proposed, there is no established therapy for liver fibrosis<sup>[10,11]</sup>. *Qianggan-Rongxian* Decoction is a traditional Chinese medicine. The aim of the present study was to investigate its protective effects on rat liver fibrosis induced by dimethylnitrosamine (DMN).

### MATERIALS AND METHODS

#### Composition of *Qianggan-Rongxian* Decoction

The compositions of *Qianggan-Rongxian* Decoction

mainly include 13 Chinese herbs, including 15 g *Pig Bile powder*, 10 g *Bupleuri*, 10 g *Baical Skullcap Root*, 10 g *Pinellia Tuber*, 10 g *Chinese Angelica*, 10 g *Barbary Wolfberry Fruit*, 10 g *Nutgrass Galingale*, 10 g *Oriental Waterplantain Rhizome*, 3 g *Pangolin Scale*, 153 g *Danshen Root*, 10 g *White Peony Alba*, 10 g *Radix Glycyrrhizae*, 15 g *Tangshen*.

### Animals and experiment protocol

Male Wistar rats weighing 175-200 g were obtained from the Experimental Animal Center of Chengde Medical College. The rats were randomly divided into control group, model group, and 3 treatment groups (12 rats in each group). Except for the normal control group, all the rats were abdominally injected with 1% DMN (10  $\mu$ L/kg body weight, i.p.), 3 times a week for 4 wk, while the rats in the control group were abdominally injected with an equivalent amount of saline. The rats in the 3 treatment groups, including a high-dose DMN group (10 mL/kg), a medium-dose DMN group (7 mL/kg), and a low-dose DMN group (4 mL/kg), were given *Qianggan-Rongxian* Decoction daily via a gastric tube, once a day for 4 wk. After 4 wk, except for the dead, all the rats were anesthetized with 200 g/L urethane (5 mL/kg, abdominal injection). Blood was taken from the abdominal aorta, centrifuged at 4°C, and plasma was kept at -20°C for assay.

### Measurement of serum levels of hyaluronic acid, type IV collagen and laminin

Quantitative enzyme-linked immunoabsorbent assay (ELISA) was used to determine serum levels of hyaluronic acid (HA), type IV collagen, and laminin (LN).

### Measurement of plasma levels of alanine aminotransferase and aspartate aminotransferase

Plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using routine laboratory methods.

### Sirius-red and HE staining

Formalin-fixed and paraffin-embedded liver tissues were cut into 4- $\mu$ m thick sections which were stained with hematoxylin and eosin (HE) and Siriusred. HE staining was used to observe liver pathologic structures, Siriusred staining and CMIAS image analysis system (Beihang, China) were used to determine the area-density percentage of collagen fibrosis in hepatic tissue. At least five high-power ( $\times 400$ ) fields were chosen and positive collagen fibrosis (red staining) was determined. The area-density percentage of collagen fibrosis was calculated by dividing the number of positive collagen fibres (positive optical density) over the total number of collagen fibres (integrated optical density).

### Electron microscopy

Fresh liver tissue sections (1 mm  $\times$  1 mm  $\times$  1 mm) were fixed in 10% paraform fixative, dehydrated and embedded in Epon-812 resin, and then stained with uranyl acetate and lead citrate for 15 min, respectively.

Table 1 Serum levels of HA, LN, and type IV collagen (mean  $\pm$  SD)

Groups	n	HA (ng/mL)	LN (ng/mL)	Type IV collagen (ng/mL)
Control	12	19.81 $\pm$ 2.86	11.02 $\pm$ 1.70	13.49 $\pm$ 2.49
Model	10	44.64 $\pm$ 3.09 <sup>c</sup>	33.27 $\pm$ 5.81 <sup>c</sup>	62.71 $\pm$ 19.16 <sup>c</sup>
High-dose DMN group	10	23.14 $\pm$ 4.58 <sup>a,c</sup>	14.02 $\pm$ 2.63 <sup>a,c</sup>	22.10 $\pm$ 2.44 <sup>a,c</sup>
Medium-dose DMN group	12	22.58 $\pm$ 3.60 <sup>a</sup>	13.87 $\pm$ 1.45 <sup>a</sup>	25.64 $\pm$ 4.68 <sup>a,c</sup>
Low-dose DMN group	10	26.08 $\pm$ 5.62 <sup>a,c</sup>	19.12 $\pm$ 5.02 <sup>a,c</sup>	27.64 $\pm$ 4.68 <sup>a,c</sup>

<sup>a</sup>P < 0.05 vs model group; <sup>c</sup>P < 0.05 vs control group.

Liver "transitional" HSC were observed under JEM-1200EX, 80 kV electron microscope (JEOL, Japan).

### Statistical analysis

Results were expressed as mean  $\pm$  SD. Quantitative data were analyzed using ANOVA with statistical software SPSS 11.0. P < 0.05 was considered statistically significant. Ridit test was used for statistical analysis of the qualitative data.

## RESULTS

### Changes in serum HA, LN levels and type IV collagen levels

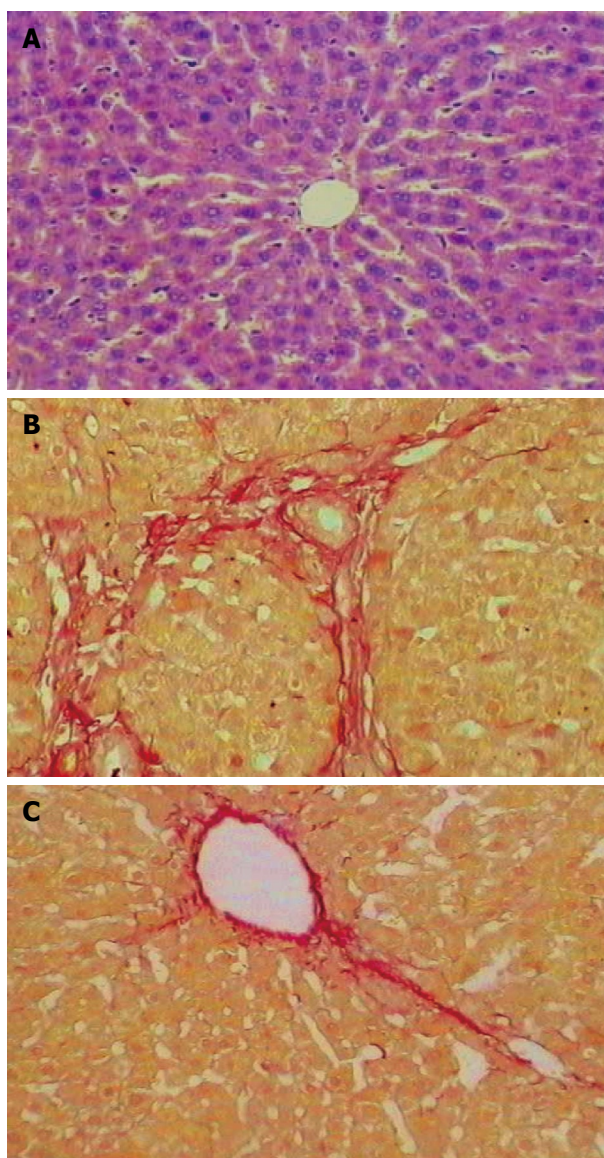
The serum levels of HA, LN, and type IV collagen were markedly increased in the model group compared with the control group (P < 0.05). Compared with the model group, the serum levels of HA, LN, and type IV collagen were significantly decreased in the 3 treatment groups (P < 0.05) (Table 1).

### Siriusred and HE staining

At the end of the study, the liver of control rats had no appreciable alterations in the model group (Figure 1A), more fibrous tissues formed and extended into the hepatic lobules to separate them incompletely and thick intralobular septa were evident (Figure 1B). While in the 3 treatment groups, especially in the medium-dose DMN group, the pathological changes in liver were rather milder, showing less fibrous tissue proliferation (Figure 1C). The rat liver was stained with Siriusred. The area occupied by the fibrotic septa was markedly increased in the model group compared with the control group (P < 0.05). Compared with the model group, the area occupied by the fibrotic septa was significantly decreased in the 3 treatment groups (P < 0.05) (Table 2).

### Plasma levels of ALT and AST

Plasma levels of ALT and AST were higher in the model group than in the control group (P < 0.05), while ALT and AST levels were significantly lower in the *Qianggan-Rongxian* Decoction treatment groups than in the model group. No difference was found in the serum levels of ALT and AST between the medium-dose DMN group and normal group (Table 3).



**Figure 1** Light microscopy showing normal liver tissue in the control group (HE staining,  $\times 100$ ) (A), liver fibrosis tissue and formation of more fibrous tissue as well as a large amount of inflammatory cells soaked in intralobules and interlobules in model group (van Gieson staining,  $\times 100$ ) (B), and liver fibrosis tissue in *Qianggan-Rongxian* Decoction treatment group (C). The pathological change in liver was rather milder compared with the model group (van Gieson staining,  $\times 100$ ).

### Ultrastructure observation under electron microscope

HSC were normal in the control group and typical myofibroblasts were observed in the fibrous septum of the model group (Figure 2A). The elongated cell body contained indented nuclei and numerous microfilaments outlined by a lamina-like structure. Collagen fibers of variable size were seen all around the myofibroblasts (Figure 2B). “Transitional” HSC could be observed under the electron microscope (Figure 2C).

## DISCUSSION

Hepatic fibrosis at the intermediate and crucial stage is characterized by reversibility. If treated properly at this stage, cirrhosis could be successfully prevented. However, it remains a problem to prevent cirrhosis or

**Table 2** Area occupied by the fibrotic septa and its ratio to the total area examined (mean  $\pm$  SD)

Groups	<i>n</i>	Area covered by fibrotic septa ( $\mu\text{m}^2$ )	Ratio (%)
Control	12	10.33 $\pm$ 6.89	0.12 $\pm$ 0.08
Model	10	221.23 $\pm$ 51.21 <sup>c</sup>	2.00 $\pm$ 0.20 <sup>c</sup>
High-dose DMN group	10	59.32 $\pm$ 10.41 <sup>a,c</sup>	1.60 $\pm$ 0.24 <sup>a,c</sup>
Medium-dose DMN group	12	21.73 $\pm$ 15.42 <sup>a,c</sup>	1.06 $\pm$ 0.13 <sup>a,c</sup>
Low-dose DMN group	10	61.73 $\pm$ 15.42 <sup>a,c</sup>	1.68 $\pm$ 0.14 <sup>a,c</sup>

<sup>a</sup>*P* < 0.05 vs model group; <sup>c</sup>*P* < 0.05 vs control group.

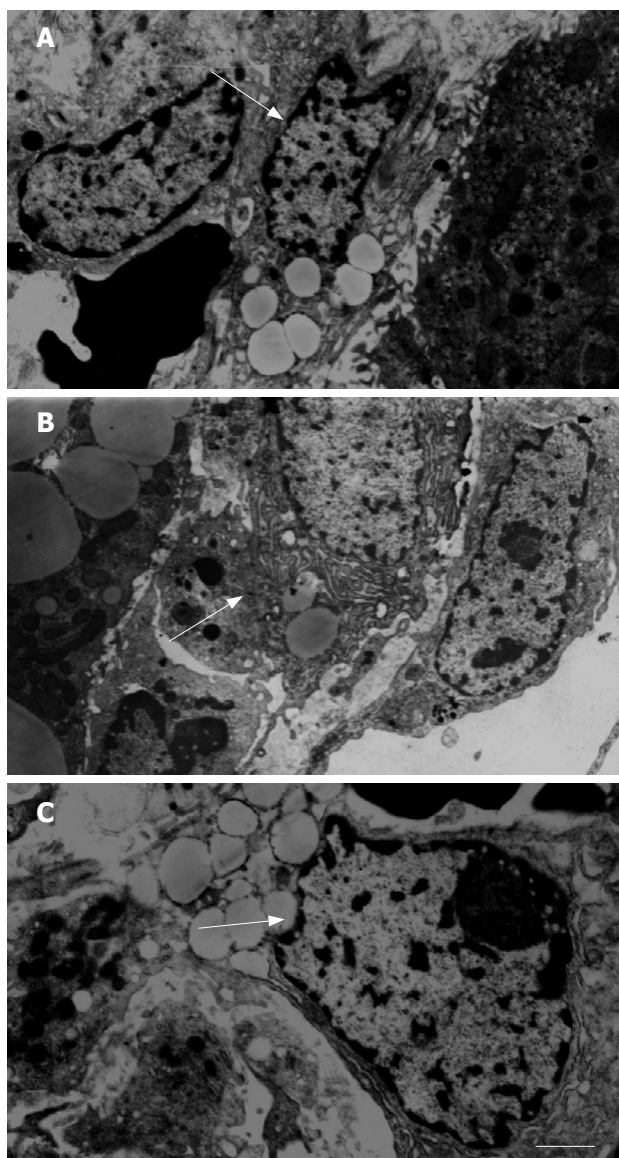
**Table 3** Serum levels of ALT and AST (mean  $\pm$  SD)

Groups	<i>n</i>	Area covered by fibrotic septa ( $\mu\text{m}^2$ )	Ratio (%)
Control	12	63.0 $\pm$ 11.9	307 $\pm$ 23
Model	10	1931 $\pm$ 552 <sup>c</sup>	2696 $\pm$ 764 <sup>c</sup>
High-dose DMN group	10	960 $\pm$ 557 <sup>a,c</sup>	1560 $\pm$ 965 <sup>a,c</sup>
Medium-dose DMN group	12	739 $\pm$ 345 <sup>a,c</sup>	1239 $\pm$ 725 <sup>a,c</sup>
Low-dose DMN group	10	983 $\pm$ 460 <sup>a,c</sup>	1631 $\pm$ 859 <sup>a,c</sup>

<sup>a</sup>*P* < 0.05 vs model group; <sup>c</sup>*P* < 0.05 vs control group.

to control its progression. Great efforts have been made to find safe and effective drugs. Recent clinical and experimental observations demonstrated that Chinese medicines have some preventive and therapeutic values against fibrosis<sup>[12-14]</sup>. *Astragalus*, one of the compositions of *Qianggan-Rongxian* Decoction, can relieve stasis by activating blood circulation, and eliminate fullness by strengthening the “spleen”, supplementing and smoothing “Qi”, reinforcing the body’s immunological function. It also could preserve the integrity of hepatocytes, eliminate toxic free radicals, inhibit lipid peroxidation of cytomembrane, relieve necrosis of hepatocytes, and reduce fibrosis<sup>[15-19]</sup>. Thoroughfare is mainly used to activate blood circulation, remove stasis, and dredge the liver<sup>[20]</sup>. *Qianggan-Rongxian* Decoction has been used for 20 years to prevent liver fibrosis in clinical practice. However, its effect and associated mechanisms need further study. DMN-induced experimental model may be helpful in understanding the relationship between liver injury and development of hepatic fibrosis<sup>[21]</sup>. As estimated by histological analysis of liver tissue stained with Sirius red<sup>[22]</sup>, it can be used to detect different degrees of hepatic fibrosis, and examination of the liver can reveal a progressive increase in fibrosis scores and expansion of fibrous septa<sup>[23]</sup>. Serum HA, LN and collagen type IV levels were significantly increased in the rats as detected by ELISA, showing that simultaneous determination of HA, LN and collagen type IV levels is an optimal choice<sup>[24]</sup>. We treated the rat liver fibrosis induced by injection of DMN with *Qianggan-Rongxian* Decoction. After 4 wk, no appreciable alterations were found in the control





**Figure 2** Electron microscopy showing normal HSC in the model group (A), typical myofibroblasts in the control group (B) ( $\times 5000$ , bar = 1  $\mu\text{m}$ ), and "transitional" HSC ( $\times 6000$ , bar = 1  $\mu\text{m}$ ) in *Qianggan-Rongxian* Decoction treatment group (C).

group. However, the rats in the model group had an almost integrity fibrosis septum, and pseudolobules could be seen in nearly all sections. While the rats that received *Qianggan-Rongxian* Decoction had less fibrosis, reticular fibrosis in the interlobular septum was limited and no pseudo-lobules could be seen. HSC play a central role in the pathogenesis of liver fibrosis and are able to regulate matrix degradation in the liver. Following liver injury, HSC become activated and express extracellular matrix. *Qianggan-Rongxian* Decoction can inhibit transition from HSC to myofibroblasts and fibroblasts. In addition, *Qianggan-Rongxian* Decoction could decrease the area-density percentage of collagen fibrosis. HA, LN, and type IV collagen are good serum markers of hepatic fibrosis. In this study, the serum levels of these 3 markers in the model group were much higher than those in the control group ( $P < 0.05$ ). The serum levels of HA, LN and type IV collagen were significantly lower

in the *Qianggan-Rongxian* Decoction treatment groups than in the control group. ALT and AST are the indexes of liver functions. Since ALT in cytoplasm of liver cells is discharged into blood when degeneration, hyper permeability and necrosis of liver cells occur, increased serum ALT levels reflect the degree of liver cell injury. Our study showed that *Qianggan-Rongxian* Decoction could decrease the serum levels of ALT and AST in rats with hepatic injury induced by DMN, indicating that *Qianggan-Rongxian* Decoction may work by protecting liver against fibrosis. The mechanism underlying rat liver fibrosis induced by DMN is associated with immune function, which is similar to the mechanism underlying human liver fibrosis<sup>[25]</sup>. Thus, DMN-induced rat liver fibrosis may be a useful model for determination of liver fibrosis during drug screening. The mechanism of *Qianggan-Rongxian* Decoction may need further study.

In summary, *Qianggan-Rongxian* Decoction may play a role in anti-fibrotic therapy by protecting liver cells and inhibiting the deposition of collagen fibers in liver, thus providing a safe and effective strategy for inhibition of cirrhosis in clinic practice.

## COMMENTS

### Background

In China, the incidence of liver cirrhosis is still high, liver fibrosis and cirrhosis are due to chronic liver injury. Although new therapeutic approaches have recently been proposed, there is no established therapy for liver fibrosis. The authors investigated the preventive effect of *Qianggan-Rongxian* Decoction on rat liver fibrosis induced by dimethylnitrosamine (DMN).

### Research frontiers

*Qianggan-Rongxian* Decoction can protect the liver against fibrosis induced by DMN in rats.

### Innovations and breakthroughs

Enzyme-linked immunosorbent assay (ELISA) and hematoxylin and eosin as well as Sirius red staining and transmission electron microscopy demonstrated that *Qianggan-Rongxian* Decoction could prevent liver against fibrosis.

### Applications

ELISA can determine the changes in serum levels of hyaluronic acid (HA), laminin (LA), and type IV collagen. Pathologic changes, particularly fibrosis can be examined by light microscopy. Hepatic stellate cells (HSC) can be examined by transmission electron microscopy.

### Terminology

*Qianggan-Rongxian* Decoction is a kind of Chinese medicine, which may protect the liver against fibrosis.

### Peer review

This is an interesting article. *Qianggan-Rongxian* Decoction can protect the liver against fibrosis and inhibit the deposition of collagen fibers in liver, thus providing a safe and effective strategy for inhibition of cirrhosis in clinical practice. The paper is well organized and the results are clearly described and commented.

## REFERENCES

- 1 Du WD, Zhang YE, Zhai WR, Zhou XM. Dynamic changes of type I, III and IV collagen synthesis and distribution of collagen-producing cells in carbon tetrachloride-induced rat liver fibrosis. *World J Gastroenterol* 1999; 5: 397-403
- 2 Canturk NZ, Canturk Z, Ozden M, Dalcik H, Yardimoglu M, Tulubas F. Protective effect of IGF-1 on experimental liver cirrhosis-induced common bile duct ligation. *Hepatogastroenterology* 2003; 50: 2061-2066
- 3 López-Lirola A, González-Reimers E, Martín Olivera R,



- Santolaria-Fernández F, Galindo-Martín L, Abreu-González P, González-Hernández T, Valladares-Parrilla F. Protein deficiency and muscle damage in carbon tetrachloride induced liver cirrhosis. *Food Chem Toxicol* 2003; **41**: 1789-1797
- 4 **Pan NS**, Li ST, Wang Y, Li MF, Han Z. [Therapeutic effect of "anti-hepatic-fibrosis 268" on hepatic fibrosis in rats] *Sichuan Daxue Xuebao Yixueban* 2004; **35**: 528-531
  - 5 **Floreani A**, Guido M, Bortolami M, Della Zentil G, Venturi C, Pennelli N, Naccarato R. Relationship between apoptosis, tumour necrosis factor, and cell proliferation in chronic cholestasis. *Dig Liver Dis* 2001; **33**: 570-575
  - 6 **Jia JD**. [Further systematize and standardize the diagnosis and treatment of liver cirrhosis] *Zhonghua Ganzhangbing Zazhi* 2005; **13**: 401-402
  - 7 **Zalatnai A**. Molecular aspects of stromal-parenchymal interactions in malignant neoplasms. *Curr Mol Med* 2006; **6**: 685-693
  - 8 **Breitkopf K**, Sawitzka I, Gressner AM. Characterization of intracellular pathways leading to coinduction of thrombospondin-1 and TGF-beta1 expression in rat hepatic stellate cells. *Growth Factors* 2005; **23**: 77-85
  - 9 **Jeong WI**, Park O, Gao B. Abrogation of the antifibrotic effects of natural killer cells/interferon-gamma contributes to alcohol acceleration of liver fibrosis. *Gastroenterology* 2008; **134**: 248-258
  - 10 **Friedman SL**. Molecular regulation of hepatic fibrosis, an integrated cellular response to tissue injury. *J Biol Chem* 2000; **275**: 2247-2250
  - 11 **Kitamura K**, Tada S, Nakamoto N, Toda K, Horikawa H, Kurita S, Tsunematsu S, Kumagai N, Ishii H, Saito H, Hibi T. Rho/Rho kinase is a key enzyme system involved in the angiotensin II signaling pathway of liver fibrosis and steatosis. *J Gastroenterol Hepatol* 2007; **22**: 2022-2033
  - 12 **Liu C**, Jiang CM, Liu CH, Liu P, Hu YY. Effect of Fuzhenghuayu decoction on vascular endothelial growth factor secretion in hepatic stellate cells. *Hepatobiliary Pancreat Dis Int* 2002; **1**: 207-210
  - 13 **Liu P**, Liu CH, Wang HN, Hu YY, Liu C. Effect of salvianolic acid B on collagen production and mitogen-activated protein kinase activity in rat hepatic stellate cells. *Acta Pharmacol Sin* 2002; **23**: 733-738
  - 14 **Kusunose M**, Qiu B, Cui T, Hamada A, Yoshioka S, Ono M, Miyamura M, Kyotani S, Nishioka Y. Effect of Shosai-ko-to extract on hepatic inflammation and fibrosis in dimethylnitrosamine induced liver injury rats. *Biol Pharm Bull* 2002; **25**: 1417-1421
  - 15 **Wang RT**, Shan BE, Li QX. [Extracorporeal experimental study on immuno-modulatory activity of Astragalus membranaceus extract] *Zhongguo Zhongxiyi Jiehe Zazhi* 2002; **22**: 453-456
  - 16 **Chu DT**, Lin JR, Wong W. [The in vitro potentiation of LAK cell cytotoxicity in cancer and aids patients induced by F3--a fractionated extract of Astragalus membranaceus] *Zhonghua Zhongliu Zazhi* 1994; **16**: 167-171
  - 17 **Zhang YD**, Shen JP, Zhu SH, Huang DK, Ding Y, Zhang XL. [Effects of astragalus (ASI, SK) on experimental liver injury] *Yaoxue Xuebao* 1992; **27**: 401-406
  - 18 **Tan YW**, Yin YM, Yu XJ. [Influence of Salvia miltiorrhizae and Astragalus membranaceus on hemodynamics and liver fibrosis indexes in liver cirrhotic patients with portal hypertension] *Zhongguo Zhongxiyi Jiehe Zazhi* 2001; **21**: 351-353
  - 19 **Liu P**, Hu YY, Liu C, Xu LM, Liu CH, Sun KW, Hu DC, Yin YK, Zhou XQ, Wan MB, Cai X, Zhang ZQ, Ye J, Zhou RX, He J, Tang BZ. Multicenter clinical study on Fuzhenghuayu capsule against liver fibrosis due to chronic hepatitis B. *World J Gastroenterol* 2005; **11**: 2892-2899
  - 20 **Chen H**, Weng L. [Comparison on efficacy in treating liver fibrosis of chronic hepatitis B between Astragalus Polygonum anti-fibrosis decoction and jinshuibao capsule] *Zhongguo Zhongxiyi Jiehe Zazhi* 2000; **20**: 255-257
  - 21 **Mancini R**, Jezequel AM, Benedetti A, Paolucci F, Trozzi L, Orlandi F. Quantitative analysis of proliferating sinusoidal cells in dimethylnitrosamine-induced cirrhosis. An immunohistochemical study. *J Hepatol* 1992; **15**: 361-366
  - 22 **Lee MH**, Yoon S, Moon JO. The flavonoid naringenin inhibits dimethylnitrosamine-induced liver damage in rats. *Biol Pharm Bull* 2004; **27**: 72-76
  - 23 **Hsu YC**, Chiu YT, Lee CY, Lin YL, Huang YT. Increases in fibrosis-related gene transcripts in livers of dimethylnitrosamine-intoxicated rats. *J Biomed Sci* 2004; **11**: 408-417
  - 24 **Liang XH**, Zheng H. [Value of simultaneous determination of serum hyaluronic acid, collagen type IV and the laminin level in diagnosing liver fibrosis] *Hunan Yike Daxue Xuebao* 2002; **27**: 67-68
  - 25 **Jezequel AM**, Mancini R, Rinaldesi ML, Ballardini G, Fallani M, Bianchi F, Orlandi F. Dimethylnitrosamine-induced cirrhosis. Evidence for an immunological mechanism. *J Hepatol* 1989; **8**: 42-52

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RAPID COMMUNICATION

## Treatment of abdominal compartment syndrome in severe acute pancreatitis patients with traditional Chinese medicine

Min-Jie Zhang, Guo-Lei Zhang, Wen-Bin Yuan, Jun Ni, Li-Feng Huang

Min-Jie Zhang, Guo-Lei Zhang, Wen-Bin Yuan, Jun Ni, Department of General surgery, Affiliated Central Hospital of Huzhou Normal College, Huzhou 313000, Zhejiang Province, China

Li-Feng Huang, Research Center of Biomedicine and Health, Hangzhou Normal University, Hangzhou 310000, Zhejiang Province, China

**Author contributions:** Zhang MJ and Zhang GL contributed equally to this work; Zhang MJ, Zhang GL, Yuan WB, and Ni J designed the research; Zhang MJ, Yuan WB, and Ni J performed the research; Huang LF provided the new reagents/analytic tools; Zhang MJ, Yuan WB, and Ni J analyzed the data; Zhang MJ, Yuan WB, and Huang LF wrote the paper.

**Correspondence to:** Min-Jie Zhang, Department of General surgery, Affiliated Central Hospital of Huzhou Normal College, Huzhou 313000, Zhejiang Province, China. [zmjys@yahoo.com.cn](mailto:zmjys@yahoo.com.cn)

Telephone: +86-572-2369815 Fax: +86-572-2369815

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( $P < 0.05$ ). On days 3-5 of treatment, acute physiology and chronic health evaluation II (APACHE II) scores for the study and control groups were significantly different ( $P < 0.05$ ). Both the effectiveness and outcome of the treatment with *Da Cheng Qi* Decoction on abdominalgia, burbulence relief time, ascites quantity, cyst formation rate and hospitalization time were quite different between the two groups ( $P < 0.05$ ). The mortality rate for the two groups had no significant difference.

**CONCLUSION:** *Da Cheng Qi* Decoction enema and external use of Glauber's salt combined with routine non-operative conservative treatment can decrease the intra-abdominal pressure (IAP) of SAP patients and have preventive and therapeutic effects on abdominal compartment syndrome of SAP.

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**Key words:** *Da Cheng Qi* Decoction; Glauber's salt; Traditional Chinese medicine; Severe acute pancreatitis; Abdominal compartment syndrome

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Zhang MJ, Zhang GL, Yuan WB, Ni J, Huang LF. Treatment of abdominal compartment syndrome in severe acute pancreatitis patients with traditional Chinese medicine. *World J Gastroenterol* 2008; 14(22): 3574-3578 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3574.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3574>

### Abstract

**AIM:** To investigate the therapeutic effect of traditional Chinese traditional medicines *Da Cheng Qi* Decoction (Timely-Purging and Yin-Preserving Decoction) and Glauber's salt combined with conservative measures on abdominal compartment syndrome (ACS) in severe acute pancreatitis (SAP) patients.

**METHODS:** Eighty consecutive SAP patients, admitted for routine non-operative conservative treatment, were randomly divided into study group and control group (40 patients in each group). Patients in the study group received *Da Cheng Qi* Decoction enema for 2 h and external use of Glauber's salt, once a day for 7 d. Patients in the control group received normal saline (NS) enema. Routine non-operative conservative treatments included non-per os nutrition (NPON), gastrointestinal decompression, life support, total parenteral nutrition (TPN), continuous peripancreatic vascular pharmaceutical infusion and drug therapy. Intra-cystic pressure (ICP) of the two groups was measured during treatment. The effectiveness and outcomes of treatment were observed and APACHE II scores were applied in analysis.

**RESULTS:** On days 4 and 5 of treatment, the ICP was lower in the study group than in the control group

### INTRODUCTION

Severe acute pancreatitis (SAP) is a serious surgical disease with a mortality of 25%-40%<sup>[1,2]</sup>. Patients with SAP tend to have elevated intra-abdominal pressure (IAP), which eventually leads to intra-abdominal hypertension (IAH). IAH causes organ dysfunctions such as respiratory, circulatory and renal failure, known as abdominal compartment syndrome (ACS)<sup>[3,4]</sup>. About 11% of SAP patients suffer from complications of ACS. SAP patients complicated by ACS, a special type of pancreatitis, tend to have a mortality of 66.7%<sup>[5]</sup>. There are certain guidelines for treatment of SAP, but no standard treatment for ACS

in SAP patients is available at present<sup>[2]</sup>. In our previous study, *Da Cheng Qi* Decoction showed beneficial effects on acute pancreatitis (AP) with no adverse effects<sup>[6]</sup>. In the present study, we used *Da Cheng Qi* Decoction and Glauber's salt combined with routine non-operative conservative treatments, including non-per os nutrition (NPON), gastrointestinal decompression, life support, total parenteral nutrition (TPN), continuous peripancreatic vascular pharmaceutical infusion and drug therapy in the treatment of ACS in SAP patients. Through comparison with the control group, we demonstrated whether the TCM-wm therapy is effective for treatment of ACS in SAP patients.

## MATERIALS AND METHODS

### Patients

A total of 80 consecutive SAP patients were treated in the Surgery Department of Affiliated Central Hospital of Huzhou Normal College. The age of the patients ranged 27-76 years (mean 54.4 years, median 47 years). There were 38 males (47.5%) and 42 females (52.5%), the male to female ratio was 0.905:1. When they were hospitalized, the severity of SAP was evaluated according to the serum level of amylase, CT serious index (CTSI)<sup>[7]</sup>, acute physiology and chronic health evaluation II (APACHE II) score<sup>[8]</sup>, and the diagnostic criteria and severity grade for AP proposed by the Japanese Ministry of Health, Labor, and Welfare (JMHLW)<sup>[9]</sup>. The diagnosis of ACS was made as previously described<sup>[10]</sup>. Demographic data, serum level of amylase, CTSI, APACHE II scores, ACS rate and severity grade ratio were not statistically different between the study and control groups (Table 1).

### Methods

Eighty patients of SAP admitted for routine non-operative conservative treatment were randomly divided into study group and control group (40 patients in each group). Patients in the study group received *Da Cheng Qi* Decoction enema (one dosage, 100 mL) for 2 h and external use of Glauber's salt (100 g) once a day for 7 d. Patients in the control group received 100 mL normal saline (NS) enema. One dosage of *Da Cheng Qi* Decoction consists of 10 g *Rheum officinale* Baill, 10 g *Sodium sulfate*, 10 g *Magnolia obavata*, 10 g *Fructus aurantii*, 10 g *Radix paeoniae rubra*, and 10 g *Raphanus sativus*. Routine non-operative conservative treatment modalities included NPON, gastrointestinal decompression, life support, TPN, continuous peripancreatic vascular pharmaceutical infusion<sup>[11,12]</sup> and drug therapy. Intracystic pressure (ICP) in the two groups could reflect the IAP conditions at admission and on days 1-7 of treatment. ICP data were defined as previously described<sup>[13]</sup>. Effectiveness of the treatment on abdominalgia, burbulence relief time, ascites quantity, cyst formation, mortality rate and hospitalization time were observed. The ascites quantity in patients was defined by B-ultrasound test on day 7 of treatment. APACHE II scores, defined using Microsoft APACHE

**Table 1** Demographic data, serum level of amylase, CTSI score, APACHE II score, ICP data and severity grade ratio of the two groups when hospitalized (mean  $\pm$  SD)

	Study group (n = 40)	Control group (n = 40)
Age (yr)	54.2 $\pm$ 7.4	54.6 $\pm$ 8.1
Sex (male/female)	20/20	18/22
serum levels of amylase (U/L)	328.23 $\pm$ 13.89	354.51 $\pm$ 14.22
ICP (cmH <sub>2</sub> O)	19.5 $\pm$ 3.4	18.7 $\pm$ 3.6
CTSI score	7.85 $\pm$ 1.1	7.90 $\pm$ 1.4
APACHE II score	17.51 $\pm$ 4.51	18.2 $\pm$ 3.87
ACS rate (%)	17.5	20.0
Severity grade (II / III)	22/8	23/7

II graded compute program version 5.1, were applied for analysis.

### Statistical analysis

All data were prepared and compiled using the SPSS computer program (version 11.0 for windows). The data were expressed as mean  $\pm$  SD. Kolmogorov-smirnov test was used for the pattern of data distribution. Student's unpaired *t*-test was used to compare data between the two groups when they were normally distributed. The Mann-Whitney *U* test was used when the data were not normally distributed. Chi square test and Fisher's exact test were used for quantitative data analysis. Step-wise regression analysis was used for multivariate analysis to determine any confounding factors. *P* < 0.05 was considered statistically significant.

## RESULTS

### ICP and APACHE II scores

On days 4 and 5 of treatment, the ICP data obtained from the study group were lower than those obtained from the control group (*P* < 0.05). On days 3-5 of treatment, the APACHE II scores of the study group and control group were significantly different (*P* < 0.05, Table 2). As shown in Table 2, the ICP data were significantly decreased from the 4th treatment day in the study group (*P* < 0.05), while significantly decreased from the 6th treatment day in the control group (*P* < 0.05). The cumulative scores of APACHE II were significantly decreased from the 3rd treatment day in the study group (*P* < 0.05), while significantly decreased from the 6th treatment day in the control group (*P* < 0.05).

### Effectiveness of treatment

As shown in Table 3, the relief time of abdominalgia and burbulence was shorter in the study group than in the control group (*P* < 0.05). The amount of ascites on day 7 of treatment was less in the study group than in the control group (*P* < 0.05).

### Outcome of treatment

As shown in Table 4, the mortality rate was lower for the study group than for the control group, and there was no significant difference between the two groups. The cyst formation rate was significantly lower for the

**Table 2** ICP and APACHE II score of the two groups ( $n = 40$ , mean  $\pm$  SD)

	ICP			APACHE II scores		
	Study group	Control group	<i>P</i>	Study group	Control group	<i>P</i>
HPd	19.5 $\pm$ 3.4	18.7 $\pm$ 3.6		17.51 $\pm$ 4.51	18.2 $\pm$ 3.87	
Day 1	16.5 $\pm$ 3.2	17.4 $\pm$ 3.8		16.44 $\pm$ 3.54	17.85 $\pm$ 4.12	
Day 2	15.8 $\pm$ 2.5	16.2 $\pm$ 1.9		14.21 $\pm$ 4.23	16.55 $\pm$ 3.98	
Day 3	15.6 $\pm$ 2.7	15.9 $\pm$ 3.1		9.66 $\pm$ 1.88 <sup>c</sup>	13.46 $\pm$ 1.93	< 0.05
Day 4	8.2 $\pm$ 1.5 <sup>a</sup>	15.2 $\pm$ 3.7	< 0.05	7.41 $\pm$ 1.72 <sup>c</sup>	12.37 $\pm$ 2.21	< 0.05
Day 5	8.7 $\pm$ 3.2 <sup>a</sup>	14.7 $\pm$ 2.9	< 0.05	4.63 $\pm$ 1.46 <sup>c</sup>	10.78 $\pm$ 2.01	< 0.05
Day 6	7.9 $\pm$ 3.9 <sup>a</sup>	7.5 $\pm$ 3.5 <sup>a</sup>		4.33 $\pm$ 2.01 <sup>c</sup>	5.23 $\pm$ 2.67 <sup>c</sup>	
Day 7	7.4 $\pm$ 2.8 <sup>a</sup>	8.1 $\pm$ 2.7 <sup>a</sup>		3.78 $\pm$ 1.53 <sup>c</sup>	4.21 $\pm$ 1.62 <sup>c</sup>	

<sup>a</sup>*P* < 0.05 vs ICP of HPd; <sup>c</sup>*P* < 0.05 vs APACHE II scores of HPd. HPd: Hospitalization day.

**Table 3** Relief time of abdominalgia and burbulence, ascites quantity on day 7 of treatment in the two groups (mean  $\pm$  SD)

	Study group ( <i>n</i> = 40)	Control group ( <i>n</i> = 40)	<i>P</i> -value
Relief time of abdominalgia (d)	4.27 $\pm$ 0.87 <sup>a</sup>	10.85 $\pm$ 1.21	0.035
Relief time of burbulence (d)	6.94 $\pm$ 1.22 <sup>a</sup>	13.27 $\pm$ 3.67	0.021
Ascites quantity (mL)	457.25 $\pm$ 34.21 <sup>a</sup>	927.84 $\pm$ 27.45	0.038

<sup>a</sup>*P* < 0.05 vs control group.

study group than for the control group (*P* < 0.05). The hospitalization time of the study group was significantly shorter than that of the control group (*P* < 0.05).

## DISCUSSION

SAP is a serious pathological condition and SAP patients tend to suffer from ACS<sup>[14,15]</sup>. It was reported that SAP can result in systemic inflammatory response syndrome (SIRS) triggered by local inflammation in the pancreas<sup>[16]</sup>. The fundamental pathophysiology of SIRS is hypercytokinemia, a pathological condition in which inflammatory cytokines are excessively released from immunocompetent cells<sup>[17]</sup>. During SIRS, activated inflammatory mediators result in the development of systemic capillary leakage syndrome (SCLS)<sup>[18]</sup>. In SCLS, vascular permeability is increased by the pathologic effects of humoral mediators, leading to interstitial edema and reduction of circulating blood volume<sup>[19]</sup>. Progressive edema of peritoneum and gut contents could rapidly increase IAP. Moreover, massive pancreatic liquid collection in the abdominal and retroperitoneal cavity and a large amount of pancreatic necrotic tissue combined with infection would lead to the formation of abscess, causing intestinal obstruction and erosion of the surrounding organs, which further result in perforation and massive bleeding and recurrence of SIRS and SCLS. Edema caused by the septic retroperitoneal necrosis pushes the peritoneum, thus rapidly increasing IAP<sup>[20]</sup>. Furthermore, for the treatment of SAP, early resuscitation with a large volume of fluid is essential to maintain organ perfusion. However, aggressive fluid

**Table 4** Mortality rate, cyst formation rate and hospitalization time of the two groups (mean  $\pm$  SD)

	Study group ( <i>n</i> = 40)	Control group ( <i>n</i> = 40)	<i>P</i> -value
Mortality rate (%)	7.5 (3/40)	20.0 (8/40)	0.194
Cyst formation rate (%)	10.0 (4/40) <sup>a</sup>	32.5 (13/40)	0.029
Hospitalization time (d)	16.59 $\pm$ 3.89 <sup>a</sup>	29.58 $\pm$ 4.52	0.037

<sup>a</sup>*P* < 0.05 vs control group.

resuscitation may aggravate intestinal edema, further increasing IAP<sup>[21]</sup>, indicating that SAP can induce retroperitoneal edema or abscess, fluid collection in the abdominal and retroperitoneal cavity, intestinal edema, and that aggressive fluid resuscitation could increase IAP and abrupt elevation of IAP eventually causes IAH. IAH causes organ dysfunctions such as respiratory, circulatory and renal failure, known as ACS<sup>[22]</sup>. Since SAP patients with complication of ACS have a very high mortality, it is very important to find a right way to prevent and treat ACS in SAP patients.

*Da Cheng Qi* Decoction is a traditional Chinese medicine documented in Shang Han Lun (Treatise on Febrile Diseases). Its major components include Rheum officinale Baill, sodium sulfate, Magnolia obovata, Fructus aurantii, Radix paeoniae rubra, and *Raphanus sativus*. Modern clinical and experimental researches have focused on the effect of such components<sup>[23-25]</sup>. Glauber's salt, a sodium sulfate dehydrate (Na<sub>2</sub>SO<sub>4</sub>), is widely used as an anti-tissue edema agent in modern medical treatment<sup>[26,27]</sup>. Non-operative conservative treatment has been widely accepted for AP<sup>[28,29]</sup>. Especially, in our previous studies<sup>[11,12,30]</sup>, continuous peripancreatic vascular pharmaceutical infusion could efficiently remove various humoral mediators and inflammatory cytokines from circulating blood, and decrease the mortality of SAP patients. In the present study, we used *Da Cheng Qi* Decoction and Glauber's salt combined with routine non-operative conservative treatments including continuous peripancreatic vascular pharmaceutical infusion for treatment of ACS in SAP patients.

In this study, the ICP data were lower in the study group than in the control group on days 4 and 5 of treatment (*P* < 0.05). The APACHE II scores in study group were significantly less in the study group than in the control group on days 3-5 of treatment (*P* < 0.05). The ICP data were significantly decreased from the 4th treatment day in the study group (*P* < 0.05) while significantly decreased from the 6th treatment day in the control group (*P* < 0.05). The cumulative scores of APACHE II were significantly decreased from the 3rd treatment day in the study group (*P* < 0.05) while significantly decreased from the 6 treatment day in the control group (*P* < 0.05). The relief time of abdominalgia and burbulence was shorter in the study group than in the control group (*P* < 0.05). The amount of ascites on day 7 of treatment was less in the study group than in the control group (*P* < 0.05). The cyst formation rate was significantly lower for the study group than for the



control group ( $P < 0.05$ ). The hospitalization time was significantly shorter in the study group than in the control group ( $P < 0.05$ ). These results suggest that *Da Cheng Qi* Decoction and Glauber's salt combined with non-operative conservative treatments including continuous peripancreatic vascular pharmaceutical infusion can treat IAH by decreasing IAP, relaxing symptoms of ACS, slowing down the pathological condition exacerbation, and accelerating the recovery of illness.

In summary, *Da Cheng Qi* Decoction and Glauber's salt combined with non-operative conservative treatments including continuous peripancreatic vascular pharmaceutical infusion (TCM-wm therapy) can prevent and treat ACS in SAP patients.

## COMMENTS

### Background

Severe acute pancreatitis (SAP) is a familiar surgical disease and about 11% of SAP patients suffer from abdominal compartment syndrome (ACS). SAP patients complicated by ACS tend to have a mortality of 66.7%. There are certain guidelines for treatment of SAP. However, no standard treatment for ACS in SAP patients is available at present.

### Research frontiers

*Da Cheng Qi* Decoction is a traditional Chinese medicine documented in Shang Han Lun (Treatise on Febrile Diseases). Modern clinical and experimental research results have proved its effect. Glauber's salt is widely used as an anti-tissue edema agent in modern medical treatment. *Da Cheng Qi* Decoction or Glauber's salt has beneficial effects on SAP with no adverse effects. Non-operative conservative treatment, especially continuous peripancreatic vascular pharmaceutical infusion for SAP could efficiently remove various humoral mediators and inflammatory cytokines from circulating blood, and decrease the mortality of SAP patients.

### Innovations and breakthroughs

In this study, *Da Cheng Qi* Decoction and Glauber's salt combined with routine non-operative conservative treatments, including continuous peripancreatic vascular pharmaceutical infusion were used in the treatment of ACS in SAP patients. Whether the TCM-wm therapy is effective for ACS in SAP patients was also studied. The results suggest that *Da Cheng Qi* Decoction and Glauber's salt can treat IAH by decreasing IAP, relaxing symptoms of ACS, slowing down the pathological condition exacerbation and accelerating the recovery of illness.

### Applications

The study results provide an important new clue to the treatment of ACS in SAP patients.

### Terminology

SAP is a serious type of acute pancreatitis (AP) complicated by pancreas necrosis and toxic shock. ACS is a syndrome due to factors that cause abrupt elevation of intra-abdominal pressure (IAP), leading to organ dysfunctions such as respiratory, circulatory and renal failure. *Da Cheng Qi* Decoction is a traditional Chinese medicine. Its major components include *Rheum officinale* Baill, sodium sulfate, *Magnolia obovata*, *Fructus aurantii*, *Radix paeoniae rubra*, and *Raphanus sativus*. Glauber's salt is a sodium sulfate dehydrate ( $\text{Na}_2\text{SO}_4$ ). Acute physiology and chronic health evaluation II (APACHE II) is a disease classification system, which is used to evaluate the severity of disease. Intra-cystic pressure (ICP) is the pressure of an empty cyst.

### Peer review

This paper describes the beneficial effect of *Da Cheng Qi* Decoction and Glauber's salt on ACS in SAP patients. Although the medication could not significantly improve the prognosis of SAP patients complicated by ACS, the data are encouraging and provide an important new clue to the treatment of ACS in SAP patients.

## REFERENCES

- 1 Isenmann R, Rau B, Beger HG. Early severe acute pancreatitis: characteristics of a new subgroup. *Pancreas* 2001; **22**: 274-278

- 2 Bosscha K, Hulstaert PF, Hennipman A, Visser MR, Gooszen HG, van Vroonhoven TJ, v d Werken C. Fulminant acute pancreatitis and infected necrosis: results of open management of the abdomen and "planned" reoperations. *J Am Coll Surg* 1998; **187**: 255-262
- 3 Saggi BH, Sugerman HJ, Ivatury RR, Bloomfield GL. Abdominal compartment syndrome. *J Trauma* 1998; **45**: 597-609
- 4 Sieh KM, Chu KM, Wong J. Intra-abdominal hypertension and abdominal compartment syndrome. *Langenbecks Arch Surg* 2001; **386**: 53-61
- 5 Gecelter G, Fahoum B, Gardezi S, Schein M. Abdominal compartment syndrome in severe acute pancreatitis: an indication for a decompressing laparotomy? *Dig Surg* 2002; **19**: 402-404; discussion 404-405
- 6 Zhang M, Zhang MJ, Yuan WB. The effect of Dachengqi Decoction on intra-abdominal pressure in patients of Acute pancreatitis. *Zhejiang Zhongyiyao Daxue Xuebao* 2007; **31**: 569-573
- 7 Balthazar EJ, Robinson DL, Megibow AJ, Ranson JH. Acute pancreatitis: value of CT in establishing prognosis. *Radiology* 1990; **174**: 331-336
- 8 Knaus WA, Draper EA, Wagner DP, Zimmerman JE. APACHE II: a severity of disease classification system. *Crit Care Med* 1985; **13**: 818-829
- 9 Yokoi H, Naganuma T, Higashiguchi T, Isaji S, Kawarada Y. Prospective study of a protocol for selection of treatment of acute pancreatitis based on scoring of severity. *Digestion* 1999; **60** Suppl 1: 14-18
- 10 Tao J, Wang C, Chen L, Yang Z, Xu Y, Xiong J, Zhou F. Diagnosis and management of severe acute pancreatitis complicated with abdominal compartment syndrome. *J Huazhong Univ Sci Technolog Med Sci* 2003; **23**: 399-402
- 11 Lu YL, Gu FY, Li HW, Zhang GL, Yao X. Regional intra arterial infusion for treatment of acute necrotizing pancreatitis: report of 116 cases. *Zhonghua Gandan Waike Zazhi* 1999; **5**: 423-425
- 12 Gu F, Liu Y, Pan R. [Local arterial infusion of 5-FU in treatment of acute necrotic pancreatitis] *Zhonghua Waike Zazhi* 1995; **33**: 339-341
- 13 Fusco MA, Martin RS, Chang MC. Estimation of intra-abdominal pressure by bladder pressure measurement: validity and methodology. *J Trauma* 2001; **50**: 297-302
- 14 Al-Bahrani AZ, Abid GH, Holt A, McCloy RF, Benson J, Eddleston J, Ammori BJ. Clinical relevance of intra-abdominal hypertension in patients with severe acute pancreatitis. *Pancreas* 2008; **36**: 39-43
- 15 De Waele JJ, Hoste E, Blot SI, Decruyenaere J, Colardyn F. Intra-abdominal hypertension in patients with severe acute pancreatitis. *Crit Care* 2005; **9**: R452-R457
- 16 Mofidi R, Duff MD, Wigmore SJ, Madhavan KK, Garden OJ, Parks RW. Association between early systemic inflammatory response, severity of multiorgan dysfunction and death in acute pancreatitis. *Br J Surg* 2006; **93**: 738-744
- 17 Norman J. The role of cytokines in the pathogenesis of acute pancreatitis. *Am J Surg* 1998; **175**: 76-83
- 18 Sanghavi R, Aneman A, Parr M, Dunlop L, Champion D. Systemic capillary leak syndrome associated with compartment syndrome and rhabdomyolysis. *Anaesth Intensive Care* 2006; **34**: 388-391
- 19 Marx G, Vangerow B, Burczyk C, Gratz KF, Maassen N, Cobas Meyer M, Leuwer M, Kuse E, Rueckholdt H. Evaluation of noninvasive determinants for capillary leakage syndrome in septic shock patients. *Intensive Care Med* 2000; **26**: 1252-1258
- 20 Moore AF, Hargest R, Martin M, Delicata RJ. Intra-abdominal hypertension and the abdominal compartment syndrome. *Br J Surg* 2004; **91**: 1102-1110
- 21 Oda S, Hirasawa H, Shiga H, Matsuda K, Nakamura M, Watanabe E, Moriguchi T. Management of intra-abdominal hypertension in patients with severe acute pancreatitis with continuous hemodiafiltration using a polymethyl methacrylate

- membrane hemofilter. *Ther Apher Dial* 2005; **9**: 355-361
- 22 **Cheatham ML**, Malbrain ML, Kirkpatrick A, Sugrue M, Parr M, De Waele J, Balogh Z, Leppaniemi A, Olvera C, Ivatury R, D'Amours S, Wendon J, Hillman K, Wilmer A. Results from the International Conference of Experts on Intra-abdominal Hypertension and Abdominal Compartment Syndrome. II. Recommendations. *Intensive Care Med* 2007; **33**: 951-962
- 23 **Lin WQ**, Zhao YF. Creatural experiment study of Dachengqi Dococotion. *Zhongyi Yanjiu* 2003; **16**: 51-54
- 24 **Tian YQ**, Ding P. The progress of pharmacology study of Dachengqi Dococotion. *Zhongyiyao Xuekan* 2006; **24**: 2134-2135
- 25 **Son BX**. The progress of molecular experimental study of Dachengqi Dococotion. *Guangmin Zhongyi* 1995; **1**: 38-39
- 26 **Wang YX**. Clinical application of Glauber's salt. *Zhonghua Linchuang Yixue Zazhi* 2006; **7**: 46-47
- 27 **Qing YJ**, Lin F. The treatment of severe acute pancreatitis with TCM- wmtherapy (30 cases). *Shiyong Zhenduan yu Zhiliao Zazhi* 2006; **20**: 838-839
- 28 **Sun ZX**, Huang HR, Zhou H. Indwelling catheter and conservative measures in the treatment of abdominal compartment syndrome in fulminant acute pancreatitis. *World J Gastroenterol* 2006; **12**: 5068-5070
- 29 **Yousaf M**, McCallion K, Diamond T. Management of severe acute pancreatitis. *Br J Surg* 2003; **90**: 407-420
- 30 **Lu YL**, Yao X, Li HW, Dai LC, Han CF, Jin C. Improvement of pancreatic blood flow in rats with acute necrotizing pancreatitis by regional intra arterial perfusion of 5 FU. *Zhonghua Gandan Waik Zazhi* 2000; **6**: 74-76

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## Ultrasonic diagnosis of biliary atresia: A retrospective analysis of 20 patients

Shi-Xing Li, Yao Zhang, Mei Sun, Bo Shi, Zhong-Yi Xu, Ying Huang, Zhi-Qin Mao

Shi-Xing Li, Yao Zhang, Bo Shi, Zhong-Yi Xu, Department of Ultrasound, Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning Province, China

Mei Sun, Zhi-Qin Mao, Department of Internal Medicine for Childhood, Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning Province, China

Ying Huang, Department of Pediatric Surgery, Shengjing Hospital of China Medical University, Shenyang 110004, Liaoning Province, China

**Author contributions:** Li SX, Sun M, and Zhang Y contributed equally to this work; Li SX, Sun M, Shi B, and Xu ZY designed the research; Li SX, Mao ZQ analyzed the data; Li SX, Sun M, and Huang Y performed the research; Li SX and Zhang Y provided the analytic tools; Li SX, Sun M, and Zhang Y wrote the paper.

**Correspondence to:** Mei Sun, Department of Internal Medicine for Childhood, Shengjing Hospital of China Medical University, No. 36, Sanhao Street, Heping District, Shenyang 110004, Liaoning Province, China. [lisx630106@vip.sina.com](mailto:lisx630106@vip.sina.com)

Telephone: +86-24-83956537 Fax: +86-24-83955092

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the porta hepatis is specific. However, it is not the only diagnostic criterion, since flat and small gallbladder and poor contraction are also of important diagnostic and differential diagnostic significance. The degree of hepatomegaly and heterogeneous echogenicity is proportional with liver fibrosis, and able to indicate the duration of course and prognosis.

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**Key words:** Biliary atresia; Ultrasonic diagnosis; The triangular cord; Maldevelopment of gallbladder; Magnetic resonance imaging

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### Abstract

**AIM:** To investigate the clinical value of ultrasonographic diagnosis of biliary atresia (BA), a retrospective analysis of the sonogram of 20 children with BA was undertaken.

**METHODS:** Ultrasonography (US) was performed in 20 neonates and infants with BA, which was confirmed with cholangiography by operation or abdominoscopy. The presence of triangular cord, the size and echo of liver, the changes in empty stomach gallbladder and postprandial gallbladder were observed and recorded.

**RESULTS:** The triangular cord could be observed at the porta hepatis (thickness: 0.3-0.6 cm) in 10 cases. Smaller triangular cord (0.2-0.26 cm) can be observed in 3 cases. The gallbladder was not observed in 2 cases, and 1 case showed a streak gallbladder without capsular space. The gallbladders of 15 cases were flat and small. The gallbladders of 2 cases were of normal size and appearance, however, there was no postprandial contraction. The livers of all cases showed hepatomegaly and heterogeneous echogenicity. Statistical analysis was performed to compare the hepatomegaly and heterogeneous echogenicity and the stage of hepatic fibrosis.

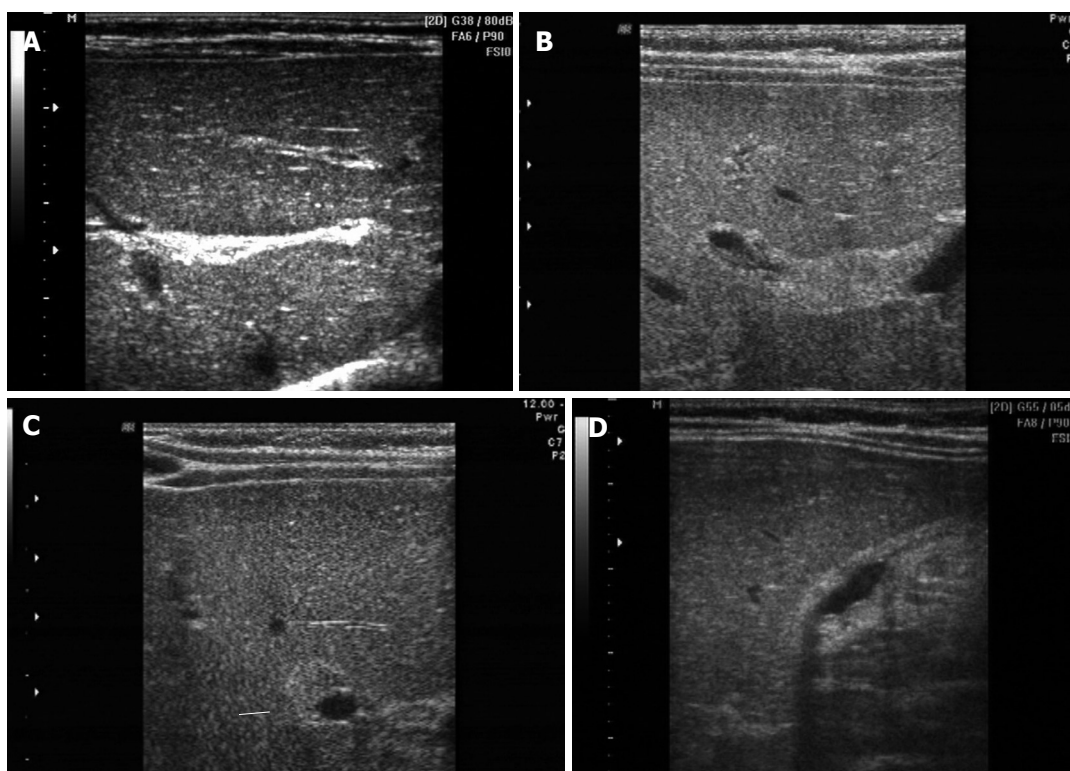
**CONCLUSION:** The presence of the triangular cord at

### INTRODUCTION

Biliary atresia (BA)<sup>[1]</sup> is a common progressive and obstructive pathological change in the intrahepatic and extrahepatic ducts, often causing immediate cirrhosis and leading to death. Prompt and accurate early diagnosis determines its optimal operation opportunity and therapeutic efficacy. With the updating of ultrasonic apparatus, especially the application of high resolution detecting head, the sonogram becomes increasingly clear, and more doctors have accumulated experience with the ultrasonic diagnosis of BA. However, no general and unitive diagnostic criteria for BA are available at present. The present study was to summarize the manifestations of sonogram for BA, and the clinical diagnostic value of ultrasonography (US) for biliary atresia, in order to provide evidence for its early diagnosis.

### MATERIALS AND METHODS

Twenty children with BA admitted to our hospital from January, 2004 to March, 2007 were included in this study. The diagnosis of BA was confirmed at operation in 17



**Figure 1** Ultrasonography. **A:** Fibrous plaque of inverted triangular shape at the confluent site of right and left hepatic ducts; **B:** Longitudinal section of streak-shaped fibrous plaque at the confluent site of right and left hepatic ducts; **C:** Cross section of streak-shaped fibrous plaque at hepatic portal vein (arrow indicates fibrous plaque surrounding the right anterior area of the right branch of portal vein); **D:** Flat and small gallbladder with low tension. The thickness of capsule wall was uneven.

children and at abdominoscopy in 3 children. There were 6 males and 14 females at the age of 37-146 d. According to Kasai classification, BA was classified as type I in 1, type II in 3 and type III in 16 children, respectively.

Voluson 730D (Kretz Company) was used. The frequency of transducer for convex and linear array was 3.5-5.5 MHz and 7.5-10 MHz, respectively. The patients were fasted for 3-4 h before examination. Examination was performed with suckling. Sedative was administered to those who kept crying. The patients were examined at a supine position. Oblique section scan under the right rib and multiple cross section scan between ribs were performed to examine liver, gallbladder, and porta hepatis. The transducer was inclined to the right branch of portal vein. Presence of triangular cord, roughness and internal echo of the liver and gallbladder was recorded. Gallbladder at empty stomach and 1, 2 and 3 h postprandial was determined. Changes in size of gallbladder before and after meal were observed dynamically to understand the contractile function of gallbladder. All the sonograms were maintained in the picture archiving and communication system (PACS).

### Statistical analysis

Pearson correlation coefficient was used to determine the correlation of liver size and heterogeneous echogenicity to the liver fibrosis stage.

## RESULTS

### Triangular cord sign at porta hepatis

Fourteen out of the 20 BA cases were accurately

diagnosed by ultrasound. A triangular cord sign was found at the porta hepatic in 13 cases and an obvious triangular cord was observed in 10 cases. The diameter was about 1.2-2.5 cm and the thickness was about 0.3-0.8 cm. An inverted triangular cord was observed (Figure 1A) and a long streak of the triangular cord was found in 7 cases (Figure 1B). The triangular cord found in 3 cases too small to be identified in 3 cases. The diameter was about 0.9-1.6 cm, and the thickness was about 0.2-0.26 cm. Cross section scan of the right branch of portal vein showed that the triangular cord sign at the porta hepatis surrounded the right anterior part of the right branch of portal vein, rather than the whole part. In addition, cross section scan of the right branch of portal vein was easier than longitudinal section scan of the triangular cord sign. As shown in Figure 1C, one case of BA had no triangular cord sign at the porta hepatis. Six cases were misdiagnosed due to the absence of triangular cord sign at the porta hepatis.

### Changes in size, appearance and contractile function of gallbladder

The manifestations of gallbladder in 20 cases of BA were as follows: (1) absence of gallbladder in 2 cases, (2) vacant and flat gallbladder in 1 case with a streak high echoic area; (3) flat and small gallbladder in 14 cases (Figure 1D), (4) gallbladder with almost a normal size and appearance.

### Size of liver and manifestations of parenchyma echo

The liver in the 20 BA cases was generally enlarged, and its length under the ribs was 1.8-4.5 cm. Rough



parenchyma echo was observed in 7 cases. General echo enhancement and roughness were observed in 13 cases. The degree of hepatomegaly and heterogeneous echogenicity was not related with age, but with the degree of liver fibrosis, suggesting that the size of liver and changes in parenchyma echo could indicate the course and prognosis of BA.

## DISCUSSION

Different diagnostic methods of BA, such as radionuclide-imaging and magnetic resonance cholangio-pancreatography (MRCP), have been reported in the literature<sup>[2-5]</sup>. However, they have different limitations. It was reported that radionuclide-imaging of liver and gallbladder can give false positive results, since there is a fully obstructive stage due to cholangitis or cholestasis in neonatal hepatitis syndrome and infantile hepatitis syndrome<sup>[6-8]</sup>. When there is deficient choleresis or the diameter of biliary tract is too small, they would be easily misdiagnosed as BA<sup>[9-11]</sup>. With the improvement and updating of ultrasonic apparatus, and the enhancement of resolution, clear image can be obtained with a high frequency detecting head. Since the abdominal wall of children is thin, ultrasonic inspection is repeatable, convenient and non-invasive, making it more predominant than other diagnostic methods<sup>[12,13]</sup> for BA.

Some studies demonstrated that the triangular cord at the porta hepatis in BA patients is a direct and specific diadynamic criterion for US, which emphasizes its importance in the diagnosis of BA and significantly increases the accurate rate of BA diagnosis<sup>[14-16]</sup>. In the present study, 13 cases were diagnosed as BA according to the triangular cord at the porta hepatis. Among them, the triangular cord in 3 cases was relatively small and difficult to identify. The diameter was only 0.2-0.26 cm. However, accurate diagnosis was made based on flat and small gallbladder, irregular shape, poor contraction, enlarged liver and parenchyma echo. Six cases were misdiagnosed due to the absence of the triangular cord. The reasons why the triangular cord is absent are as follows: (1) there is no triangular cord sign at the porta hepatic, (2) the triangular cord is too small to be identified, and (3) the doctor cannot show the triangular cord due to his or her inexperience with US<sup>[17-19]</sup>. These problems suggest that the triangular cord is not the only diagnostic criterion for BA. We should not ignore some important indirect changes in liver and gallbladder when diagnosing BA<sup>[20,21]</sup>. When the triangular cord at the porta hepatis is relatively small or does not exist, the size and appearance of gallbladder and liver<sup>[22]</sup>, changes in their contractile function<sup>[23]</sup> and parenchyma echo will help to diagnose BA<sup>[24,25]</sup>.

In this study, the triangular cord at the porta hepatis did not surround the whole right branch of portal vein. Cross section scan of the right branch of portal vein showed that the triangular cord adhered tightly to its right anterior area and was easier to show the triangular cord than longitudinal section scan.

In conclusion, the manifestations of sonograms on

BA can be summarized as follows. (1) The triangular cord sign at the porta hepatis is one of the direct and specific objective criteria for BA, but not the only one. (2) The manifestations of gallbladder can be classified into four types: absence of gall bladder, vacant and flat gallbladder, flat and small gallbladder, and gallbladder with almost normal size and appearance. (3) The degree of hepatomegaly and heterogeneous echogenicity of liver parenchyma echo are positively correlated to liver fibrosis, which is able to indicate duration and prognosis of BA and provide reliable clinical evidence for choosing operation opportunity.

## COMMENTS

### Background

The present research was to summarize the manifestations of sonogram on biliary atresia (BA), and the clinical diagnostic value of ultrasonography (US) for BA. The presence of triangular cord in the right and left hepatic ducts can become an objective index of the ultrasonic diagnosis of BA. However, it is not the only diagnostic criterion. Flat and small gallbladder and poor contraction are also important for diagnosis and differential diagnosis of BA.

### Research frontiers

Studies demonstrated that the triangular cord at the porta hepatis in BA patients is a direct and specific criterion for US, which emphasizes its importance in the diagnosis of BA and significantly increases the accurate BA diagnosis rate. It has become an indispensable evidence for the diagnosis of BA.

### Innovations and breakthroughs

The triangular cord at the porta hepatis did not surround the whole right branch of portal vein. Cross section scan of the right branch of portal vein revealed that the triangular cord adhered tightly to its right anterior area and was easier to show the triangular cord than longitudinal section scan.

### Applications

The manifestations of sonogram on BA and the clinical diagnostic value of US for BA can provide strong evidence for its early diagnosis. The presence of triangular cord in the right and left hepatic ducts was specific, and can become an objective index for the ultrasonic diagnosis of BA. However, it was not the only diagnostic criterion. Flat and small gallbladder and poor contraction were also important in the diagnosis and differential diagnosis of BA.

### Peer review

The authors investigated the clinical value of ultrasonographic diagnosis of BA and a retrospective analysis of the sonogram of 20 children with BA was undertaken. The manuscript is very interesting and the study was well designed.

## REFERENCES

- 1 Shi WJ, Wang QY, Zhang SD, Fei DK, Gao JT, Wang DZ, Liu GL, Xiao SD, Yin JZ, Zhang CL, Lin SC, Fang SD, Jiang GJ, Xu CS, Zhou XS. Biliary tract surgery. 1st ed. Shanghai: Shanghai Science and Technology Publishing Company, 1993: 127
- 2 Norton KI, Glass RB, Kogan D, Lee JS, Emre S, Shneider BL. MR cholangiography in the evaluation of neonatal cholestasis: initial results. *Radiology* 2002; **222**: 687-691
- 3 Cox KL, Stadalnik RC, McGahan JP, Sanders K, Cannon RA, Ruebner BH. Hepatobiliary scintigraphy with technetium-99m disofenin in the evaluation of neonatal cholestasis. *J Pediatr Gastroenterol Nutr* 1987; **6**: 885-891
- 4 Berdon WE, Condon VR, Currarino G, Flitz CR, Leonides JC, Parker B, Slovis T, Wood B. Caffey's Pediatric X-Ray Diagnosis. St Louis, Missouri: Elsevier Mosby, 1993: 1055
- 5 Choi SO, Park WH, Lee HJ, Woo SK. 'Triangular cord': a sonographic finding applicable in the diagnosis of biliary atresia. *J Pediatr Surg* 1996; **31**: 363-366
- 6 Kamińska A, Pawłowska J, Jankowska I, Swiatek-Rawa E, Socha P, Kamiński A, Teisseyre M, Czubkowski P, Toth K.

- Hepatobiliary scanning in the diagnosis of biliary atresia. *Med Sci Monit* 2001; **7** Suppl 1: 110-113
- 7 **Ge YB**, Xu H, Chen J, Lie RH, Xie JQ, Huang H, Wang D. Diagnostic value of 99mTc-EHIDA liver and gall imaging for congenital biliary atresia. *Ji'nan Daxue Xuebao* 2002; **23**: 108
- 8 **Duan QJ**, Wei GF, Cao QH. Differential diagnosis for infantile hepatitis syndrome and congenital biliary atresia using radionuclide dynamic imaging of liver and gall. *Weifang Yixueyuan Xuebao* 1998; **20**: 145
- 9 **Jaw TS**, Kuo YT, Liu GC, Chen SH, Wang CK. MR cholangiography in the evaluation of neonatal cholestasis. *Radiology* 1999; **212**: 249-256
- 10 **Guibaud L**, Lachaud A, Touraine R, Guibal AL, Pelizzari M, Basset T, Pracros JP. MR cholangiography in neonates and infants: feasibility and preliminary applications. *AJR Am J Roentgenol* 1998; **170**: 27-31
- 11 **Fan GM**, Chen LY, Guo QY, Hou Y, Sun CP. MRI diagnosis for biliary atresia. *Zhongguo Yixue Yingxiang Zazhi* 2004; **12**: 244-246
- 12 **Carroll BA**, Oppenheimer DA, Muller HH. High-frequency real-time ultrasound of the neonatal biliary system. *Radiology* 1982; **145**: 437-440
- 13 **Lin WY**, Lin CC, Changlai SP, Shen YY, Wang SJ. Comparison technetium of Tc-99m disofenin cholescintigraphy with ultrasonography in the differentiation of biliary atresia from other forms of neonatal jaundice. *Pediatr Surg Int* 1997; **12**: 30-33
- 14 **Kim MJ**, Park YN, Han SJ, Yoon CS, Yoo HS, Hwang EH, Chung KS. Biliary atresia in neonates and infants: triangular area of high signal intensity in the porta hepatis at T2-weighted MR cholangiography with US and histopathologic correlation. *Radiology* 2000; **215**: 395-401
- 15 **Xie YR**, Lv MD, Zhang ZD, Li GS, Liu JD. Echo of portal fibrous plaque: reliable diagnostic evidence for infantile biliary atresia. *Zhonghua Yixue Chaosheng Zazhi* 1997; **13**: 38-39
- 16 **Park WH**, Choi SO, Lee HJ, Kim SP, Zeon SK, Lee SL. A new diagnostic approach to biliary atresia with emphasis on the ultrasonographic triangular cord sign: comparison of ultrasonography, hepatobiliary scintigraphy, and liver needle biopsy in the evaluation of infantile cholestasis. *J Pediatr Surg* 1997; **32**: 1555-1559
- 17 **Azuma T**, Nakamura T, Nakahira M, Harumoto K, Nakaoka T, Moriuchi T. Pre-operative ultrasonographic diagnosis of biliary atresia--with reference to the presence or absence of the extrahepatic bile duct. *Pediatr Surg Int* 2003; **19**: 475-477
- 18 **Ma JD**, Zhang YL, Ma RB, Yao SQ. Type-B ultrasonic diagnosis and clinical validation for biliary atresia. *Zhongguo Chaosheng Yixue Zazhi* 1996; **12**: 38-40
- 19 **Tan Kendrick AP**, Phua KB, Ooi BC, Subramaniam R, Tan CE, Goh AS. Making the diagnosis of biliary atresia using the triangular cord sign and gallbladder length. *Pediatr Radiol* 2000; **30**: 69-73
- 20 **Tan Kendrick AP**, Phua KB, Ooi BC, Tan CE. Biliary atresia: making the diagnosis by the gallbladder ghost triad. *Pediatr Radiol* 2003; **33**: 311-315
- 21 **Huang ZH**, Yue GR, Dong YT. The differential diagnostic significance of ultrasonic inspection on gallbladder and duodenal juice draining examination for infantile jaundice. *Zhonghua Yixue Zazhi* 2004; **28**: 134-136
- 22 **Huang YJ**, Huang ZH, Yue GR, Dong YS, Wang G. The diagnostic value of ultrasonic inspection on changes of gallbladder size before and after breast-feeding for infantile hepatitis syndrome and biliary atresia. *Guang dong Yixue* 2004; **25**: 39-40
- 23 **Park WH**, Choi SO, Lee HJ. The ultrasonographic 'triangular cord' coupled with gallbladder images in the diagnostic prediction of biliary atresia from infantile intrahepatic cholestasis. *J Pediatr Surg* 1999; **34**: 1706-1710
- 24 **Bates MD**, Bucuvalas JC, Alonso MH, Ryckman FC. Biliary atresia: pathogenesis and treatment. *Semin Liver Dis* 1998; **18**: 281-293
- 25 **Mieli-Vergani G**, Howard ER, Portman B, Mowat AP. Late referral for biliary atresia--missed opportunities for effective surgery. *Lancet* 1989; **1**: 421-423

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## Subclinical peritonitis due to perforated sigmoid diverticulitis 14 years after heart-lung transplantation

Haridimos Markogiannakis, Manousos Konstadoulakis, Dimitrios Tzertzemelis, Pantelis Antonakis, Ilias Gomatos, Constantinos Bramis, Andreas Manouras

Haridimos Markogiannakis, Manousos Konstadoulakis, Dimitrios Tzertzemelis, Pantelis Antonakis, Ilias Gomatos, Constantinos Bramis, Andreas Manouras, 1st Department of Propaedeutic Surgery, Hippokration Hospital, Athens Medical School, University of Athens, Vasilissis Sofias 114 Avenue, Athens 11527, Greece

Author contributions: Markogiannakis H, Tzertzemelis D, Antonakis P, Gomatos I, and Bramis C contributed to the conception and design of the paper and acquisition of data and literature research; Markogiannakis H, Konstadoulakis M, and Manouras A drafted, wrote and revised the manuscript critically for important intellectual content.

Correspondence to: Haridimos Markogiannakis, MD, 1st Department of Propaedeutic Surgery, Hippokration Hospital, Athens Medical School, University of Athens, Aristeidou 239 street, Kallithea, Athens 17673, Greece. [hmarkogiannakis@mycosmos.gr](mailto:hmarkogiannakis@mycosmos.gr)

Telephone: +30-697-6788806 Fax: +30-210-7707574

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of even vague abdominal symptoms, adjustment of immunosuppression, broad-spectrum antibiotics, and immediate surgical treatment are critical. Moreover, strategies to reduce the risk of this complication should be implemented. Pretransplantation colon screening, prophylactic pretransplantation sigmoid resection in patients with diverticulosis, and elective surgical intervention in patients with nonoperatively treated acute diverticulitis after transplantation deserve consideration and further studies.

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**Key words:** Heart-lung transplantation; Acute diverticulitis; Colon perforation; Subclinical peritonitis

**Peer reviewer:** Mauro Bernardi, Professor, Internal Medicine, Cardioangiology, Hepatology, University of Bologna, Semeiotica Medica-Policlinico S. Orsola-Malpighi-Via Massarenti, 9, Bologna 40138, Italy

### Abstract

Acute complicated diverticulitis, particularly with colon perforation, is a rare but serious condition in transplant recipients with high morbidity and mortality. Neither acute diverticulitis nor colon perforation has been reported in young heart-lung grafted patients. A case of subclinical peritonitis due to perforated acute sigmoid diverticulitis 14 years after heart-lung transplantation is reported. A 26-year-old woman, who received heart-lung transplantation 14 years ago, presented with vague abdominal pain. Physical examination was normal. Blood tests revealed leukocytosis. Abdominal X-ray showed air-fluid levels while CT demonstrated peritonitis due to perforated sigmoid diverticulitis. Sigmoidectomy and end-colostomy (Hartmann's procedure) were performed. Histopathology confirmed perforated acute sigmoid diverticulitis. The patient was discharged on the 8th postoperative day after an uneventful postoperative course. This is the first report of acute diverticulitis resulting in colon perforation in a young heart-lung transplanted patient. Clinical presentation, even in peritonitis, may be atypical due to the masking effects of immunosuppression. A high index of suspicion, urgent aggressive diagnostic investigation

Markogiannakis H, Konstadoulakis M, Tzertzemelis D, Antonakis P, Gomatos I, Bramis C, Manouras A. Subclinical peritonitis due to perforated sigmoid diverticulitis 14 years after heart-lung transplantation. *World J Gastroenterol* 2008; 14(22): 3583-3586 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3583.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3583>

### INTRODUCTION

Abdominal complications are common in solid organ transplant recipients. They include esophagitis, gastritis, duodenitis, hernia, cholelithiasis or diverticulosis with minor morbidity and peptic ulcer disease, choledocholithiasis, acute cholecystitis, acute cholangitis, acute pancreatitis, hepatitis, hepatic or pancreatic abscess, bowel obstruction, megacolon, colitis, perirectal abscess, acute appendicitis, acute diverticulitis, gastrointestinal tract hemorrhage and viscus perforation with significant morbidity and mortality<sup>[1-12]</sup>. They may occur early after transplantation or even years later<sup>[4,5,7,8,10,12]</sup>. Successful management requires a high index of suspicion, early diagnosis and prompt treatment; wherever doubt exists, laparotomy is the appropriate way to establish a definite diagnosis<sup>[1,2-4,7,9,10,12]</sup>.



**Figure 1** Abdominal X-ray showed air-fluid levels.



**Figure 2** Abdominal CT scan revealed a markedly dilated sigmoid colon, extrasceral free air, and peritonitis.

Colon perforations may also occur in the early to late post-transplantation period<sup>[4,5,9,12]</sup>. Early-onset perforations are secondary to perioperative hypoperfusion, increased intraluminal pressure from narcotics, use of bowel stimulants, and high-dose immunosuppression while late perforations are often related to acute diverticulitis and invasive fungal or viral colonic disease<sup>[4,5,9,12]</sup>.

Acute complicated diverticulitis, particularly with colon perforation, is a rare but very serious condition in transplant recipients and remains a challenging surgical problem. Furthermore, neither acute diverticulitis nor colon perforation has been reported until now in heart-lung transplanted young patients. We herein report a case of subclinical peritonitis due to perforated acute sigmoid diverticulitis 14 years after heart-lung transplantation in a 26-year-old woman.

## CASE REPORT

A 26-year-old female patient presented with vague abdominal pain of 24 h duration. The patient received combined heart-lung transplantation because of Eisenmenger's syndrome 14 years ago. During the post-transplantation period, her medical history was uneventful. She was treated with azathioprine and cyclosporine. On admission, vital signs and physical examination were normal while blood tests

revealed leukocytosis (WBC:  $19 \times 10^9/L$ ). Upright chest radiography was normal and abdominal X-ray showed air-fluid levels but no free air (Figure 1). Tests were negative for acute cytomegalovirus (CMV) disease. Abdominal computed tomography (CT) scan demonstrated sigmoid colon perforation resulting in peritonitis (Figure 2).

Operative intervention was carried out immediately under broad spectrum antibiotic coverage. Laparotomy revealed diffuse fecal contamination of the abdomen due to perforated sigmoid diverticulitis. Meticulous lavage of the peritoneal cavity, sigmoidectomy and an end-colostomy (Hartmann's procedure) were performed. Histopathologic examination of the resected specimen confirmed sigmoid colon diverticula, acute diverticulitis, and perforation of one diverticulum. No signs of bacterial, fungal, or viral infection were detected.

The postoperative course was uneventful and the patient was discharged on the 8th postoperative day. The colostomy was closed 6 months later. At a follow-up of 6 months, she is alive with no recurrence of colonic disease.

## DISCUSSION

Abdominal and, particularly, gastrointestinal complications are common in transplanted patients representing an important source of morbidity and mortality<sup>[2-4]</sup>. They are reported in 6.3%-51% of solid organ transplanted patients with a mortality rate, particularly of major complications, of 7.7%-63%<sup>[1-6,8,9,13-16]</sup>. Clinical presentation, even in peritonitis, may be atypical in the immunosuppressed transplant recipient due to the masking effects of the immunosuppression, particularly steroid medication on symptoms and signs<sup>[5,8,16]</sup>. This may result in delayed presentation and diagnosis or even misdiagnosis<sup>[15]</sup>. This patient, although not receiving corticosteroids, but azathioprine and cyclosporine, presented with vague abdominal pain and normal abdominal clinical examination despite suffering from peritonitis due to colonic perforation.

The high mortality of gastrointestinal complications appears to be related to the effects of the immunosuppression and the associated response to sepsis. Reduction of these complications and associated morbidity and mortality can be achieved by improved preventive measures, prompt diagnosis, immediate surgical management, and reduced or steroid-sparing immunosuppressive protocols<sup>[1,2,9,10,12,16]</sup>. Increased dosages of maintenance steroid regimens may correlate with the occurrence of gastrointestinal complications<sup>[16]</sup> while the time interval between onset of symptoms and operative intervention and mean corticosteroid dose are associated with mortality<sup>[10]</sup>.

Asymptomatic diverticulosis has been reported in 39.8% of heart transplanted patients<sup>[7]</sup>. In a study of renal transplant recipients, the incidence of diverticulosis was 12.7%; major colonic complications eventually developed in 57.1% of these patients resulting in a mortality of 75%<sup>[17]</sup>. In patients with diverticulosis and



a previous history of diverticulitis, consideration for elective segmental colectomy prior to transplantation was, thus, advocated<sup>[17]</sup>. It is worth mentioning, however, that the presence of colonic diverticula is deemed unlikely in young patients such as this case, and this may lead to late diagnosis. Although uncommon, the presented complication should, therefore be included in the differential diagnosis of young transplanted patients presenting with vague abdominal complaints.

Acute diverticulitis is a relatively rare, but serious problem in transplanted patients<sup>[6,11,12,15]</sup>. It is reported in 0.4%-8% of solid organ transplanted patients with a 0%-83.3% mortality rate<sup>[4,8,11,16,18-20]</sup>. However, no case of acute diverticulitis in patients with heart-lung transplantation has been reported before, particularly in young patients. Compared to the general population, heart and lung transplant recipients are at substantially increased risk of experiencing acute diverticulitis<sup>[18,20]</sup>. In the study of Qasabian *et al.*, all patients with severe diverticulitis survived and death occurred only in a conservatively treated patient in whom a definite diagnosis was not reached until post-mortem examination was made<sup>[18]</sup>.

Diagnosis of diverticulitis poses a significant challenge in the immunosuppressed patients<sup>[11,18,19]</sup>. Symptoms and physical examination usually do not reflect the severity of the disease while signs of infection, such as fever and tachycardia, may be absent. Laboratory testing is often unreliable and misleading in estimating the severity of the disease. Abdominal radiographs may be non-specific such as in our patient. Abdominal CT scan is the most reliable modality in determining the location and degree of pathology. A high index of suspicion, prompt diagnosis, reduction of immunosuppression, and early administration of broad spectrum antibiotics are essential for management and prognosis of these patients<sup>[11,18-20]</sup>. If bowel perforation is suspected, immediate operative intervention with bowel resection and colostomy is indicated. In the setting of uncomplicated diverticulitis, it is prudent to proceed with colectomy following resolution of the acute episode, provided the patient is a reasonable operative candidate<sup>[11,18-20]</sup>.

Colon perforation has been observed in 0.5%-5.3% of solid organ recipients with a mortality of 0%-100%<sup>[1,3-6,13-15]</sup>. Moreover, colon perforation due to acute diverticulitis is a rare complication with a high mortality<sup>[2,4,9,10,12]</sup>. It has been described in kidney, heart, and lung transplant recipients<sup>[2,4,9,10,12,14]</sup>. Regarding colon perforation due to acute diverticulitis in renal graft recipients, 0.5%-3% have been reported to sustain perforation with a mortality of 12.5%-83.3%<sup>[2,9,10]</sup>. In heart and lung grafted patients, this incidence is 3.4% and 4.8%-6.7%, respectively, with a mortality of 0%-50%<sup>[12,16]</sup>. However, up to now, there has been no report of colon perforation due to acute diverticulitis in heart-lung transplanted patients.

History of diverticula<sup>[2]</sup> or acute diverticulitis<sup>[10]</sup> and steroid therapy<sup>[16]</sup> have been found to be risk factors for colon perforation. The time between onset of symptoms and operation, intensity of immunosuppression (mean corticosteroid dose), and nutritional status (levels of serum

albumin) are associated with the mortality of patients with colon perforation<sup>[9,12]</sup>. Immediate diagnosis and early, aggressive care consisting of surgical intervention, broad spectrum antibiotics, and reduced or steroid-sparing immunosuppression are crucial to outcome<sup>[8,10,12]</sup>. Furthermore, although not uniformly supported in the literature, pretransplantation colon screening and prophylactic sigmoidectomy in patients with diverticulosis, and elective surgical intervention in patients with a nonoperatively treated episode of acute diverticulitis after transplantation have been suggested<sup>[11,12,18,20]</sup>.

Even though in the presence of an acute abdomen laparotomy is the appropriate approach to establish a definitive diagnosis and to treat these patients, abdominal operations for urgent surgical conditions are associated with increased morbidity and mortality<sup>[4,12]</sup>. Significant reduction of morbidity and mortality can be achieved by prompt diagnosis and early surgical management<sup>[1,2,9,10]</sup>.

In conclusion, acute complicated diverticulitis, particularly with colon perforation, is a rare but serious condition in transplanted patients with high morbidity and mortality. Our patient is the first case of acute diverticulitis resulting in colon perforation in a young heart-lung graft recipient in the literature. Clinical presentation may be atypical. A high index of suspicion, urgent aggressive diagnostic investigation of even vague abdominal symptoms, adjustment of immunosuppression, adequate selection of antimicrobial agents, and prompt surgical treatment are critical. Strategies to reduce the risk of this complication should also be implemented in transplantation centres; thus, pretransplantation colon screening, prophylactic pretransplantation sigmoidectomy in patients with diverticulosis and elective surgical intervention in patients with a prior episode of acute diverticulitis after transplantation deserve further consideration and studies.

## REFERENCES

- 1 **Merrell SW**, Ames SA, Nelson EW, Renlund DG, Karwande SV, Burton NA, Sullivan JJ, Jones KW, Gay WA Jr. Major abdominal complications following cardiac transplantation. Utah Transplantation Affiliated Hospitals Cardiac Transplant Program. *Arch Surg* 1989; **124**: 889-894
- 2 **Benoit G**, Moukarzel M, Verdelli G, Hiesse C, Buffet C, Bensadoun H, Charpentier B, Jardin A, Fries D. Gastrointestinal complications in renal transplantation. *Transpl Int* 1993; **6**: 45-49
- 3 **Smith PC**, Slaughter MS, Petty MG, Shumway SJ, Kshetry VR, Bolman RM 3rd. Abdominal complications after lung transplantation. *J Heart Lung Transplant* 1995; **14**: 44-51
- 4 **Maurer JR**. The spectrum of colonic complications in a lung transplant population. *Ann Transplant* 2000; **5**: 54-57
- 5 **Watson CJ**, Jamieson NV, Johnston PS, Wreghitt T, Large S, Wallwork J, English TA. Early abdominal complications following heart and heart-lung transplantation. *Br J Surg* 1991; **78**: 699-704
- 6 **Augustine SM**, Yeo CJ, Buchman TG, Achuff SC, Baumgartner WA. Gastrointestinal complications in heart and in heart-lung transplant patients. *J Heart Lung Transplant* 1991; **10**: 547-555; discussion 555-556
- 7 **Rodriguez-Larrain JM**, Ziebert JJ, Kfoury AG, Kuwada S, Taylor DO, Renlund DG. Incidence of adenomatous colorectal polyps in cardiac transplant recipients.

- Transplantation* 1997; **64**: 528-530
- 8 **Lederman ED**, Conti DJ, Lempert N, Singh TP, Lee EC. Complicated diverticulitis following renal transplantation. *Dis Colon Rectum* 1998; **41**: 613-618
- 9 **Stelzner M**, Vlahakos DV, Milford EL, Tilney NL. Colonic perforations after renal transplantation. *J Am Coll Surg* 1997; **184**: 63-69
- 10 **Pirenne J**, Lledo-Garcia E, Benedetti E, West M, Hakim NS, Sutherland DE, Gruessner RW, Najarian JS, Matas AJ. Colon perforation after renal transplantation: a single-institution review. *Clin Transplant* 1997; **11**: 88-93
- 11 **Detry O**, Defraigne JO, Meurisse M, Bertrand O, Demoulin JC, Honore P, Jacquet N, Limet R. Acute diverticulitis in heart transplant recipients. *Transpl Int* 1996; **9**: 376-379
- 12 **Beaver TM**, Fullerton DA, Zamora MR, Badesch DB, Weill D, Brown JM, Campbell DN, Grover FL. Colon perforation after lung transplantation. *Ann Thorac Surg* 1996; **62**: 839-843
- 13 **Owens ML**, Wilson SE, Saltzman R, Gordon HE. Gastrointestinal complications after renal transplantation: predictive factors and morbidity. *Arch Surg* 1976; **111**: 467-471
- 14 **Faro RS**, Corry RJ. Management of surgical gastrointestinal complications in renal transplant recipients. *Arch Surg* 1979; **114**: 310-312
- 15 **Steed DL**, Brown B, Reilly JJ, Peitzman AB, Griffith BP, Hardesty RL, Webster MW. General surgical complications in heart and heart-lung transplantation. *Surgery* 1985; **98**: 739-745
- 16 **Mueller XM**, Tevæarai HT, Stumpe F, Hurni M, Ruchat P, Fischer AP, Seydoux C, Goy JJ, von Segesser LK. Gastrointestinal disease following heart transplantation. *World J Surg* 1999; **23**: 650-655; discussion 655-656
- 17 **Sawyer OI**, Garvin PJ, Codd JE, Graff RJ, Newton WT, Willman VL. Colorectal complications of renal allograft transplantation. *Arch Surg* 1978; **113**: 84-86
- 18 **Qasabian RA**, Meagher AP, Lee R, Dore GJ, Keogh A. Severe diverticulitis after heart, lung, and heart-lung transplantation. *J Heart Lung Transplant* 2004; **23**: 845-849
- 19 **Khan S**, Eppstein AC, Anderson GK, Dengal MK, Eggenberger JC, Lee CS, Szilagy EJ, Margolin DA. Acute diverticulitis in heart- and lung transplant patients. *Transpl Int* 2001; **14**: 12-15
- 20 **Goldberg HJ**, Hertz MI, Ricciardi R, Madoff RD, Baxter NN, Bullard KM. Colon and rectal complications after heart and lung transplantation. *J Am Coll Surg* 2006; **202**: 55-61

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## Resection of a locally advanced hilar tumor and the hepatic artery after stepwise hepatic arterial embolization: A case report

Takuya Miura, Kenichi Hakamada, Takashi Ohata, Shunji Narumi, Yoshikazu Toyoki, Masaki Nara, Keinosuke Ishido, Motonari Ohashi, Harue Akasaka, Hiroyuki Jin, Norihito Kubo, Shuichi Ono, Hiroshi Kijima, Mutsuo Sasaki

Takuya Miura, Kenichi Hakamada, Shunji Narumi, Yoshikazu Toyoki, Masaki Nara, Keinosuke Ishido, Motonari Ohashi, Harue Akasaka, Hiroyuki Jin, Norihito Kubo, Mutsuo Sasaki, Department of Gastroenterological Surgery, Hirosaki University Graduate School of Medicine, Hirosaki 036-8562, Japan

Takashi Ohata, Shuichi Ono, Department of Radiology and Radiation Oncology, Hirosaki University Graduate School of Medicine, Hirosaki 036-8562, Japan

Motonari Ohashi, Harue Akasaka, Hiroyuki Jin, Hiroshi Kijima, Department of Pathology and Bioscience, Hirosaki University Graduate School of Medicine, Hirosaki 036-8562, Japan

**Author contributions:** Hakamada K, Narumi S, Toyoki Y, Nara M, Ishido K, Kubo N and Sasaki M followed the patient; Ohata T and Ono S performed the radiological intervention; Ohashi M, Akasaka H, Jin H and Kijima H performed the pathologic evaluation; Miura T wrote the paper; Hakamada K supervised the writing.

**Correspondence to:** Kenichi Hakamada, MD, Department of Gastroenterological Surgery, Hirosaki University Graduate School of Medicine, 5 Zaifu-cho, Hirosaki 036-8562, Japan. [hakamada@cc.hirosaki-u.ac.jp](mailto:hakamada@cc.hirosaki-u.ac.jp)

Telephone: +81-172-395079 Fax: +81-172-395080

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of the posterior and anterior hepatic arteries two weeks apart. Finally, the proper hepatic artery was occluded after formation of collateral flow from the inferior phrenic and superior mesenteric arteries was confirmed. One month later, a left hepatectomy with hepatic arterial resection was successfully performed without any major complications.

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**Key words:** Hepatic arterial embolization; Stepwise; Hilar tumor; Arterial resection; Collateral artery

**Peer reviewer:** Tadatoshiki Takayama, Professor, Department of Digestive Surgery, Nihon University School of Medicine, 30-1 Oyaguchikami-machi, Itabashi-ku, Tokyo 173-8610, Japan

Miura T, Hakamada K, Ohata T, Narumi S, Toyoki Y, Nara M, Ishido K, Ohashi M, Akasaka H, Jin H, Kubo N, Ono S, Kijima H, Sasaki M. Resection of a locally advanced hilar tumor and the hepatic artery after stepwise hepatic arterial embolization: A case report. *World J Gastroenterol* 2008; 14(22): 3587-3590 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3587.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3587>

### Abstract

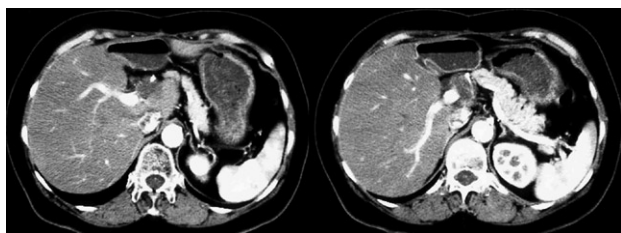
We herein report a case of a hilar tumor with extensive invasion to the proper hepatic artery, which was successfully treated with a radical resection in a 57-year-old female patient after a stepwise hepatic arterial embolization. She underwent right colectomy and partial hepatectomy for advanced colon cancer two years ago and radiofrequency ablation therapy for a liver metastasis one year ago, respectively. A recurrent tumor was noted around the proper hepatic artery with invasion to the left hepatic duct and right hepatic artery 7 mo previously. We planned a radical resection for the patient 5 mo after the absence of tumor progression was confirmed while he was undergoing chemotherapy. To avoid surgery-related liver failure, we tried to promote the formation of collateral hepatic arteries after stepwise arterial embolization

### INTRODUCTION

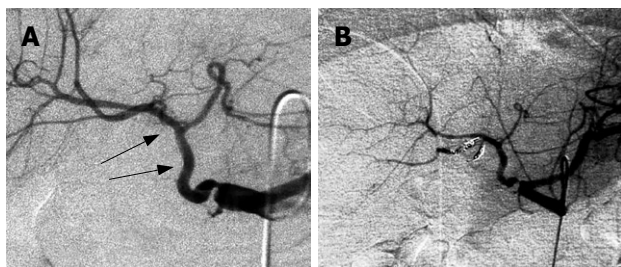
Locally advanced hilar tumor often involves the hepatic artery (HA) within the hepatoduodenal ligament, and a concomitant resection may be required for its cure. However, hepatic arterial resection is uncommon, because the reconstruction requires microvascular techniques<sup>[1-3]</sup> and major complications may occur<sup>[4,5]</sup>. Arterioportal shunting has reported as an alternative to microvascular reconstruction<sup>[6,7]</sup>. However, this approach also requires vascular reconstruction and could cause an occlusion. We herein report the use of stepwise arterial embolization as another alternative to microvascular reconstruction and arterioportal shunting after performing a hepatic arterial resection.

### CASE REPORT

A 57-year-old female patient underwent a right



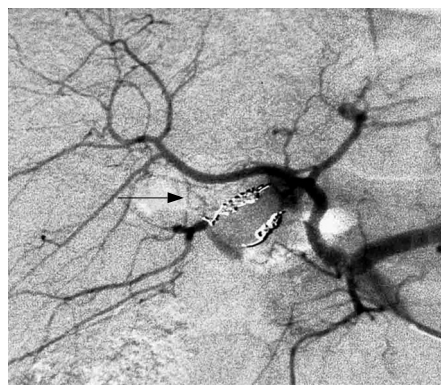
**Figure 1** A CT during hepatic angiography showing a recurrent tumor with extensive invasion to the proper HA.



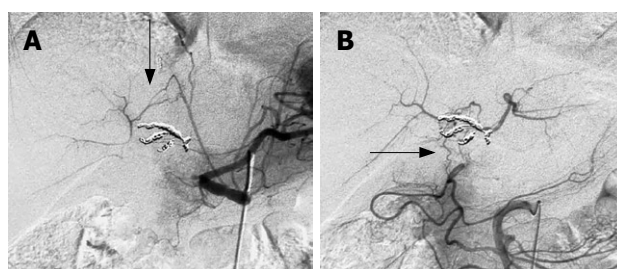
**Figure 2** An angiogram of the celiac artery showing the proper and right arterial encasements (arrows) (A) and PRHA embolized with coils (B).

colectomy for ascending colon cancer in March, 2001. In January, 2003, liver metastasis was noted in the left medial segment (Couinaud's Segment 4; S4). She underwent a partial hepatectomy and ligation of the gastroduodenal and right gastric arteries for hepatic arterial infusion. In September, 2004, a recurrence in S4 was identified and a partial hepatectomy was carried out again. In September, 2005, a metastatic tumor appeared in S4 again and radiofrequency ablation therapy was performed.

In April, 2006, a recurrent tumor was noted around the proper HA with invasion to the left hepatic duct and the proper and right HAs (Figure 1). After 5 mo of continuing chemotherapy, we decided to perform a radical resection since no tumor progression or other metastatic lesions were observed. For the radical resection, left hepatectomy and proper hepatic arterial resection were needed. To avoid surgery-related liver failure, we tried to form collateral hepatic arteries after stepwise arterial embolization of the HA. At first, the posterior right hepatic artery (PRHA) was embolized with interlocking detachable coils (IDC) and fibered platinum coils (FPC; Boston Scientific, USA) (Figure 2). We placed seven IDC with their diameter ranging from 2 mm to 4 mm and length ranging from 4 cm to 8 cm, and 3 FPC (straight) measuring 5 mm in length. Two weeks later, the branches of the PRHA were enhanced from the branches of the anterior right hepatic artery (ARHA) (Figure 3). After the development of collateral arteries was confirmed in the right liver, the ARHA was embolized with IDC and FPC. We placed nine IDC, with their diameter ranging from 2 mm to 5 mm and length ranging from 4 cm to 6 cm, and 4 FPC (straight) measuring 5 mm in length. Finally, the proper HA was occluded with IDC after confirming the formation of collateral flow from the inferior phrenic artery (IPA) and the superior mesenteric artery (SMA)



**Figure 3** An embolized angiogram of the celiac artery 2 wk after enlargement of PRHA with its branches supplied from branches of the ARHA (arrow).



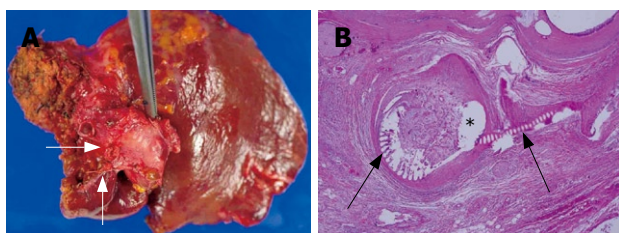
**Figure 4** An angiogram of the celiac artery after the proper HA embolized with coils showing the collateral pathways from IPA to the branches of PRHA (arrow) (A), and an angiogram of the SMA after the proper hepatic artery embolized with coils showing the collateral pathways from SMA to the branches of ARHA (arrow) (B).

was confirmed (Figure 4). We placed two IDC, 2 mm and 4 mm in diameter, 4 cm and 8 cm in length, respectively. After such embolization, the patient had no complications including hepatic dysfunction or liver abscess.

One month later, a left hepatectomy was performed with hepatic arterial resection. The tumor was located around the common, proper, anterior right and posterior right HA. Fortunately, the portal vein and bile duct were not resected because the tumor did not invade them. The operating time was 325 min. The operative blood loss was 1380 mL and no red blood cell transfusion was required. The serum concentration of aspartate aminotransferase and alanine aminotransferase one day after the operation was 381 U/L and 259 U/L, respectively, which returned to normal within 5 d. The maximum postoperative concentration of total serum bilirubin was 1.5  $\mu\text{mol/L}$ . Fourteen days after the operation, a biloma from the cut margin of the liver was noticed and treated with percutaneous drainage. Thereafter, it improved and the patient was discharged 30 d after the operation without major complications.

The tumor measured 3.0 cm  $\times$  2.5 cm  $\times$  4.0 cm (Figure 5A). Histological examination of the resected specimen revealed tumor invasion of the HA (Figure 5B). Neural invasion also was observed. The radial margin was free of tumor. The histological diagnosis was mucinous adenocarcinoma compatible with metastasis of colon cancer.





**Figure 5** Tumor around the proper HA showing the anterior and posterior right hepatic arterial stump (arrows) (A) and histology revealing direct invasion of the tumor into the vascular wall of proper HA (\*) (HE staining, x 200) with traces of the coils in vascular lumen (arrow) (B).

## DISCUSSION

Because hilar tumor approximates the HA, it easily spreads to it. It is necessary to excise the tumor with negative margins to cure it. A concomitant vascular resection could be employed whenever the tumor invades the HA supplying the remnant liver. It is necessary to preserve the hepatic arterial flow, as an interruption of the arterial flow can cause major complications, such as liver failure and liver abscess<sup>[8,9]</sup>. It was reported that arterial reconstruction using a microvascular anastomosis is useful<sup>[1-3]</sup>. However, the approach is difficult in cases in which the arterial diameter is small due to with multiple arterial stumps or the deep operative field is deep<sup>[4]</sup>. As an alternative, an arteriportal shunt has been reported with favorable results<sup>[6,7]</sup>. This procedure also requires vascular reconstruction with the possibility of fatal complications, including occlusion, aneurysm, or dissection<sup>[4-7]</sup>.

Stepwise hepatic arterial embolization does not require a vascular surgical procedure, can eliminate the possibility of the above complications and decrease the complexity of the surgical procedure and the operating time. In addition, it makes possible to perform an en bloc resection of the tumor and lymph nodes around the proper and common HA. If an extra-hepatic bile duct resection with removal of the HA can sufficiently eradicate the tumor, an extensive hepatectomy would be unnecessary. Therefore, in regard to safety and potential cure, this approach is acceptable for a hilar tumor with hepatic arterial invasion.

Wachsberg *et al.*<sup>[10]</sup> reported a case in which liver necrosis occurred after the right HA was occluded. It was reported that in most patients who undergo angiography within 1 or 2 wk after hepatic arterial ligation, collateral arteries are present and subsequent angiograms show progressive enlargement<sup>[11]</sup>. We scheduled embolization of the PRHA, ARHA, and proper HA at 2 week intervals. As a result, collateral pathways from ARHA to PRHA, IPA to PRHA, and SMA to ARHA were formed without complication.

This procedure also has some limitations. For example, whether collateral arteries form as expected. Takeuchi *et al.*<sup>[12]</sup> reported that almost all patients have an extra-hepatic arterial supply from the IPA and SMA. Since intersegmental hepatic collateral arteries are formed after hepatic arterial embolization<sup>[13]</sup>, collateral vessels should form as expected. However, if no collateral vessels are formed, an arteriportal shunt may be required.

Though this case involved a metastatic tumor, almost all cases of hilar tumor are associated with biliary cancer. Usually, the bile duct is excised and a bilioenteric anastomosis is performed. In the case of stepwise hepatic arterial embolization, the bile duct stump lacks of arterial blood flow *via* the hepatoduodenal ligament and is only supplied from collateral arteries through the liver. An impairment of the blood supply to the bile duct stump could cause disruption of the bilioenteric anastomosis. However, Plengvanit *et al.*<sup>[11]</sup> showed that collateral vessels enter the liver peripherally after ligation of HA, and the blood fills the branches of HA to the occluded portion in a retrograde fashion. It is presumed that a breakdown of the bilioenteric anastomosis does not occur because sufficient arterial blood flow can reach the bile duct stump *via* the intrahepatic shunts which mainly come from the IPA. Because the left and right IPA contact the bare area of the liver segments 1, 2, and 7<sup>[14]</sup>, this method is useful not only for the left trisegmentectomy with resection of the right hepatic artery but also for the right trisegmentectomy with resection of the left hepatic artery.

In conclusion, stepwise hepatic arterial embolization is one of the useful methods for performing resection of locally advanced hilar tumor invading HA, although the safety of the procedure needs to be confirmed in other cases.

## REFERENCES

- 1 Yamanaka N, Yasui C, Yamanaka J, Ando T, Kuroda N, Maeda S, Ito T, Okamoto E. Left hemihepatectomy with microsurgical reconstruction of the right-sided hepatic vasculature. A strategy for preserving hepatic function in patients with proximal bile duct cancer. *Langenbecks Arch Surg* 2001; **386**: 364-368
- 2 Yamamoto Y, Sugihara T, Sasaki S, Furukawa H, Furukawa H, Okushiba S, Nohira K. Microsurgical reconstruction of the hepatic and superior mesenteric arteries using a back wall technique. *J Reconstr Microsurg* 1999; **15**: 321-325
- 3 Sakamoto Y, Sano T, Shimada K, Kosuge T, Kimata Y, Sakuraba M, Yamamoto J, Ojima H. Clinical significance of reconstruction of the right hepatic artery for biliary malignancy. *Langenbecks Arch Surg* 2006; **391**: 203-208
- 4 Inomoto T, Nishizawa F, Sasaki H, Terajima H, Shirakata Y, Miyamoto S, Nagata I, Fujimoto M, Moriyasu F, Tanaka K, Yamaoka Y. Experiences of 120 microsurgical reconstructions of hepatic artery in living related liver transplantation. *Surgery* 1996; **119**: 20-26
- 5 Matsuda H, Yagi T, Sadamori H, Matsukawa H, Shinoura S, Murata H, Umeda Y, Tanaka N. Complications of arterial reconstruction in living donor liver transplantation: a single-center experience. *Surg Today* 2006; **36**: 245-251
- 6 Kondo S, Hirano S, Ambo Y, Tanaka E, Kubota T, Katoh H. Arteriportal shunting as an alternative to microvascular reconstruction after hepatic artery resection. *Br J Surg* 2004; **91**: 248-251
- 7 Iseki J, Noie T, Touyama K, Nakagami K, Takagi M, Ori T, Ooba N, Ito K. Mesenteric arteriportal shunt after hepatic artery interruption. *Surgery* 1998; **123**: 58-66
- 8 Miyazaki M, Ito H, Nakagawa K, Ambiru S, Shimizu H, Yoshidome H, Shimizu Y, Okaya T, Mitsuhashi N, Wakabayashi Y, Nakajima N. Unilateral hepatic artery reconstruction is unnecessary in biliary tract carcinomas involving lobar hepatic artery: implications of interlobar hepatic artery and its preservation. *Hepatogastroenterology*

- 2000; **47**: 1526-1530
- 9 **Tsuge H**, Mimura H, Hamazaki K, Mori M, Kawata N, Orita K. Interruption of hepatic arterial blood flow after resection of pancreaticobiliary carcinoma. *Hepatogastroenterology* 1995; **42**: 966-974
- 10 **Wachsberg RH**, Cho KC, Raina S. Liver infarction following unrecognized right hepatic artery ligation at laparoscopic cholecystectomy. *Abdom Imaging* 1994; **19**: 53-54
- 11 **Plengvanit U**, Chearanai O, Sindhvananda K, Dambrongsak D, Tuchinda S, Viranuvatti V. Collateral arterial blood supply of the liver after hepatic artery ligation, angiographic study of twenty patients. *Ann Surg* 1972; **175**: 105-110
- 12 **Takeuchi Y**, Arai Y, Inaba Y, Ohno K, Maeda T, Itai Y. Extrahepatic arterial supply to the liver: observation with a unified CT and angiography system during temporary balloon occlusion of the proper hepatic artery. *Radiology* 1998; **209**: 121-128
- 13 **Charnsangavej C**, Chuang VP, Wallace S, Soo CS, Bowers T. Angiographic classification of hepatic arterial collaterals. *Radiology* 1982; **144**: 485-494
- 14 **Gwon DI**, Ko GY, Yoon HK, Sung KB, Lee JM, Ryu SJ, Seo MH, Shim JC, Lee GJ, Kim HK. Inferior phrenic artery: anatomy, variations, pathologic conditions, and interventional management. *Radiographics* 2007; **27**: 687-705

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## Ischemic colitis associated with intestinal vasculitis: Histological proof in systemic lupus erythematosus

Jeong Rok Lee, Chang Nyol Paik, Jin Dong Kim, Woo Chul Chung, Kang-Moon Lee, Jin Mo Yang

Jeong Rok Lee, Chang Nyol Paik, Jin Dong Kim, Woo Chul Chung, Kang-Moon Lee, Jin Mo Yang, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, St. Vincent's Hospital, Suwon Si, Paldal-gu, Gyeonggi-Do 442-723, South Korea

**Author contributions:** Paik CN and Lee JR collected data, design the research; Paik CN, Kim JD, Chung WC, Lee KM, and Yang JM contributed to conception, carried out the literature research; Paik CN, Lee JR, Chung WC, and Lee KM prepared the manuscript. Paik CN and Lee JR wrote/draft the paper.

**Correspondence to:** Chang Nyol Paik, MD, Department of Internal Medicine, College of Medicine, The Catholic University of Korea, St. Vincent's Hospital Ji-dong, Suwon Si, Paldal-gu, Gyeonggi-Do 442-723, South Korea. [cmcu@catholic.ac.kr](mailto:cmcu@catholic.ac.kr)

Telephone: +82-31-2497137 Fax: +82-31-2538898

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### INTRODUCTION

Ischemic colitis, an acute abdominal disease, is caused by various predisposing factors. Although patients with connective tissue disorders, such as systemic lupus erythematosus (SLE), are at risk for various forms of colonic ischemia due to impairment of small vessel circulation to the large bowel caused by widespread vasculitis, ischemic colitis is an uncommon gastrointestinal complication in patients with SLE<sup>[1-8]</sup>. We report a case of ischemic colitis associated with intestinal vasculitis and presented with increased SLE activity.

### CASE REPORT

A 32-year-old male with a history of SLE was admitted to our department due to a two-day history of abdominal pain and bloody stool. He was diagnosed as having SLE. He had received cyclophosphamide and steroid, had been in clinical remission since last 13 mo. At 3 mo before admission, immunologic tests showed stable SLE disease activity. On the day of admission, the vital signs were normal except for body temperature of 37.8°C. Physical examination revealed mild tenderness of the left lower abdomen but no peritoneal signs or organomegaly. Initial laboratory test results were as follows: white blood cell count,  $8.7 \times 10^9/L$  (normal,  $4.5 \times 10^9/L$ - $10.5 \times 10^9/L$ ); hemoglobin, 14.7 g/dL; platelet count,  $195 \times 10^9/L$  (normal,  $150 \times 10^9/L$ - $350 \times 10^9/L$ ); blood urea nitrogen, 27 mg/dL; creatinine, 1.1 mg/dL. The results of electrolytes and coagulation were normal. Immunologic test showed increased activity of SLE: ESR, 48 mm/h (normal, 0-20 mm/h); CRP, 6.79 mg/dL (normal, 0-0.3 mg/dL); antinuclear antibody (ANA), a titer of 1:200 (normal, < 1:50); C3, 108 mg/dL (normal, 84-151 mg/dL); C4, 23 mg/dL (normal, 17-40 mg/dL), antiphospholipid antibody, 2.0 U/mL (normal, < 10 U/mL); anti-Smith antibody, positive; anti-double stranded DNA antibody, 43.91 IU/mL (normal, < 5.30 IU/mL). Flexible sigmoidoscopy demonstrated severe inflammation of the mucosa extending from rectosigmoid junction to the splenic

### Abstract

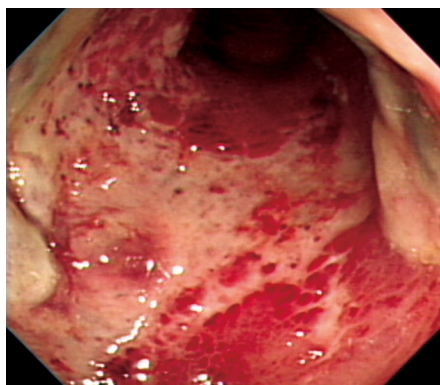
Ischemic colitis is an uncommon complication in patients with systemic lupus erythematosus (SLE). In previously reported cases of colitis caused by SLE, intestinal vasculitis is implicated as the causative process, but is rarely confirmed histologically. We described a case of a 32-year-old man with increased activity of SLE, who presented with hematochezia and abdominal pain due to ischemic colitis with small vessel vasculitis which was proven by sigmoidoscopic biopsy. The clinical course of the patient was improved after steroid and conservative management.

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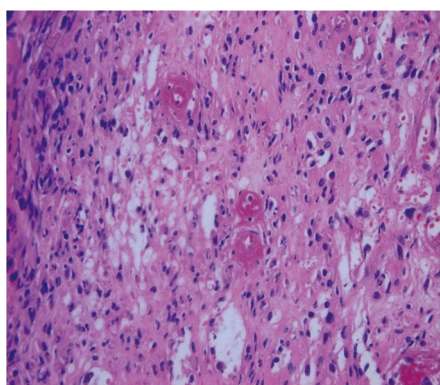
**Key words:** Systemic lupus erythematosus; Ischemic colitis; Vasculitis

**Peer reviewers:** Francesco Costa, MD, Dipartimento di Medicina Interna-U.O. di Gastroenterologia, Università di Pisa-Via Roma, 67-56122-Pisa, Italy; Rami Eliakim, Professor, Department of Gastroenterology, Rambam Medical Center, p.o.b. 9602, Haifa 31096, Israel; Jean-Noel Freund, MD, INSERM 682, Str Asbourg, France, 3 Avenue Molière, Strasbourg 67200, France

Lee JR, Paik CN, Kim JD, Chung WC, Lee KM, Yang JM. Ischemic colitis associated with intestinal vasculitis: Histological proof in systemic lupus erythematosus. *World J*



**Figure 1** Sigmoidoscopy shows the severe erythematous friable mucosa and diffuse ulceration with dirty exudates.



**Figure 2** Microscopy of colon biopsies shows the thickening of small vessel wall and lymphocyte infiltration around vessels (HE, x 400).

flexure, with contact bleeding and irregularly shaped ulceration (Figure 1). Rectum appeared relatively free from disease. Biopsy specimens from the sigmoid colon revealed inflammatory cell infiltration and hemorrhage in the mucosa, and small vessel wall thickening with lymphocyte infiltration, and vasculitis was considered (Figure 2). An ischemic colitis with increased SLE activity was confirmed and conservative management was done with fluids, intravenous antibiotics and intravenous steroid therapy. Clinical improvement was observed over the next three days, with stopped bloody stool and loss of abdominal pain. On the seventh day, follow-up flexible sigmoidoscopy demonstrated marked improvement of inflammation and ulceration in the sigmoid colon. On the ninth day, the patient was discharged.

## DISCUSSION

The development of ischemic colitis in patients with SLE is an uncommon complication<sup>[1-8]</sup>. But widespread fibrinoid vasculitis, typical of SLE, is thought to be a likely predisposing factor<sup>[1]</sup>. If this vasculitis involves the colon, ischemic colitis occurs. Gastrointestinal vasculitis is one of the most serious complications of SLE, even though the occurrence of colonic lesions is rare (0.2%)<sup>[4-6]</sup>. The gastrointestinal vasculitis of SLE is consequence of tissue damage from vasculopathy mediated by immune complexes, and has been associated with SLE activity<sup>[4,5]</sup>.

There are no pathognomic and histopathologic findings in SLE; however, pathologic changes associated with gastrointestinal vasculitis occur in the small vessels of the intestinal wall rather than in medium-sized mesenteric arteries<sup>[4,6]</sup>. Ischemic colitis in patients with SLE is caused by decreased blood perfusion of mesenteric vasculatures. The predisposing factors are embolism, thrombosis, vasospasm, drugs (steroids and immunosuppressive agents), vasculitis, performed colonoscopy, and enema<sup>[1,7,8]</sup>. Management of abdominal manifestations of SLE, in the absence of compelling radiographic or clinical findings suggestive of infarction or perforation, are steroid, antibiotics, and fluid therapy<sup>[2]</sup>.

The present case reveals ischemic change of edematous, erythematous mucosa and ulcerations with normal mucosa except for lesions on flexible sigmoidoscopy<sup>[9,10]</sup>. Even though the patient has received immunosuppressive agents, he had stable disease activity during last 13 mo without using immunosuppressant or steroid and no procedure, such as colonoscopy or enema has been performed. Comparing with previous laboratory findings, several tests concerned with SLE disease activity demonstrated increased activity; elevated ESR, elevated CRP, positive antinuclear antibody, elevated anti-double strand DNA antibody. Under this condition, we could diagnose ischemic colitis associated with increased SLE activity and vasculitis by histopathology. Generally, a typical pattern of vasculitis is quite difficult to prove by endoscopic biopsy<sup>[11]</sup>. Therefore, some literature used the term "lupus enteritis" rather than "gastrointestinal vasculitis" in SLE patients<sup>[5]</sup>.

In conclusion, the present case is ischemic colitis associated with intestinal vasculitis and increased SLE activity, and the patient showed clinical improvement with steroid and conservative treatment. In fact, there is no simple gauge to assess the extent and degree of intestinal ischemia<sup>[2]</sup>. However, if there are abdominal manifestations, such as pain or bloody stool in patients with active SLE, ischemic colitis should be considered and suspected.

## REFERENCES

- 1 **Versaci A**, Macri A, Scuderi G, Bartolone S, Familiari L, Lupattelli T, Famulari C. Ischemic colitis following colonoscopy in a systemic lupus erythematosus patient: report of a case. *Dis Colon Rectum* 2005; **48**: 866-869
- 2 **Gore RM**, Marn CS, Ujiki GT, Craig RM, Marquardt J. Ischemic colitis associated with systemic lupus erythematosus. *Dis Colon Rectum* 1983; **26**: 449-451
- 3 **Reissman P**, Weiss EG, Teoh TA, Lucas FV, Wexner SD. Gangrenous ischemic colitis of the rectum: a rare complication of systemic lupus erythematosus. *Am J Gastroenterol* 1994; **89**: 2234-2236
- 4 **Miyahara S**, Ito S, Soeda A, Chino Y, Hayashi T, Takahashi R, Goto D, Matsumoto I, Tsutsumi A, Sumida T. Two cases of systemic lupus erythematosus complicated with colonic ulcers. *Intern Med* 2005; **44**: 1298-1306
- 5 **Lee CK**, Ahn MS, Lee EY, Shin JH, Cho YS, Ha HK, Yoo B, Moon HB. Acute abdominal pain in systemic lupus erythematosus: focus on lupus enteritis (gastrointestinal vasculitis). *Ann Rheum Dis* 2002; **61**: 547-550



- 6 **Grimbacher B**, Huber M, von Kempis J, Kalden P, Uhl M, Kohler G, Blum HE, Peter HH. Successful treatment of gastrointestinal vasculitis due to systemic lupus erythematosus with intravenous pulse cyclophosphamide: a clinical case report and review of the literature. *Br J Rheumatol* 1998; **37**: 1023-1028
- 7 **Cappell MS**. Intestinal (mesenteric) vasculopathy. II. Ischemic colitis and chronic mesenteric ischemia. *Gastroenterol Clin North Am* 1998; **27**: 827-860, vi
- 8 **Lafsky RD**. Colonoscopy in ischemic enterocolitis. *Gastrointest Endosc* 2000; **52**: 310-311
- 9 **Byun JY**, Ha HK, Yu SY, Min JK, Park SH, Kim HY, Chun KA, Choi KH, Ko BH, Shinn KS. CT features of systemic lupus erythematosus in patients with acute abdominal pain: emphasis on ischemic bowel disease. *Radiology* 1999; **211**: 203-209
- 10 **Kim YG**, Ha HK, Nah SS, Lee CK, Moon HB, Yoo B. Acute abdominal pain in systemic lupus erythematosus: factors contributing to recurrence of lupus enteritis. *Ann Rheum Dis* 2006; **65**: 1537-1538
- 11 **Teramoto J**, Takahashi Y, Katsuki S, Sato T, Sakamaki S, Kobayashi D, Watanabe N, Niitsu Y. Systemic lupus erythematosus with a giant rectal ulcer and perforation. *Intern Med* 1999; **38**: 643-649

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## CASE REPORT

# Massive ascites as a presenting manifestation of chronic lymphocytic leukemia

Neelam Siddiqui, Saeed Al-Amoudi, Aamer Aleem, Maha Arafah, Layla Al-Gwaiz

Neelam Siddiqui, Aamer Aleem, Department of Medicine, College of Medicine, King Khalid University Hospital, King Saud University, Riyadh, Saudi Arabia

Saeed Al-Amoudi, Maha Arafah, Layla Al-Gwaiz, Department of Pathology, College of Medicine, King Khalid University Hospital, King Saud University, Riyadh, Saudi Arabia

Author contributions: Siddiqui N, Al-Amoudi S, Aleem A wrote the manuscript; Arafah M and Al-Gwaiz L prepared the figures and critically revised the manuscript.

Correspondence to: Dr. Aamer Aleem, MBBS, MRCP, MRCPATH, Assistant Professor & Consultant Hematologist, King Khalid University Hospital & College of Medicine, PO Box 7805, Riyadh 11472, Saudi Arabia. [aameralaleem@hotmail.com](mailto:aameralaleem@hotmail.com)

Telephone: +966-1-4671771 Fax: +966-1-4679277

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## Abstract

Ascites is not an uncommon manifestation of certain solid tumors like gastrointestinal malignancies, ovarian cancer and breast cancer. However, it is unusual to encounter ascites in patients with hematological malignancies especially chronic leukemia. The patient described here presented with massive ascites and blood lymphocytosis. Further studies confirmed the diagnosis of chronic lymphocytic leukemia with ascites. The ascitic fluid was exudative, consisting of mature-looking B-lymphocytes, which were morphologically and immunophenotypically similar to peripheral blood and bone marrow cells. The patient was treated with chemotherapy and achieved a good response and diminution of ascitic fluid accumulation.

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**Key words:** Ascites; Chronic lymphocytic leukemia

**Peer reviewer:** Sammy Saab, MD, UCLA, 200 Medical Plaza, Suite 214, Los Angeles, CA 90095, United States

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## INTRODUCTION

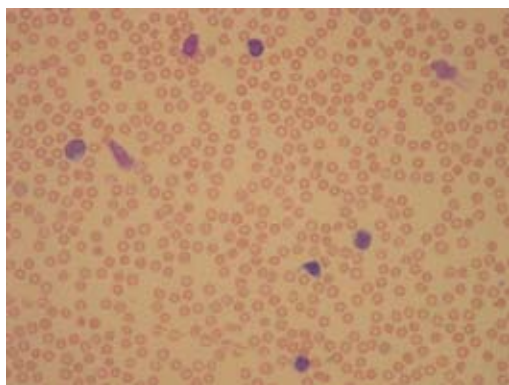
Chronic lymphocytic leukemia (CLL) is the most common hematological malignancy in the elderly. The disease is often indolent or slowly progressive. Approximately 40% of the patients are asymptomatic in the initial stages and the diagnosis is first made on the basis of a complete blood count when more than  $5 \times 10^9/L$  lymphocytes are found on differential white cell count and confirmed by a characteristic immunophenotyping pattern of peripheral blood or bone marrow lymphocytes. The common clinical features of the disease are lymphadenopathy, hepatosplenomegaly, malaise and weight loss. Bacterial infections, most often pneumonias, are fairly common in these patients. Less common complications are infiltration of various organs and transformation of the leukemia into other hematological malignancies. Ascites, however, is very rare in CLL patients particularly at the time of initial diagnosis. Development of ascites in CLL has been described at the time of relapse or transformation into prolymphocytic leukemia or Richter's syndrome<sup>[1-3]</sup>. It was only once reported as an initial manifestation of the disease and also as a case of chylous ascites<sup>[4]</sup>.

## CASE REPORT

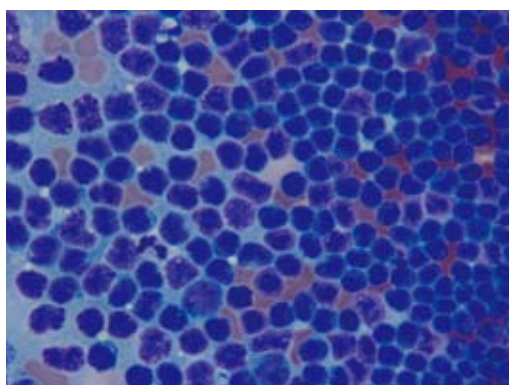
A 75-year-old man was referred to our institute in March 2004 because of progressively increasing abdominal distension over a four-month period. More recently, he had also noticed weight loss, fatigue and night sweats. There was no history of change in bowel or bladder function, use of tobacco or alcohol. There was no history of diabetes mellitus, hypertension or heart disease. He had never required hospitalization prior to this admission.

Physical examination revealed an elderly man in obvious discomfort due to abdominal distension. He had generalized symmetrical lymphadenopathy including bilateral cervical, axillary and inguinal lymph nodes. There was minimal peripheral edema. His abdomen was protuberant with bulging flanks and a positive fluid thrill. There was no hepatosplenomegaly and no stigmata of chronic liver disease.

On admission, his complete blood count revealed a white blood cell count of  $45.2 \times 10^9/L$ , hemoglobin of 119 g/L, and platelet count of  $62 \times 10^9/L$ . The absolute lymphocyte count was  $33 \times 10^9/L$ . Peripheral blood smear examination showed lymphocytosis with



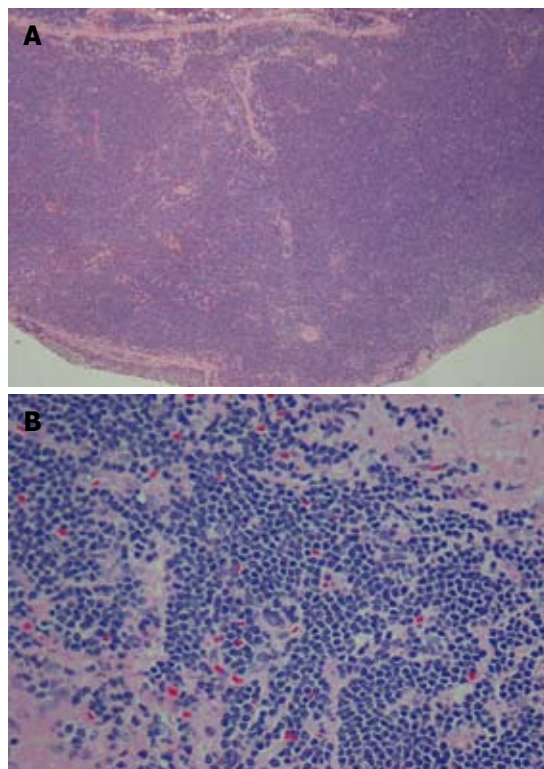
**Figure 1** Peripheral blood film showing lymphocytosis and several smear cells.



**Figure 2** Cytospin of ascitic fluid showing infiltration by a large number of small, mature looking lymphocytes (DQ staining,  $\times 400$ ).

mature lymphocytes and many smear cells (Figure 1). The lymphocytes were of variable small to medium size and most revealed round nuclei with some displaying nuclear irregularity. Prolymphocytes were only 8%. Flow cytometry performed on the peripheral blood showed the lymphocytes to be positive for CD5, CD19, CD20, CD23 and FMC7 and negative for CD10 and CD7. Bone marrow examination showed diffuse infiltration of the marrow with around 95% of the cells being lymphocytes. Many smear cells were seen. Normal hematopoiesis was markedly reduced. Cytogenetic studies did not reveal any chromosomal abnormalities. Serum protein electrophoresis disclosed low total protein with a normal percentage of albumin (56%). Serum immunoelectrophoresis revealed a decrease in both the serum IgG level (6.88 g/L; normal, 8-18) and IgM level (0.30 g/L; normal, 0.6-2.5).

His electrocardiogram was normal and a chest X-ray showed mediastinal lymphadenopathy. CT scan of the thorax and abdomen disclosed multiple enlarged lymph nodes in the axilla, mediastinum, abdomen and inguinal region. There was gross ascites with edema of the abdominal wall. Liver, spleen and the peritoneum appeared normal with no evidence of portal hypertension. At this stage, two liters of clear yellowish ascitic fluid was removed for symptomatic relief and laboratory analysis. Biochemistry of the ascitic fluid revealed the total protein was high, 13 g/L, and glucose



**Figure 3** A: Lymph node section in low power showing total effacement of normal architecture (HE staining,  $\times 40$ ); B: Lymph node section in higher power showing infiltration with small lymphocytes (HE staining,  $\times 400$ ).

and lactate dehydrogenase were normal. Thus, the fluid was exudative with a low serum ascitic albumin gradient. On microscopic evaluation, it was a highly cellular specimen consisting of numerous non-cohesive, small monomorphic lymphoid cells containing hyperchromatic nuclei with coarsely granular chromatin and mesothelial cells seen in the background (Figure 2). Morphological picture and immunophenotyping by flow cytometry of the ascitic fluid were consistent with small lymphocytic lymphoma/leukemia cells similar to blood and bone marrow. The gram-staining, culture and acid fast bacilli were negative.

Upper and lower gastrointestinal endoscopies were performed to rule out a coexisting gastrointestinal malignancy as a cause of ascites. The endoscopic findings along with serum  $\alpha$ -fetoprotein were normal. Hepatitis B and C studies were negative.

The lymph node biopsy showed total nodal effacement, with perinodal tumor infiltration and diffuse proliferation of small lymphoid cells (Figure 3). These cells were small and round with minimal degree of nuclear irregularity and scant cytoplasm. Several proliferating centers containing paraimmunoblasts were also present. These paraimmunoblasts were larger in size than the background lymphocytes having round nuclei with a vesicular chromatic pattern and medium to large nucleoli. These were found in aggregates that appeared pale and scattered in between the small lymphocytes.

Based on CD5/CD19 positive lymphocytosis, lymphadenopathy and thrombocytopenia, a diagnosis of chronic lymphocytic leukemia, Rai stage V was made<sup>[5]</sup>.

It was interesting to notice that the peripheral blood, bone marrow and ascitic fluid studies showed a clonal proliferation of mature lymphocytes.

In view of the above diagnosis, the patient was commenced on cyclophosphamide, vincristine and prednisolone (CVP) chemotherapy. He received three cycles of chemotherapy with a good response. His blood count normalized with disappearance of the lymph nodes and there was a remarkable reduction in the rate of accumulation of ascites. Unfortunately, after the third cycle of therapy, the patient failed to attend the oncology clinic (having had to travel more than 1000 kilometers on each occasion) and was lost to follow-up.

## DISCUSSION

In 1982, May and Costanzi reported a known case of CLL whose relapse was heralded by development of splenomegaly with massive ascites<sup>[6]</sup>. The ascitic fluid was a transudate with polymorphonuclear leukocytes and mononuclear cells. On cytopathology, there were no lymphocytes or abnormal cells. They attributed the cause of ascites to be periportal infiltration of the liver and marked infiltration of the spleen leading to portal hypertension. A similar case of portal hypertension caused by intra-hepatic block in CLL was later reported by Mouly<sup>[7]</sup>.

Clonal evolution is common in CLL during the course of the disease. This disease may convert to prolymphocytic leukemia, diffuse large-cell lymphoma or acute leukemia. "Prolymphocytoid" transformation of CLL is characterized by appearance of increasing number of large cells with irregular nuclei, i.e., prolymphocytes and development of hepatosplenomegaly. Ascites and pleural effusion have occasionally been described in this setting<sup>[2]</sup>. The ascitic fluid consists of prolymphocytes and is an exudate with low albumin gradient. Our patient had a very small population of prolymphocytes in the peripheral blood and none in the ascitic fluid, therefore transformation of CLL to "prolymphocytic leukemia" was not considered.

About 3%-10% cases of CLL may transform to diffuse large-cell lymphoma when it is called "Richter's syndrome". This is usually a terminal event with very poor prognosis. A few cases of ascites developing at the time of Richter's transformation have been described in the literature<sup>[3,8,9]</sup>. The ascitic fluid in these cases showed large blast-like lymphoma cells similar to the cells in the lymph node biopsy. In our patient, the ascitic fluid consisted of mostly small mature-looking lymphocytes consistent with CLL and not diffuses large cell lymphoma or acute leukemia.

Other possible causes of ascites which could be related to CLL are infection leading to subacute bacterial peritonitis, another malignancy or portal hypertension. Although patients with CLL are at high risk of infection, in this patient there was no evidence of a specific organism causing bacterial peritonitis, nor was there any indication of tuberculous peritonitis. There have

been some reports of increased incidence of second solid tumors in patients with CLL. Carcinoma of gastrointestinal tract and lung were ruled out in our patient by normal results of endoscopies and CT scans. Since serum  $\alpha$ -fetoprotein was normal and there was no hepatic lesion on CT, hepatocellular carcinoma was also unlikely as a cause of ascites in this patient. The remaining differential diagnosis in this patient may be hepatic cirrhosis, heart failure, or renal disease. However, all of these possibilities were systematically screened and excluded.

In conclusion, the patient described here had an unusual presentation of CLL. The ascitic fluid consisted of mature B-cell type lymphocytes, morphologically and immunophenotypically similar to the peripheral blood and bone marrow cells. The mechanism of ascites in this case can only be speculated. It is possible that despite the normal radiological studies, there may be microscopic peritoneal deposition by leukemia cells or portal system lymphatic obstruction due to extensive lymphatic infiltration. The fact that the ascitic fluid was sterile and most of the cells were mature lymphocytes, like in the blood, suggests that the ascites in this patient was due to infiltration of the peritoneum and was malignant ascites not significantly different from that found in solid tumors. A liver biopsy in this case might have been helpful in confirming a normal histology but it was not done as it was not indicated in the opinion of the managing team. It is interesting to note that there was a good response to chemotherapy with marked reduction in ascites. Unfortunately, nothing further can be commented in this regard because of the lack of follow-up after three cycles of chemotherapy. In view of the continued increase in the number of cases diagnosed with CLL, a greater awareness of the possibility of development of ascites as a presenting manifestation of this disease may be beneficial to the physicians caring for patients with ascites.

## REFERENCES

- 1 **Mamode C**, Beauregard P, Langevin S, Mongeau CJ. [Chronic lymphoid leukemia complicated with ascites] *Can J Gastroenterol* 2000; **14** Suppl D: 181D-184D
- 2 **Shimoni A**, Shvidel L, Shtalrid M, Klepfish A, Berrebi A. Prolymphocytic transformation of B-chronic lymphocytic leukemia presenting as malignant ascites and pleural effusion. *Am J Hematol* 1998; **59**: 316-318
- 3 **Cuneo A**, de Angeli C, Roberti MG, Piva N, Bigoni R, Gandini D, Rigolin GM, Moretti S, Cavazzini P, del Senno L, Castoldi G. Richter's syndrome in a case of atypical chronic lymphocytic leukaemia with the t(11;14)(q13;q32): role for a p53 exon 7 gene mutation. *Br J Haematol* 1996; **92**: 375-381
- 4 **Davis MN**, Alloy AM, Chiesa JC, Pecora AA. Chronic lymphocytic leukemia presenting with massive chylous ascites. *Am J Gastroenterol* 1990; **85**: 593-596
- 5 **Rai KR**, Sawitsky A, Cronkite EP, Chanana AD, Levy RN, Pasternack BS. Clinical staging of chronic lymphocytic leukemia. *Blood* 1975; **46**: 219-234
- 6 **May JT**, Costanzi JJ. Ascites in chronic leukemia: a case report and review of the literature. *Oncology* 1982; **39**: 55-58
- 7 **Mouly S**, Cochand-Priollet B, Halimi C, Bergmann JF.



- [Portal hypertension caused by intra-hepatic block in chronic lymphoid leukemia] *Presse Med* 1996; **25**: 497-498
- 8 **Desablens B**, Gineston JL, Joly JP, Piprot-Choffat C, Sevestre H, Capron JP. [Immunoblastic lymphoma of the ileocecal region in chronic lymphoid leukemia. Richter's syndrome localized in the intestine and disclosed by ascites] *Gastroenterol Clin Biol* 1987; **11**: 901-903
- 9 **Case records of the Massachusetts General Hospital. Weekly clinicopathological exercises.** Case 31-1983. Chronic lymphocytic leukemia with the recent development of hepatosplenomegaly and ascites. *N Engl J Med* 1983; **309**: 297-305

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## CASE REPORT

# Successful endoscopic sclerotherapy for bleeding gastric varices with combined cyanoacrylate and aethoxysklerol

Bei Shi, Wei Wu, Hui Zhu, Yun-Lin Wu

Bei Shi, Wei Wu, Yun-Lin Wu, Department of Gastroenterology, Ruijin Hospital Affiliated to Shanghai Jiaotong University, Shanghai 200025, China

Hui Zhu, Department of Radiology, Ruijin Hospital Affiliated to Shanghai Jiaotong University, Shanghai 200025, China

Author contributions: Wu YL and Shi B designed and performed the research; Zhu H made the radiologic studies; Shi B and Wu W wrote the paper.

Correspondence to: Yun-Lin Wu, Department of Gastroenterology, Ruijin Hospital Affiliated to Shanghai Jiaotong University, 197 Ruijin Er Road, Shanghai 200025, China. [wuyunlin1951@163.com](mailto:wuyunlin1951@163.com)

Telephone: +86-21-64370045 Fax: +86-21-64150773

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cyanoacrylate and aethoxysklerol. *World J Gastroenterol* 2008; 14(22): 3598-3601 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3598.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3598>

## INTRODUCTION

Gastric varices have been increasingly recognized as a major cause of gastrointestinal bleeding in patients with portal hypertension. Compared with esophageal variceal bleeding, hemorrhage caused by gastric varices is usually more severe and hemostatic control is reported to be more difficult<sup>[1]</sup>.

Management for gastric varices usually include vasoactive agents, endoscopic therapy and surgery<sup>[2]</sup>. Rupture of gastric fundal varices is often lethal because of massive bleeding<sup>[3,4]</sup>. Three-channel double-balloon catheter might be ineffective for gastric fundal varices when acute bleeding occurs. Endoscopic injection with tissue adhesives such as histoacryl can effectively control active bleeding<sup>[5,6]</sup>. However, gastric varices cannot disappear due to the unsatisfactory control of inflammation and fibrous organization caused by adhesives. Although endoscopic variceal ligation therapy (EVL) has shown its benefit for esophageal varices, it cannot achieve a similar success in the management of gastric varices<sup>[6]</sup>. It was reported that balloon retrograde transvenous obliteration (B-RTO) is a useful treatment for gastric fundal varices, but it requires a gastroduodenal shunt as the draining vein<sup>[7]</sup>. We report two cases of marked gastric varices with no gastroduodenal shunt, who were successfully treated with endoscopic therapy with combined adhesives and sclerosants.

## Abstract

Two patients with liver cirrhosis and portal hypertension related to hepatitis infection were admitted to Shanghai Ruijin Hospital due to recurrent melena and hematemesis. Isolated gastric varices were observed in the gastric fundus during the retroflexion of gastroscopy. We carried out endoscopic sclerotherapy successfully for bleeding gastric varices with combined cyanoacrylate and aethoxysklerol, which disappeared dramatically several months after two courses of sclerotherapy for each patient. No complication and clinical signs of gastrointestinal re-bleeding were observed during the 6-mo endoscopic follow-up. CT portal angiography (CTPA) has been widely used in the assessment of variceal treatment and improves the results of endoscopic injection therapy.

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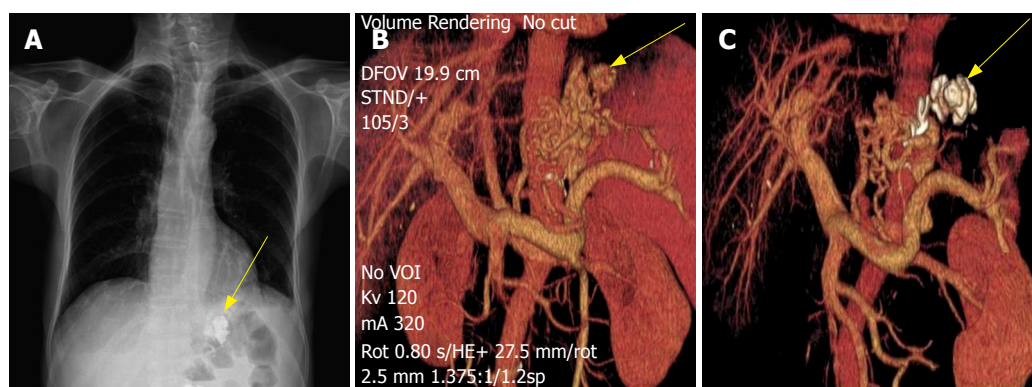
**Key words:** Endoscopic sclerotherapy; Cyanoacrylate; Aethoxysklerol; Gastric varices; CT portal angiography

**Peer reviewers:** Volker F Eckardt, Chief, MD, Professor, Department of Gastroenterology, Deutsche Klinik für Diagnostik, Aukammallee 33, Wiesbaden 65191, Germany; Hiroshi Yoshida, MD, First Department of Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8603, Japan

Shi B, Wu W, Zhu H, Wu YL. Successful endoscopic sclerotherapy for bleeding gastric varices with combined

## CASE REPORT

Patient one was a 69-year-old male suffering from recurrent melena for 3 mo. He had a 20-year history of hepatitis B virus (HBV) infection and a 8-year history of hepatitis C virus (HCV) infection and was diagnosed having viral liver cirrhosis and portal hypertension two years ago. His Child-Pugh was class B. Patient two was a 61-year-old female with liver cirrhosis and portal hypertension. She was admitted to the gastroenterology ward due to the onset of hematemesis. Her Child-Pugh was class A. Upper gastrointestinal (GI) endoscopy



**Figure 1** Subsequent chest X-ray on the 69-year-old male demonstrating marked retention of the cyanoacrylate/lipiodol mixture within the variceal bed in gastric fundus (arrow) (A), CTPA-reconstructed image showing abundant portal venous collaterals with no gastroduodenal shunt and gastric fundal varices (arrow) communicating with the confluence through the left gastric vein (B) and shrinkage of gastric fundal varices and deposition of cyanoacrylate/lipiodol mixture (arrow), blocking the draining veins (C) after sclerotherapy.

showed massive isolated gastric varices of the gastric fundus with red flat spots on their surface in both patients. No esophageal varices were observed. CTPA revealed dilated portal vein and tortuous splenic vein in both patients while gastric fundal varices were noticed to communicate with the confluence through the left gastric vein. No evidence of gastroduodenal shunt was shown. Written informed consent was obtained from the two patients before endoscopic sclerotherapy.

Both patients received endoscopic injection sclerotherapy with combined cyanoacrylate and aethoxysklerol. Routine preparation was carried out and anti-foam agent, simethicone, was administered 20 min before endoscopy. An intravenous infusion of somatostatin at 250 µg/h was performed 2 h before and 72 h after the therapy. The endoscopic therapy procedure required a retroflex view for variceal visualization. During the treatment, 10 mL of 1% aethoxysklerol (Polidocanol, Germany) was injected into the varix followed by conventional endoscopic injection sclerotherapy with a mixture of 1.5 mL  $\alpha$ -cyanoacrylate alkyl (Compoint, China) and 1.5 mL iodinated oil (Lipiodol, French). When no active bleeding or oozing of the varix occurred, the procedure was terminated.

Both patients tolerated the procedure well with no immediate complication. Subsequent chest X-ray one day after sclerotherapy demonstrated a marked collection of cyanoacrylate/lipiodol mixture within the variceal bed in the gastric fundus, but no sign of ectopic infarction. CTPA 3 wk after sclerotherapy showed shrinkage of gastric fundal varices and deposition of cyanoacrylate/lipiodol mixture blocking the draining veins (Figure 1). A repeated sclerotherapy was performed 3 wk later. A 6-mo endoscopic follow-up after the second treatment revealed dramatic disappearance of gastric varices in both patients (Figure 2). The two patients were free from re-bleeding after the last endoscopic treatment.

## DISCUSSION

The incidence of gastric variceal bleeding is lower than that of esophageal varices. However, rupture of gastric

varices usually results in more severe hemorrhage and a higher mortality<sup>[1]</sup>.

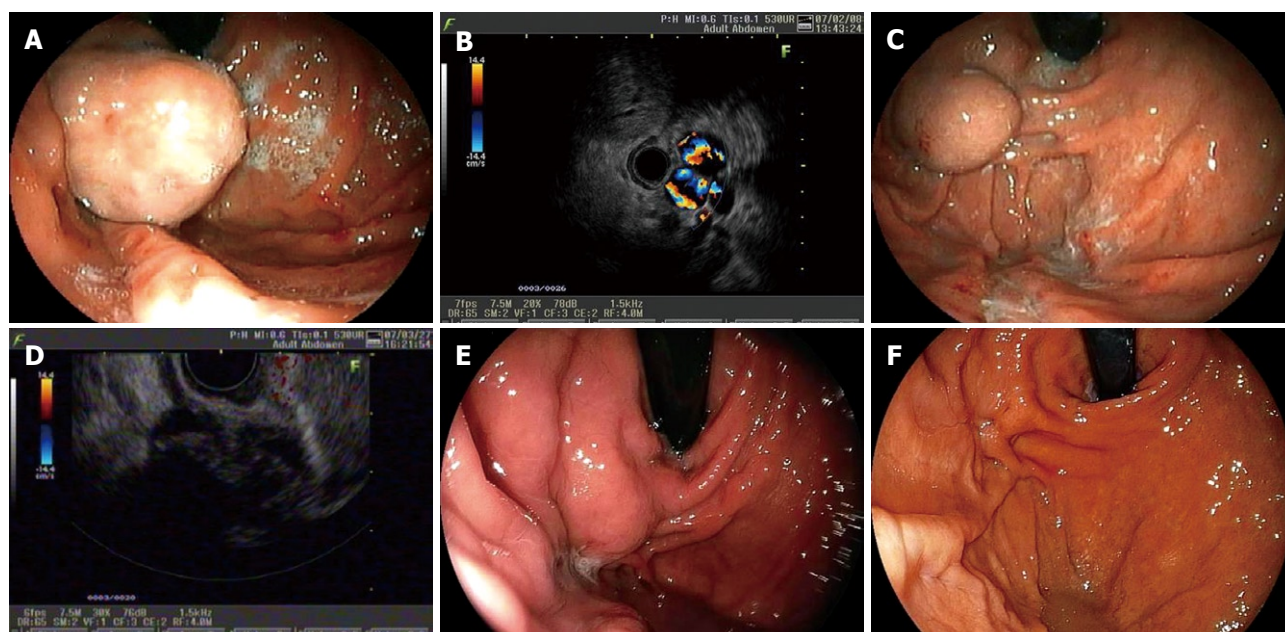
Since the description of experience with the use of cyanoacrylate compounds in endoscopic treatment of gastric varices in an experimental dog model in 1975<sup>[8]</sup>, adhesives such as N-butyl-2-cyanoacrylate (histoacryl) have been proved safe and efficient in the treatment of active bleeding of gastric varices. N-butyl-2-cyanoacrylate has not been approved for its use in treatment of variceal bleeding by China State Food and Drug Administration (SFDA). In our cases,  $\alpha$ -cyanoacrylate alkyl, an analogue of N-butyl-2-cyanoacrylate approved by SFDA, showed a similar pharmaceutical effect to N-butyl-2-cyanoacrylate. The adhesive polymer, an unabsorbable foreign body in the bed of varices after injection, could block the ruptured vein yet had a weak effect on promoting organization of the veins. Ulceration may occur and the polymer extrudes from the vessel bed several weeks after injection, during which fatal re-bleeding may occur<sup>[9]</sup>. The similar extrusion process was performed for the 69-year-old male patient during the endoscopic follow-up period (Figure 3).

Polidocanol is a well-accepted sclerosant for obliteration of gastric varices. It can produce significant thrombosis, inflammatory reaction and fibrous degeneration, thus finally eliminating the varices. Though sclerosing agents are widely used in the treatment of esophageal varices, they are far less promising for the treatment of gastric varices<sup>[10,11]</sup>. In Japan, ethanolamine oleate (EO) remains the first choice of sclerotherapeutic agents for eradicating esophageal varices, yet it has not been greatly used in the management of gastric varices. Oho and colleagues<sup>[12]</sup> reported that the rate of initial hemostasis is significantly lower in patients with bleeding gastric varices treated with EO than in those treated with butyl cyanoacrylate.

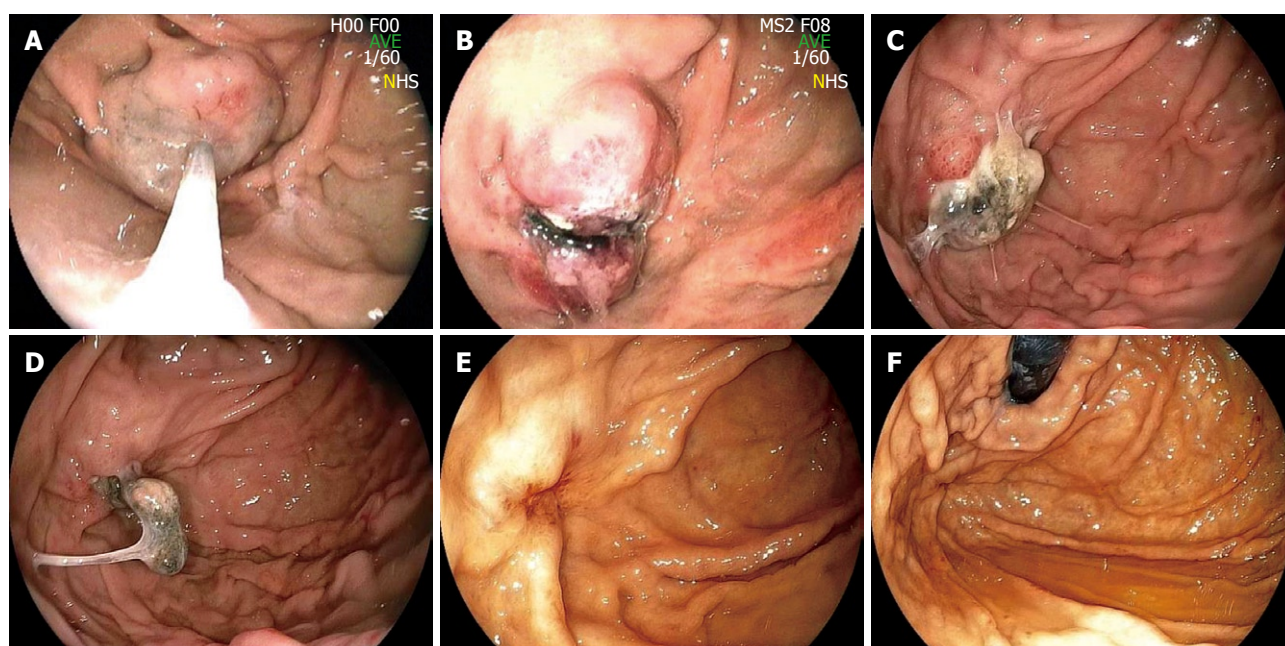
In our cases, the effect of  $\alpha$ -cyanoacrylate alkyl and aethoxysklerol on gastric varices was satisfactory and their gastric varices disappeared six months after treatment.

Rare but serious complications of endoscopic sclerotherapy may include cerebral stroke, pulmonary





**Figure 2** Endoscopy showing isolated gastric varices in gastric fundus (A) and abundant blood flow (B) in gastric varices before treatment, and shrinkage of gastric varices (C) and no blood flow signals (D), flat gastric varices with slight red spots on the top (E), and eradication of gastric varices (F) in the 69-year-old male patient after treatment.



**Figure 3** Endoscopic findings after the first injection treatment with combined cyanoacrylate and aethoxysklerol (A), an marked ulcer noticed on the surface of varices 3 d after the first injection with no stigmata bleeding (B), an adhesive extrusion process 3 wk after the initial treatment (C), adhesive found on the surface of flat gastric varices 3 wk after the secondary injection (D), disappearance of the former massive gastric varices three and a half months after the secondary injection (E, F).

embolism, portal vein embolism and splenic infarction<sup>[13,14]</sup>. Gastric varices are characterized by an enlarged caliber of the vessel bed, large draining veins and rapid blood flow, which may exacerbate the situation. Fortunately, no complication was found in these two patients.

After sclerotherapy, both patients were given antacids to suppress the secretion of gastric acid, thus protecting the gastric mucosa and promoting the healing of ulcer. The dosage of rabeprazole, a proton-pump inhibitor,

may be increased to 20 mg once a day per oral depending on the severity of ulcer on the surface of varices. Meanwhile, the patients were given propranolol and isosorbide mononitrate to reduce their portal pressure. After sclerotherapy, re-bleeding, especially during the process of adhesive extrusion, should be prevented.

CTPA can serve as an important supplement and an alternative technique for gastric varices. It can be applied in choosing appropriate candidates, evaluating and following up patients with gastric varices<sup>[15]</sup>. We detected



the varices and made assessments of portosystemic collaterals through CTPA before sclerotherapy, which demonstrated the afferent and efferent vessels of gastric varices. Willmann and colleagues<sup>[16]</sup> demonstrated that CTPA is able to differentiate between submucosal and perigastric fundal varices, which is of paramount interest since only the former may cause gastrointestinal hemorrhage. CTPA revealed the vessels blocked by adhesive polymer, obliteration and elimination of gastric varices, suggesting that injection of adhesives in combination with sclerosants is an effective treatment modality for gastric varices.

In conclusion, although the optimal treatment for gastric fundal variceal bleeding remains controversial, sclerotherapy with combined  $\alpha$ -cyanoacrylate alkyl and aethoxysklerol is an alternative and feasible treatment modality for gastric fundal varices.

## REFERENCES

- 1 **Sarin SK**, Lahoti D, Saxena SP, Murthy NS, Makwana UK. Prevalence, classification and natural history of gastric varices: a long-term follow-up study in 568 portal hypertension patients. *Hepatology* 1992; **16**: 1343-1349
- 2 **Garcia-Tsao G**, Sanyal AJ, Grace ND, Carey WD. Prevention and management of gastroesophageal varices and variceal hemorrhage in cirrhosis. *Am J Gastroenterol* 2007; **102**: 2086-2102
- 3 **Watanabe K**, Kimura K, Matsutani S, Ohto M, Okuda K. Portal hemodynamics in patients with gastric varices. A study in 230 patients with esophageal and/or gastric varices using portal vein catheterization. *Gastroenterology* 1988; **95**: 434-440
- 4 **Akahoshi T**, Hashizume M, Shimabukuro R, Tanoue K, Tomikawa M, Okita K, Gotoh N, Konishi K, Tsutsumi N, Sugimachi K. Long-term results of endoscopic Histoacryl injection sclerotherapy for gastric variceal bleeding: a 10-year experience. *Surgery* 2002; **131**: S176-S181
- 5 **Sarin SK**, Jain AK, Jain M, Gupta R. A randomized controlled trial of cyanoacrylate versus alcohol injection in patients with isolated fundic varices. *Am J Gastroenterol* 2002; **97**: 1010-1015
- 6 **Lo GH**, Lai KH, Cheng JS, Chen MH, Chiang HT. A prospective, randomized trial of butyl cyanoacrylate injection versus band ligation in the management of bleeding gastric varices. *Hepatology* 2001; **33**: 1060-1064
- 7 **Ninoi T**, Nishida N, Kaminou T, Sakai Y, Kitayama T, Hamuro M, Yamada R, Nakamura K, Arakawa T, Inoue Y. Balloon-occluded retrograde transvenous obliteration of gastric varices with gastroduodenal shunt: long-term follow-up in 78 patients. *AJR Am J Roentgenol* 2005; **184**: 1340-1346
- 8 **Rosch J**, Goldman ML, Dotter CT. Experimental catheter obstruction of the gastric coronary vein. Possible technique for percutaneous intravascular tamponade of the gastroesophageal varices. *Invest Radiol* 1975; **10**: 206-211
- 9 **Soehendra N**, Grimm H, Nam VC, Berger B. N-butyl-2-cyanoacrylate: a supplement to endoscopic sclerotherapy. *Endoscopy* 1987; **19**: 221-224
- 10 **Trudeau W**, Prindiville T. Endoscopic injection sclerosis in bleeding gastric varices. *Gastrointest Endosc* 1986; **32**: 264-268
- 11 **Sarin SK**. Long-term follow-up of gastric variceal sclerotherapy: an eleven-year experience. *Gastrointest Endosc* 1997; **46**: 8-14
- 12 **Oho K**, Iwao T, Sumino M, Toyonaga A, Tanikawa K. Ethanolamine oleate versus butyl cyanoacrylate for bleeding gastric varices: a nonrandomized study. *Endoscopy* 1995; **27**: 349-354
- 13 **Binmoeller KF**. Glue for gastric varices: some sticky issues. *Gastrointest Endosc* 2000; **52**: 298-301
- 14 **Feretis C**, Dimopoulos C, Benakis P, Kalliakmanis B, Apostolidis N. N-butyl-2-cyanoacrylate (Histoacryl) plus sclerotherapy versus sclerotherapy alone in the treatment of bleeding esophageal varices: a randomized prospective study. *Endoscopy* 1995; **27**: 355-357
- 15 **Matsumoto A**, Kitamoto M, Imamura M, Nakanishi T, Ono C, Ito K, Kajiyama G. Three-dimensional portography using multislice helical CT is clinically useful for management of gastric fundic varices. *AJR Am J Roentgenol* 2001; **176**: 899-905
- 16 **Willmann JK**, Weishaupt D, Bohm T, Pfammatter T, Seifert B, Marincek B, Bauerfeind P. Detection of submucosal gastric fundal varices with multi-detector row CT angiography. *Gut* 2003; **52**: 886-892

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LETTERS TO THE EDITOR

## Pleiotropic effects of bombesin and neurotensin on intestinal mucosa: Not just trefoil peptides

Stelios F Assimakopoulos, Chrisoula D Scopa, Vassiliki N Nikolopoulou, Constantine E Vagianos

Stelios F Assimakopoulos, Department of Internal Medicine, School of Medicine, University of Patras, Patras 26110, Greece  
Chrisoula D Scopa, Department of Pathology, School of Medicine, University of Patras, Patras 26110, Greece  
Vassiliki N Nikolopoulou, Division of Gastroenterology, Department of Internal Medicine, School of Medicine, University of Patras, Patras 26110, Greece  
Constantine E Vagianos, First Surgical Department, "Saint Panteleimon" General Hospital of Nikaia, Piraeus 18454, Greece

**Author contributions:** Assimakopoulos SF generated the idea for the commentary; all authors wrote, revised and approved the final form of this manuscript.

**Correspondence to:** Stelios F Assimakopoulos, MD, PhD, Department of Internal Medicine, School of Medicine, University of Patras, Vironos 18, Patras 26224, Greece. [sassim@upatras.gr](mailto:sassim@upatras.gr)

Telephone: +30-2610-346946 Fax: +30-2610-990775

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**Peer reviewers:** Tsuneo Kitamura, Associate Professor, Department of Gastroenterology, Juntendo University Urayasu Hospital, Juntendo University School of Medicine, 2-1-1 Tomioka, Urayasu-shi, Chiba 279-0021, Japan; Yoshiharu Motoo, MD, PhD, FACP, FACP, Professor and Chairman, Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

Assimakopoulos SF, Scopa CD, Nikolopoulou VN, Vagianos CE. Pleiotropic effects of bombesin and neurotensin on intestinal mucosa: Not just trefoil peptides. *World J Gastroenterol* 2008; 14(22): 3602-3603 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3602.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3602>

### Abstract

Bombesin and neurotensin are neuropeptides which exert a wide spectrum of biological actions on gastrointestinal tissues influencing intestinal growth and adaptation, intestinal motility, blood flow, secretion, nutrient absorption and immune response. Based mainly on their well-established potent enterotrophic effect, numerous experimental studies investigated their potential positive effect on the atrophic or injured intestinal mucosa. These peptides proved to be effective mucosa-healing factors, but the potential molecular and cellular mechanisms for this action remained unresolved. In a recently published study (*World J Gastroenterol* 2008; 14(8): 1222-1230), it was shown that their protective effect on the intestine in experimentally induced inflammatory bowel disease was related to anti-inflammatory, antioxidant and antiapoptotic actions. These results are in close agreement with our previous studies on jaundiced and hepatectomized rats that showed a regulatory effect of bombesin and neurotensin on critical cellular processes such as enterocyte proliferation and death, oxidative stress and redox equilibrium, tight junctions' formation and function, and inflammatory response. The pleiotropic effects of bombesin and neurotensin on diverse types of intestinal injury may justify their consideration for clinical trials.

### TO THE EDITOR

We read with great interest the recently published article (*World J Gastroenterol* 2008; 14(8): 1222-1230) by Dr. Akcan and colleagues<sup>[1]</sup>, on the effect of neuropeptides Bombesin (BBS) and Neurotensin (NTS) on trinitrobenzene sulphonic acid-induced colitis in rats, an experimental model of colonic inflammatory bowel disease. In this nice set of experiments, the authors demonstrated the beneficial effects of both BBS and NTS on the preservation of intestinal macroscopic and microscopic integrity in experimental colitis. Most importantly, it was shown that this positive effect on the intestinal mucosa was related to anti-inflammatory, antioxidant and antiapoptotic actions.

It has been two decades since the issue of the potential beneficial role of BBS and NTS on preservation of intestinal homeostasis arose, based on peptides' well-established potent enterotrophic effect<sup>[2,3]</sup>. Up to now, numerous experimental studies have demonstrated the protective effect of BBS and NTS against diverse types of intestinal injury, such as administration of elemental diets or methotrexate, induction of chemical colitis, burns, radiation therapy, ischemia/reperfusion and small bowel resection<sup>[3-9]</sup>. However, the molecular and cellular mechanisms implicated in their intestinal mucosa-healing effect remained unresolved for a long period. Our recent studies with jaundiced and hepatectomized rats showed

that BBS and NT exert regulatory effects on critical cellular processes of enterocytes such as proliferation and death, oxidative stress, redox equilibrium, tight junctions' formation and function, and inflammatory response<sup>[10-13]</sup>. The results presented in this study by Dr. Akcan *et al* are in close agreement with those previously reported by us and add further support to the hypothesis of a multifactorial mode of action of BBS and NT on the intestinal mucosa, beyond their trophic effect. The pleiotropic (mitogenic, antioxidant, antiapoptotic, anti-inflammatory and tight-junction modulating) effects of BBS and NT on intestinal mucosa and the wide range of intestinal injuries that could be healed or prevented by these peptides render BBS and NTS potential pivotal "gut-regulatory peptides" for many intestinal diseases. Although the authors very precisely state that the results of laboratory experiments are not readily applicable to the clinical situation, we feel that there is already a substantial experimental body of evidence supporting their consideration for clinical trials.

## REFERENCES

- 1 Akcan A, Muhtaroglu S, Akgun H, Akyildiz H, Kucuk C, Sozuer E, Yurci A, Yilmaz N. Ameliorative effects of bombesin and neurotensin on trinitrobenzene sulphonic acid-induced colitis, oxidative damage and apoptosis in rats. *World J Gastroenterol* 2008; **14**: 1222-1230
- 2 Wood JG, Hoang HD, Bussjaeger LJ, Solomon TE. Neurotensin stimulates growth of small intestine in rats. *Am J Physiol* 1988; **255**: G813-G817
- 3 Evers BM, Izukura M, Townsend CM Jr, Uchida T, Thompson JC. Differential effects of gut hormones on pancreatic and intestinal growth during administration of an elemental diet. *Ann Surg* 1990; **211**: 630-636; discussion 636-638
- 4 Chu KU, Evers BM, Ishizuka J, Townsend CM Jr, Thompson JC. Role of bombesin on gut mucosal growth. *Ann Surg* 1995; **222**: 94-100
- 5 Gulluoglu BM, Kurtel H, Gulluoglu MG, Aktan AO, Yegen BC, Dizdaroglu F, Yalin R. Bombesin ameliorates colonic damage in experimental colitis. *Dig Dis Sci* 1999; **44**: 1531-1538
- 6 Alican I, Unluer EE, Yegen C, Yegen BC. Bombesin improves burn-induced intestinal injury in the rat. *Peptides* 2000; **21**: 1265-1269
- 7 Vagianos C, Karatzas T, Scopa CD, Panagopoulos C, Tsoni I, Spiliopoulou I, Kalfarentzos F. Neurotensin reduces microbial translocation and improves intestinal mucosa integrity after abdominal radiation. *Eur Surg Res* 1992; **24**: 77-83
- 8 Ryan CK, Miller JH, Seydel AS, de Mesy Jensen K, Sax HC. Epidermal growth factor and neurotensin induce microvillus hypertrophy following massive enterectomy. *J Gastrointest Surg* 1997; **1**: 467-473
- 9 Heuser M, Pfaar O, Gralla O, Grone HJ, Nustede R, Post S. Impact of gastrin-releasing peptide on intestinal microcirculation after ischemia-reperfusion in rats. *Digestion* 2000; **61**: 172-180
- 10 Assimakopoulos SF, Scopa CD, Charonis A, Spiliopoulou I, Georgiou C, Nikolopoulou V, Vagianos CE. Experimental obstructive jaundice disrupts intestinal mucosal barrier by altering occludin expression: beneficial effect of bombesin and neurotensin. *J Am Coll Surg* 2004; **198**: 748-757
- 11 Assimakopoulos SF, Scopa CD, Zervoudakis G, Mylonas PG, Georgiou C, Nikolopoulou V, Vagianos CE. Bombesin and neurotensin reduce endotoxemia, intestinal oxidative stress, and apoptosis in experimental obstructive jaundice. *Ann Surg* 2005; **241**: 159-167
- 12 Assimakopoulos SF, Alexandris IH, Scopa CD, Mylonas PG, Thomopoulos KC, Georgiou CD, Nikolopoulou VN, Vagianos CE. Effect of bombesin and neurotensin on gut barrier function in partially hepatectomized rats. *World J Gastroenterol* 2005; **11**: 6757-6764
- 13 Assimakopoulos SF, Vagianos CE, Charonis AS, Alexandris IH, Spiliopoulou I, Thomopoulos KC, Nikolopoulou VN, Scopa CD. Experimental obstructive jaundice alters claudin-4 expression in intestinal mucosa: effect of bombesin and neurotensin. *World J Gastroenterol* 2006; **12**: 3410-3415

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**Manal F Abdelmalek, MD, MPH, Associate Professor of Medicine**  
Duke University, PO Box 3913, Durham, NC 27710, United States

**Hitoshi Asakura, Director, Emeritus Professor**  
International Medical Information Center, Shinanomachi Renga Bldg.35,  
Shinanomachi, Shinjuku, Tokyo 160-0016, Japan

**Andrew V Biankin, BMedSc, MB, BS, PhD, Associate Professor**  
Cancer Research Program, Garvan Institute of Medical Research, 384 Victoria St,  
Darlinghurst, NSW, 2010, Australia

**Dr. Milan Jirsa**  
Laboratory of Experimental Medicine-building Z1, Institute for Clinical and  
Experimental Medicine, Videnska 1958/9, Praha 4, 14000, Czech

**Rosemar Joyce Burnett, PhD**  
Department of Epidemiology National School of Public Health, University of  
Limpopo, Medunsa Campus PO Box 173, MEDUNSA, Pretoria 0204,  
South Africa

**Dr. Wang-Xue Chen**  
Institute for Biological Sciences, National Research Council Canada, 100 Sussex  
Drive, Room 3100, Ottawa, Ontario K1A 0R6, Canada

**Ana J Coito, Associate Professor of Surgery**  
Department of Surgery, The Dumont, UCLA Transplant Center, 77-120 CHS,  
Box 957054, Los Angeles, CA 90095-7054, United States

**Dario Conte, Professor**  
GI Unit-IRCCS Osp. Maggiore, Chair of Gastroenterology, Via F. Sforza, 35,  
Milano 20122, Italy

**Arno J Dormann, PD, MED**  
Habil, Medizinische Klinik, Krankenhaus Holweide, Kliniken der Stadt Köln  
gmbH, Neufelder St. 32, 51067 Köln, Germany

**Emad M El-Omar, Professor**  
Department of Medicine & Therapeutics, Aberdeen AB25 2ZD, United Kingdom

**Isabel Fabregat, PhD, Associate Professor**  
Laboratori d'Oncologia Molecular, Institut d'Investigació Biomèdica de Bellvitge,  
Gran Via, Km 2, 7, L'Hospitalet, 08907 Barcelona, Spain

**Francesco Feo, Professor**  
Dipartimento di Scienze Biomediche, Sezione di Patologia Sperimentale e  
Oncologia, Università di Sassari, Via P. Manzella 4, 07100 Sassari, Italy

**Julia B Greer, MD, MPH**  
Department of Gastroenterology, Hepatology and Nutrition, University of  
Pittsburgh Medical Center, M2, Presbyterian University Hospital, 200 Lothrop  
Street, Pittsburgh, Pa 15213, United States

**Salvatore Gruttadauria, MD, Assistant Professor**  
Abdominal Transplant Surgery, ISMETT, Via E. Tricomi, 190127 Palermo, Italy

**Toru Hiyama, MD, PhD**  
Health Service Center, Hiroshima University, 1-7-1 Kagamiyama, Higashihiroshima  
739-8521, Japan

**Dr. Serdar Karakose, Professor**  
Department of Radiology, Meram Medical Faculty, Selcuk University, Konya  
42080, Turkey

**Tom H Karlsen, MD**  
Institute of Immunology, Rikshospitalet University Hospital, N-0027 Oslo,  
Norway

**Jin-Hong Kim, Professor,**  
Department of Gastroenterology, Ajou University Hospital, San 5, Wonchong,  
Yongtong-gu, Suwon 442-721, South Korea

**Leonidas G Koniaris, Professor**  
Alan Livingstone Chair in Surgical Oncology, 3550 Sylvester Comprehensive  
Cancer Center (310T), 1475 NW 12th Ave., Miami, FL 33136, United States

**Ezio Laconi, MD, PhD, Professor of General Pathology**  
Department of Sciences and Biomedical Technologies, Unit of Experimental  
Pathology, University of Cagliari, Via Porcell, 4 -IV Piano, 09125- Cagliari, Italy

**Yasushi Matsuzaki, Associated Professor**  
Division of Gastroenterology and Hepatology, Graduate School of Comprehensive  
Human Sciences and University Hospital, 1-1-1, Tennodai, Tsukuba 305-8575,  
Japan

**Didier Merlin, PhD, Associate Professor**  
Department of Medicine Division of Digestive Diseases, Emory University, 615  
Michael Street, Atlanta, GA 30322, United States

**Peter L Moses, MD, FACP, AGAF, Professor**  
University of Vermont College of Medicine Section of Gastroenterology &  
Hepatology, 111 Colchester Avenue, Smith 237B, MCHV, Burlington, VT 05401,  
United States

**Raymund R Razonable, MD**  
Division of Infectious Diseases, Mayo Clinic, 200 First Street SW, Rochester,  
Minnesota 55905, United States

**Barry Rosser, MD**  
Department of Transplantation, Mayo Clinic Jacksonville, Suite 1100, 4205 Belfort  
Road, Jacksonville, Florida 32216, United States

**Henning Schulze-Bergkamen, MD**  
First Medical Department, University of Mainz, Langenbeckstr, 1, 55101 Mainz,  
Germany

**Tomohiko Shimatani, Assistant Professor**  
Department of General Medicine, Hiroshima University Hospital, 1-2-3 Kasumi,  
Minami-ku, Hiroshima 7348551, Japan

**Ned Snyder, MD, FACP, AGAF, Professor of Medicine, Chief of Clinical  
Gastroenterology and Hepatology**  
Department of Internal Medicine, The University of Texas Medical Branch, 301  
University Blvd., Galveston, Texas 77555-0764, United States

**Michael Torbenson, MD, Associate Professor of Pathology**  
Room B314 1503 E Jefferson (Bond Street Building), The Johns Hopkins  
University School of Medicine, Baltimore, MD 21231, United States

**Sun-Lung Tsai, MD, PhD, Professor, Director**  
Hepatogastroenterology Section, Department of Internal Medicine and Liver  
Research Unit, Department of Medical Research, Chi Mei Medical Center, 901  
Chung Hwa Road, Young-Kang City, Tainan County 710, Taiwan, China

**Yvan Vandenplas, Professor**  
Department of Pediatrics, AZ-VUB, Laarbeeklaan 101, Brussels 1090, Belgium

**Saúl Villa-Treviño, MD, PhD**  
Departamento de Biología Celular, Centro de Investigación y Estudios  
Avanzados del IPN (Cinvestav), Ave. IPN No. 2508. Col. San Pedro, Zacatenco,  
CP 07360, México, DF, México

**Eric M Yoshida, MD**  
Department of Medicine, University of British Columbia, 100-2647 Willow Street,  
Vancouver V5Z 3P1, Canada





## Meetings

### Events Calendar 2008-2009

**FALK SYMPOSIA 2008**  
 January 24-25, Frankfurt, Germany  
 Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008  
 February 14-16, Paris, France  
 EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
 8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
 3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation  
 Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

February 29, Québec, Canada  
 Canadian Association of Gastroenterology  
 E-mail: [general@cag-acg.org](mailto:general@cag-acg.org)

March 10-13, Birmingham, UK  
 British Society of Gastroenterology Annual Meeting  
 E-mail: [BSG@mailbox.ulcc.ac.uk](mailto:BSG@mailbox.ulcc.ac.uk)

March 14-15, HangZhou, China  
 Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
 Asian Pacific Association for the Study of the Liver  
 18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
 Shanghai-Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
 OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas  
 E-mail: [robert.giuli@oeso.org](mailto:robert.giuli@oeso.org)

April 9-12, Los Angeles, USA  
 SAGES 2008 Annual Meeting - part of Surgical Spring Week  
[www.sages.org/08program/html/](http://www.sages.org/08program/html/)

April 18-22, Buenos Aires, Argentina  
 9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
 Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
 43<sup>rd</sup> Annual Meeting of the European Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary

Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA  
 Digestive Disease Week 2008

May 21-22, California, USA  
 ASGE Annual Postgraduate Course  
 Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion  
 E-mail: [education@#97;sgge.org](mailto:education@#97;sgge.org)

June 4-7, Helsinki, Finland  
 The 39<sup>th</sup> Nordic Meeting of Gastroenterology  
[www.congex.com/ngc2008](http://www.congex.com/ngc2008)

June 5-8, Sitges (Barcelona), Spain  
 Semana de las Enfermedades Digestivas  
 E-mail: [sepd@sepd.es](mailto:sepd@sepd.es)

June 6-8, Prague, Czech Republic  
 3<sup>rd</sup> Annual European Meeting: Perspectives in Inflammatory Bowel Diseases  
 E-mail: [meetings@imedex.com](mailto:meetings@imedex.com)

June 10-13, Istanbul, Turkey  
 ESGAR 2008 19<sup>th</sup> Annual Meeting and Postgraduate Course  
 E-mail: [fca@netvisao.pt](mailto:fca@netvisao.pt)

June 11-13, Stockholm, Sweden  
 16<sup>th</sup> International Congress of the European Association for Endoscopic Surgery  
 E-mail: [info@#101;aes-eur.org](mailto:info@#101;aes-eur.org)

June 13-14, Amsterdam, Netherlands  
 Falk Symposium 165: XX International Bile Acid Meeting. Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic  
 Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management  
 E-mail: [idla2008@guarant.cz](mailto:idla2008@guarant.cz)

June 25-28, Barcelona, Spain  
 10<sup>th</sup> World Congress on Gastrointestinal Cancer  
 Imedex and ESMO  
 E-mail: [meetings@imedex.com](mailto:meetings@imedex.com)

June 25-28, Lodz, Poland  
 Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatologists (IAP)  
 E-mail: [office@epc-iap2008.org](mailto:office@epc-iap2008.org)  
[www.e-p-c.org](http://www.e-p-c.org)  
[www.pancreatology.org](http://www.pancreatology.org)

June 26-28, Bratislava, Slovakia  
 5<sup>th</sup> Central European Gastroenterology Meeting  
[www.ceurgem2008.cz](http://www.ceurgem2008.cz)

July 9-12, Paris, France  
 ILTS 14<sup>th</sup> Annual International Congress  
[www.iltis.org](http://www.iltis.org)

September 10-13, Budapest, Hungary  
 11<sup>th</sup> World Congress of the International Society for Diseases of the Esophagus  
 E-mail: [isde@isde.net](mailto:isde@isde.net)

September 13-16, New Delhi, India  
 Asia Pacific Digestive Week  
 E-mail: [apdw@apdw2008.net](mailto:apdw@apdw2008.net)

III FALK GASTRO-CONFERENCE  
 September 17, Mainz, Germany

Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany  
 Falk Symposium 166:  
 GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic  
 Prague Hepatology Meeting 2008  
[www.czech-hepatology.cz/phm2008](http://www.czech-hepatology.cz/phm2008)

September 20-21, Mainz, Germany  
 Falk Symposium 167:  
 Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France  
 Third Annual Meeting  
 European Society of Coloproctology  
[www.escp.eu.com](http://www.escp.eu.com)



October 8-11, Istanbul, Turkey  
 18<sup>th</sup> World Congress of the International Association of Surgeons,  
 Gastroenterologists and Oncologists  
 E-mail: [orkun.sahin@serenas.com.tr](mailto:orkun.sahin@serenas.com.tr)

October 18-22, Vienna, Austria  
 16<sup>th</sup> United European Gastroenterology Week  
[www.negf.org](http://www.negf.org)  
[www.acv.at](http://www.acv.at)

October 22-25, Minnesota, USA  
 Anstralian Gastroenterology Week 2008  
 E-mail: [gesa@gesa.org.au](mailto:gesa@gesa.org.au)

October 22-25, Brisbane, Australia  
 71<sup>st</sup> Annual Colon and Rectal Surgery Conference  
 E-mail: [info@colonrectalcourse.org](mailto:info@colonrectalcourse.org)

October 31-November 4, Moscone West Convention Center, San Francisco, CA  
 59<sup>th</sup> AASLD Annual Meeting and Postgraduate Course  
 The Liver Meeting  
 Information: [www.aasld.org](http://www.aasld.org)

November 6-9, Lucerne, Switzerland  
 Neurogastroenterology & Motility Joint International Meeting 2008  
 E-mail: [ngm2008@mci-group.com](mailto:ngm2008@mci-group.com)  
[www.ngm2008.com](http://www.ngm2008.com)

November 12, Santiago de Chile, Chile  
 Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt  
 1<sup>st</sup> Hepatology and Gastroenterology Post Graduate Course  
[www.egyptgastrohep.com](http://www.egyptgastrohep.com)

December 7-9, Seoul, Korea  
 6<sup>th</sup> International Meeting  
 Hepatocellular Carcinoma: Eastern and Western Experiences  
 E-mail: [sglee@amc.seoul.kr](mailto:sglee@amc.seoul.kr)

INFORMATION FOR ALL  
 FALK FOUNDATION e.V.  
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[www.falkfoundation.de](http://www.falkfoundation.de)

Advanced Courses - European

Institute of Telesurgery EITS - 2008  
 Strasbourg, France  
 January 18-19, March 28-29, June 6-7, October 3-4

N.O.T.E.S  
 April 3-5, November 27-29  
 Laparoscopic Digestive Surgery

June 27-28, November 7-8  
 Laparoscopic Colorectal Surgery

July 3-5  
 Interventional GI Endoscopy Techniques  
 Contact address for all courses:  
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International Gastroenterological Congresses 2009  
 March 23-26, Glasgow, Scotland  
 Meeting of the British Society of Gastroenterology (BSG)  
 E-mail: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

May 17-20, Denver, Colorado, USA  
 Digestive Disease Week 2009

November 21-25, London, UK  
 Gastro 2009 UEGW/World Congress of Gastroenterology  
[www.gastro2009.org](http://www.gastro2009.org)



### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



## Instructions to authors

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*World Journal of Gastroenterology* (*World J Gastroenterol* ISSN 1007-9327 CN 14-1219/R) is a weekly open access peer-reviewed journal supported by an editorial board consisting of 1215 experts in gastroenterology and hepatology from 60 countries. The aim of the journal is to deliver the most clinically relevant original and commentary articles to readers, and to make the full text publicly available to all clinicians, scientists, patients and biomedical students on an unrestricted platform, so that they can access and learn about the most recent key advances in the field.

In addition to the open access nature, another key characteristic of *WJG* is its reading guidance for each article which includes background, research frontier, related reports, breakthroughs, applications, terminology, and comments of peer reviewers for the general readers.

*WJG* publishes articles on esophageal, gastrointestinal, hepatobiliary and pancreatic tumors, and other esophageal, gastrointestinal, hepatic-biliary and pancreatic diseases in relation to epidemiology, immunology, microbiology, motility & nerve-gut interaction, endocrinology, nutrition & obesity, endoscopy, imaging and advanced hi-technology.

The main goal of *WJG* is to publish high quality commentary articles contributed by leading experts in gastroenterology and hepatology and original articles that combine the clinical practice and advanced basic research, to provide an interactive platform for clinicians and researchers in internal medicine, surgery, infectious diseases, traditional Chinese medicine, oncology, integrated Chinese and Western medicine, imaging, endoscopy, interventional therapy, pathology and other basic medical specialties, and thus eventually improving the clinical practice and healthcare for patients.

### Indexed and abstracted in

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### Published by

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An informative, structured abstract of no more than 350 words should accompany each manuscript. Abstracts for original contributions should be structured into the following sections: AIM: Only the purpose should be included. METHODS: The materials, techniques, instruments and equipment, and the experimental procedures should be included. RESULTS: The observed and experimental results, including data, effects, outcome, *etc.* should be included. Authors should present *P* value where necessary, and also include any significant data. CONCLUSION: Accurate view and the value of the results should be included.

The format for structured abstracts can be found at: <http://www.wjgnet.com/wjg/help/11.doc>.

#### Key words

Please list 5-10 key words, selected mainly from *Index Medicus*, which reflect the content of the study.

#### Text

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and case reports, the main text should be structured into the following sections: INTRODUCTION, MATERIALS AND METHODS, RESULTS and DISCUSSION, and should include appropriate Figures and Tables. Data should be presented in the body text or in Figures and Tables, but not in both. The main text format of these sections, editorial, topic highlight, case report, letters to the editors, should be found at: <http://www.wjgnet.com/wjg/help/instructions.jsp>.

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Figures should be numbered as 1, 2, 3, *etc.*, and mentioned clearly in the main text. Provide a brief title for each figure on a separate page. Detailed legends should not be provided under the figures. This part should be added into the text where the figures are applicable. Figures should be either Photoshop or Illustrator files (in tiff, eps, jpeg formats) at high-resolution. Examples can be found at: <http://www.wjgnet.com/1007-9327/13/4520.pdf>; <http://www.wjgnet.com/1007-9327/13/4554.pdf>; <http://www.wjgnet.com/1007-9327/13/4891.pdf>; <http://www.wjgnet.com/1007-9327/13/4986.pdf>; <http://www.wjgnet.com/1007-9327/13/4498.pdf>. Keeping all elements compiled is necessary in line-art image. Scale bars should be used rather than magnification factors, with the length of the bar defined in the legend rather than on the bar itself. File names should identify the figure and panel. Avoid layering type directly over shaded or textured areas. Please use uniform legends for the same subjects. For example: Figure 1 Pathological changes in atrophic gastritis after treatment. A: ...; B: ...; C: ...; D: ...; E: ...; F: ...; G: ... *etc.* It is our principle to publish high resolution-figures for the printed and E-versions.

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### Notes in tables and illustrations

Data that are not statistically significant should not be noted. <sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01 should be noted (*P* > 0.05 should not be noted). If there are other series of *P* values, <sup>c</sup>*P* < 0.05 and <sup>d</sup>*P* < 0.01 are used. A third series of *P* values can be expressed as <sup>e</sup>*P* < 0.05 and <sup>f</sup>*P* < 0.01. Other notes in tables or under illustrations should be expressed as <sup>1</sup>F, <sup>2</sup>F, <sup>3</sup>F; or sometimes as other symbols with a superscript (Arabic numerals) in the upper left corner. In a multi-curve illustration, each curve should be labeled with ●, ○, ■, □, ▲, △, *etc.*, in a certain sequence.

### Acknowledgments

Brief acknowledgments of persons who have made genuine contributions to the manuscripts and who endorse the data and conclusions should be included. Authors are responsible for obtaining written permission to use any copyrighted text and/or illustrations.

## REFERENCES

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The author should number the references in Arabic numerals according to the citation order in the text. Put reference numbers in square brackets in superscript at the end of citation content or after the cited author's name. For citation content which is part of the narration, the coding number and square brackets should be typeset normally. For example, "Crohn's disease (CD) is associated with increased intestinal permeability<sup>[1,2]</sup>". If references are cited directly in the text, they should be put together within the text, for example, "From references<sup>[19,22-24]</sup>, we know that..."

When the authors write the references, please ensure that the order in text is the same as in the references section, and also ensure the spelling accuracy of the first author's name. Do not list the same citation twice.

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### Format

#### Journals

*English journal article (list all authors and include the PMID where applicable)*

- 1 **Jung EM**, Clevert DA, Schreyer AG, Schmitt S, Rennert J, Kubale R, Feuerbach S, Jung F. Evaluation of quantitative contrast harmonic imaging to assess malignancy of liver tumors: A prospective controlled two-center study. *World J Gastroenterol* 2007; **13**: 6356-6364 [PMID: 18081224 DOI: 10.3748/wjg.13.6356]

*Chinese journal article (list all authors and include the PMID where applicable)*

- 2 **Lin GZ**, Wang XZ, Wang P, Lin J, Yang FD. Immunologic effect of Jianpi Yishen decoction in treatment of Pixu-diarrhoea. *Shijie Huaren Xiaohua Zazhi* 1999; **7**: 285-287

*In press*

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

*Organization as author*

- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; **40**: 679-686 [PMID: 12411462]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; **169**: 2257-2261 [PMID: 12771764]

*No author given*

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; **325**: 184 [PMID: 12142303]

*Volume with supplement*

- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment



of migraine and in comparison with sumatriptan. *Headache* 2002; 42 Suppl 2: S93-99 [PMID: 12028325]

*Issue with no volume*

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (401): 230-238 [PMID: 12151900]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS/A Careaction* 2002; 1-6 [PMID: 12154804]

## Books

*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 **Breedlove GK**, Schorffheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

*Conference paper*

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

**Electronic journal** (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. Emerg Infect Dis serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

**Patent** (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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Use SI units. For example: body mass, *m* (B) = 78 kg; blood pressure, *p* (B) = 16.2/12.3 kPa; incubation time, *t* (incubation) = 96 h, blood glucose concentration, *c* (glucose) 6.4  $\pm$  2.1 mmol/L; blood CEA mass concentration, *p* (CEA) = 8.6 24.5  $\mu$ g/L; CO<sub>2</sub> volume fraction, 50 mL/L CO<sub>2</sub>, not 5% CO<sub>2</sub>; likewise for 40 g/L formaldehyde, not 10% formalin; and mass fraction, 8 ng/g, etc. Arabic numerals such as 23, 243, 641 should be read 23 243 641.

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## Minimal hepatic encephalopathy matters in daily life

Jasmohan S Bajaj

Jasmohan S Bajaj, Division of Gastroenterology and Hepatology, Medical College of Wisconsin, Milwaukee, WI 53226, United States

Author contributions: Bajaj JS contributed all to this paper.

Correspondence to: Jasmohan S Bajaj, MBBS, MD, MS, Division of Gastroenterology and Hepatology, Medical College of Wisconsin, Milwaukee, WI 53226, United States. [jasmohan@gmail.com](mailto:jasmohan@gmail.com)

Telephone: +1-414-4566825 Fax: +1-414-4566214

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Department of Clinical and Experimental Medicine, Federico II University Medical School, VIA S. PANSINI, 5, Naples 80131, Italy

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### Abstract

Minimal hepatic encephalopathy is a neuro-cognitive dysfunction which occurs in an epidemic proportion of cirrhotic patients, estimated as high as 80% of the population tested. It is characterized by a specific, complex cognitive dysfunction which is independent of sleep dysfunction or problems with overall intelligence. Although named “minimal”, minimal hepatic encephalopathy (MHE) can have a far-reaching impact on quality of life, ability to function in daily life and progression to overt hepatic encephalopathy. Importantly, MHE has a profound negative impact on the ability to drive a car and may be a significant factor behind motor vehicle accidents. A crucial aspect of the clinical care of MHE patients is their driving history, which is often ignored in routine care and can add a vital dimension to the overall disease assessment. Driving history should be an integral part of care in patients with MHE. The lack of specific signs and symptoms, the preserved communication skills and lack of insight make MHE a difficult condition to diagnose. Diagnostic strategies for MHE abound, but are usually limited by financial, normative or time constraints. Recent studies into the inhibitory control and critical flicker frequency tests are encouraging since these tests can increase the rates of MHE diagnosis without requiring a psychologist. Although testing for MHE and subsequent therapy is not standard of care at this time, it is important to consider this in cirrhotics in order to improve their ability to live their life to the fullest.

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**Key words:** Minimal hepatic encephalopathy; Quality of life; Driving impairment; Diagnosis; Therapy; Prognosis

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### INTRODUCTION

Minimal hepatic encephalopathy is a neuro-cognitive dysfunction which occurs in an epidemic proportion of cirrhotic patients, estimated as high as 80% of the population tested. It is characterized by a specific, complex cognitive dysfunction which is independent of sleep dysfunction or problems with overall intelligence<sup>[1]</sup>. Although named “minimal”, minimal hepatic encephalopathy (MHE) can have a far-reaching impact on quality of life, ability to function in daily life and progression to overt hepatic encephalopathy (OHE)<sup>[2]</sup>. Importantly, MHE has a profound negative impact on the ability to drive a car and may be a significant factor behind motor vehicle accidents. Research in this field is expanding rapidly, but little consensus has emerged regarding standard diagnostic strategies or therapeutic options<sup>[3]</sup>. The current editorial will focus on the relevance of MHE as an important clinical entity that is ready for evaluation and regular detection not only in research centers but in routine hepatology practice.

### PREVALENCE AND IMPORTANCE

With improving management of cirrhotic patients, including those with end-stage liver disease, the neuro-psychological care of these patients is being recognized as an unmet need<sup>[4-6]</sup>. The importance of MHE has been recognized by hepatologists worldwide and of late an explosion of research in this field has occurred<sup>[7,8]</sup>.

Since its first description in the 1970s, MHE has been diagnosed in several countries around the world at a rate of 30%-80%<sup>[9,10]</sup>. The European experience has shown a high prevalence of MHE in patients who are predominantly non-alcoholic and without any psychoactive drug use<sup>[11,12]</sup>. The diagnostic methodologies were a combination of neuro-psychometric and neuro-physiologic testing strategies<sup>[11-13]</sup>. In the United States, the rate of MHE in several research series has been reported to

be 60%-80%, again using a combination of psychometric and neuro-physiologic techniques<sup>[14,15]</sup>.

Experience in Asian countries, especially India, Japan and China, has reconfirmed the high prevalence using locally modified tools<sup>[10,16-22]</sup>. The diagnostic tools were adapted to the local language and to include illiterate subjects<sup>[17,19]</sup>. The patient population in these series has included a higher number of patients with viral hepatitis compared to Western series<sup>[21,22]</sup>.

## CHARACTERIZATION OF MINIMAL HEPATIC ENCEPHALOPATHY

The importance of MHE lies in its specific deficits. As outlined by Weissenborn *et al*, patients with MHE have defects in attention, vigilance and orientation<sup>[23]</sup>. These attention deficits in turn lead to learning impairment and difficulties in working memory<sup>[12,24]</sup>. Psychometric testing in patients with MHE has consistently demonstrated a preservation of overall IQ compared to age-matched controls, indicating that the defects are restricted to certain aspects only<sup>[11]</sup>. Patients with cirrhosis can also exhibit motor impairments that include Parkinsonian features and features of hepatic myelopathy<sup>[25,26]</sup>. However, these motor deficits are not included in the typical impairment seen in MHE.

Importantly, deficits in MHE do not extend to the verbal and communication spheres<sup>[11]</sup>. Similar to OHE patients, there is evidence that patients with MHE have poor insight into their psychometric impairments<sup>[27-29]</sup>. The preservation of communication skills, lack of symptoms and the poor insight make MHE patients a difficult group to identify with simple questioning in the office.

## CONTRIBUTION OF CONCOMITANT DISEASES TO MHE

Patients with specific etiologies of cirrhosis are more likely to exhibit psychometric impairment, specifically chronic hepatitis C. Investigations in the chronic hepatitis C infected groups (both with and without cirrhosis) show a worse psychometric performance compared to patients without chronic hepatitis C in selected studies<sup>[30-33]</sup>. However, other studies have not demonstrated a difference in psychometric performance of cirrhotics with chronic hepatitis C compared to those without it<sup>[30,34]</sup>. In addition, a recent detailed study before and after interferon therapy in chronic hepatitis C cirrhotics failed to find an improvement or deterioration during and after therapy completion<sup>[35]</sup>.

Diabetes mellitus is an important correlate of patients with cirrhosis, with the increasing importance of non-alcoholic steatohepatitis, and is also correlated with chronic hepatitis C in the general population<sup>[36]</sup>. Diabetes mellitus, possibly due to its adverse effect on gastrointestinal motility, has been associated with hepatic encephalopathy<sup>[34,37]</sup>.

Most studies of MHE exclude patients with alcoholic liver disease; therefore, excluding patients with chronic

hepatitis C and diabetes mellitus will seriously hinder the generalization of the study. Therefore, a subgroup analysis of chronic hepatitis C and diabetes mellitus within the cirrhosis group or regression adjustment would be necessary for MHE investigation.

## CONCOMITANT SLEEP DISTURBANCES

Hepatic encephalopathy is associated with adverse effects on the sleep-wake cycle, especially causing fragmentation of sleep, sleep deprivation and reports of drowsiness during the day<sup>[38]</sup>. Sleep deprivation *per se* can result in impaired psychometric test performance and as is evidenced in the case of obstructive sleep apnea, and can independently lead to poor driving outcomes<sup>[39]</sup>. There is debate whether the MHE-associated psychometric impairment is partly due to the inherent sleep-wake cycle disturbances in this condition. Validated sleep and quality of life questionnaire such as Sickness Impact Profile (SIP) sleep scales, Pittsburgh Sleep Quality index and Epworth Sleepiness scale evaluation demonstrate a worse sleep quality and effect on quality of life in patients with MHE<sup>[17,38,40]</sup>. Steindl *et al* demonstrated a disrupted melatonin cycle in patients with cirrhosis and MHE which was independent of psychometric performance<sup>[41]</sup>. However, reports have demonstrated that cirrhotics with MHE and sleep disruption do not have worse psychometric performance compared to those who do not have sleep disruption<sup>[38,42,43]</sup>. This implies that although there is a significant disruption of the sleep-wake and circadian rhythm in patients with MHE, this phenomenon co-exists with the psychometric impairment and is not the cause of it.

## QUALITY OF LIFE AND MHE

Quality of life is an essential assessment component of patients with chronic diseases. Issues pertaining to quality of life are also central to most patient complaints in cirrhosis<sup>[44]</sup>. Groeneweg *et al* studied the Sickness Impact Profile (SIP) in a cohort of cirrhotics being tested for MHE (Medical Outcomes Trust, Boston, MA)<sup>[40]</sup>. The SIP consists of 136 items which questions patients about 12 sections; sleep and rest, eating, work, home management, recreation and pastimes, ambulation, mobility, body care and movement (the last three generate a physical sub score), social interaction, alertness behavior, emotional behavior, and communication (comprising the psychosocial sub score). All scales were significantly impaired in MHE patients compared to others. A recent study by Prasad *et al* confirmed these findings in MHE patients in all spheres apart from communication, which was similar between patients with or without MHE<sup>[17]</sup>. Impaired quality of life has also been demonstrated using the Short Form 36 (SF-36) in MHE populations in several studies across the world<sup>[45,46]</sup>.

Short form-36 (SF-36) is a 36-part questionnaire that has been used in several studies to characterize chronic liver disease and the chronic liver disease questionnaire

has also been used in patients with liver disease<sup>[44-47]</sup>.

The SIP is an extensive survey which requires several minutes to complete, in contrast to the relatively short SF-36. The SF-36, therefore, may be a better tool for clinical practice. However, since quality of life changes in MHE are subtle, the SIP is perhaps the questionnaire better suited for research studies since it can differentiate between small changes in several aspects of QOL.

## WORK CAPACITY AND MHE

The specific nature of cognitive dysfunction in MHE results in a disproportionate impairment of workers engaged in “blue-collar” professions compared to “white-collar” professionals. This is essential to remember because cirrhotics engaged in professions that require constant vigilance and coordination, e.g. machinery operators and drivers are affected by MHE more severely compared to those who have predominantly verbal and intellectual functions, such as administrative and company executives<sup>[29]</sup>. Therefore, MHE not only has the potential to endanger the patients and co-workers during complex occupational tasks, it also can adversely affect their socio-economic status by interfering with work performance<sup>[48]</sup>.

## MHE AND PROGRESSION TO OVERT HEPATIC ENCEPHALOPATHY

Overt hepatic encephalopathy portends a poor prognosis and overall survival<sup>[49,50]</sup>. Patients with MHE have a higher likelihood of development of OHE<sup>[14,49,51,52]</sup>. Specific subgroups that are more likely to progress to OHE are males, those with a history of OHE, those with alcoholic etiology of cirrhosis and those with varices<sup>[19,53,54]</sup>. Positive responders to the glutamine tolerance test are also more likely to develop OHE<sup>[53]</sup>. It is not clear, however, which individual MHE patient will go on to develop OHE. The relative contributions of precipitating factors, such as gastrointestinal bleeding, for OHE development in the context of MHE *versus* no MHE have also not been fully elucidated. Therefore, patients with MHE may be a subgroup requiring close follow-up clinically for OHE development, especially when potential precipitating factors are encountered.

## MHE AND DRIVING CAPABILITY

The ability to drive a motor vehicle requires coordination of visual, auditory and vestibular inputs and has the potential to be impaired by metabolic encephalopathies<sup>[55]</sup>.

MHE affects attention, psychomotor function and working memory, all of which are essential for safe driving<sup>[56]</sup>. Most studies of driving ability using on-road driving tests have demonstrated that MHE patients have significant defects in reaction time, resulting in their pronouncement as unsafe drivers in studies from Germany and Japan<sup>[49,57]</sup>. Wein *et al* studied the driving ability and rating of driving behavior of 44 patients with cirrhosis

using instructors masked to their status. Fourteen of these patients had been diagnosed with MHE<sup>[58]</sup>. Results showed that patients with MHE required interventions by the driving instructor to prevent an accident at a rate 10 times higher compared to those with MHE and controls. Specific driving behaviors were also rated worse in patients with MHE, especially car handling, adaptation, cautiousness and maneuvering<sup>[58]</sup>.

Another essential skill required for safe driving is navigation, which ensures that the subject is in the right place at the right time<sup>[55]</sup>. Given the working memory abnormalities in cirrhotic patients, the study of navigation in MHE is important<sup>[24]</sup>. Our group recently published a study evaluating the performance of cirrhotics to age and education-matched controls on a driving simulator. Navigation skills in MHE are adversely affected<sup>[59]</sup>. All patients underwent a driving simulation which also included a navigation task. This task consisted of driving through a “virtual city” and illegal turns off the marked path were recorded. There was a significantly higher rate of illegal turns in the MHE group compared to those without MHE and controls. Illegal turns were proportionate to impairment in psychometric performance in cirrhotic group<sup>[59]</sup>. Therefore, driving difficulties in patients with MHE are likely multi-dimensional and includes impairment in reaction time and navigation skills.

## DRIVING OUTCOMES IN PATIENTS WITH CIRRHOSIS AND MHE

Traffic accidents are one the leading causes of death worldwide, especially in young adults, the most productive age group of any society. It is important to determine whether patients with MHE also have poor driving outcomes compared to those without MHE and controls. This would be essential in formulating public health decisions regarding licensing and therapy for MHE. A study published by our group sent an anonymous driving outcome questionnaire to controls, cirrhotics tested for MHE and cirrhotics not tested for MHE due to concurrent psychoactive drug use<sup>[60]</sup>. As many as 33% of MHE patients reported having a traffic accident or violation within the last year compared to only 4% of MHE negative patients and 12% of the patients using psychoactive drugs. When 5 year data were analyzed a significant majority of MHE patients (53%) reported a traffic accident or violation compared to only 23% of MHE negative patients and 22% of those on psychoactive drugs. This is even more significant since none of the MHE patients were drinking alcohol. On multi-variate analysis, MHE emerged as the sole factor associated with traffic violations [odds ratio 6.0 (CI 1.2-31.3)], motor vehicle accidents [odds ratio 7.3 (CI 2.1-33.2)] and both [odds ratio 7.6 (CI 1.5-37.3)]<sup>[60]</sup>. However, despite these striking numbers, there is still the need to analyze driving data prospectively using identified records before making specific recommendations regarding driving capability<sup>[3]</sup>.

Patients with cirrhosis have a poor prognosis after trauma and surgery, especially with increasing Child-



Pugh score<sup>[61]</sup>. A combination of coagulation impairment, sepsis and hepatic dysfunction has been noted as contributing factors to this worse prognosis<sup>[61]</sup>. A study of a large inpatient sample from the United States showed that patients with cirrhosis who were involved in a motor vehicle crash had a higher mortality than those who were admitted for motor vehicle crashes only<sup>[62]</sup>. Despite a younger average age, patients with cirrhosis and crash had a similar mortality compared to those admitted with cirrhosis only. Hospitalization charges and inpatient stay were also significantly higher in cirrhotics with crash compared to patients admitted for cirrhosis only and those admitted for motor vehicle accidents only. On multi-variate regression within the patients admitted with motor vehicle accidents, age > 65 years and cirrhosis were the variables most significantly associated with mortality<sup>[62]</sup>.

Therefore, not only are patients with MHE more likely to get into an accident, they are also more likely to die from it and utilize greater resources as a result of the accident. All these factors make it essential for a clinician to take a driving history when evaluating patients for cirrhosis and chronic liver disease.

## MHE: INSIGHT INTO THE DISEASE PROCESS AND DRIVING SKILLS

Insight into personal deficits is essential in patients in order to seek medical intervention. Anosognosia, defined as the unawareness of a disease, is a key component of the disease process in several metabolic and vascular cerebral disorders<sup>[63,64]</sup>. This phenomenon is clearly observed in patients with OHE, in which it is the persons in the environment who detect changes in the patients' sensorium rather than the patient<sup>[27]</sup>. A recent report extended this lack of insight into driving impairment. This study demonstrated that patients with MHE rated themselves as significantly better drivers compared to those without MHE and controls when they were evaluated by independent observers<sup>[28]</sup>. Therefore, similar to patients with OHE, it may be important to elicit a complete driving history and assessment from relatives familiar with the MHE patients' driving rather than relying on the patients' history alone.

## THE HISTORY NOT TAKEN: DRIVING HISTORY

The standard of care of patients with cirrhosis without any ongoing acute issues is focused on strategies aimed to prevent decompensation. These strategies are aimed at reducing mortality and morbidity from a liver disease standpoint. However, as evidenced by recent reports, patients with cirrhosis and MHE also are at risk for developing morbidity and mortality behind the steering wheel<sup>[58,60]</sup>. An objective driving history, including confirmation from the local supervisory agency, and corroboration of driving skills by relatives is also an essential aspect of patient care. The driving history to the clinical

history would arguably be a vital addition to the overall understanding of the disease severity from a clinical and psychosocial view in cirrhosis.

## TESTING FOR MHE DURING CLINIC VISITS

Although the majority of surveyed hepatologists in Spain and the United States agreed that MHE was a significant problem requiring testing, the minority were able to actually test for MHE as part of their clinical practice<sup>[7,8]</sup>. Main barriers to MHE testing were inability to get tests paid for by insurance, adding time to clinic visits and lack of standardized norms for the United States<sup>[8]</sup>.

The psychometric battery recommended by the Working Group on Hepatic Encephalopathy is the PSE-syndrome test published by Weissenborn *et al*<sup>[2,11]</sup>. This test battery, although quite efficient in diagnosing MHE, requires a psychologist and valid population norms. The difficulty of applying these tests in the United States is the lack of background population norms and the need for a licensed psychologist to order and administer these tests. In addition, these are still not routinely covered by private health care insurance. These logistic barriers have effectively prevented routine clinic diagnosis of MHE.

The AASLD survey also highlighted the need for simpler and rapid testing that can take the place of cumbersome and copyrighted psychometric testing<sup>[8]</sup>. The inhibitory control and the critical flicker frequency have emerged as tests that can be applied in clinical practice without the need for psychological expertise<sup>[3,13,14,20,65]</sup>. However, detailed validation studies are still underway for these tests.

## POPULATION TO BE TESTED

Patients with cirrhosis who are ambulatory and capable of independent living are the ones most affected by MHE and should definitely be tested. Previous recommendations have been split regarding the specific population to be targeted for testing. Ortiz *et al* and Stewart *et al* have specifically recommended certain patient populations be tested<sup>[1,48]</sup>. Psychometric performance can be affected by current alcohol use, use of psychoactive drugs and pre-existing neurological disorders<sup>[11]</sup>. In cirrhotics who do not fulfill these criteria, it is in the best interest of the patient to be offered testing at the initial visit regardless of their subsequent activities. There is no consensus regarding the frequency of testing, but experience has shown relative similarity in psychometric scores at 6 mo intervals in the absence of acute clinical and neurological events such as development of OHE<sup>[19]</sup>.

## THERAPY FOR MHE

Treatment of MHE improves psychometric performance and quality of life<sup>[16,17]</sup>.

A recent consensus conference promulgated lactulose as the first choice of therapy for MHE in concor-

dance with the previous study data and the AASLD survey<sup>[3,8]</sup>. However, whether this would have any effect on development of OHE, driving capability or overall survival remains to be investigated. Since driving and psychometric impairments are highly correlated, it is reasonable to expect that driving performance would also improve after MHE therapy.

However, the adherence rate of lactulose in patients with OHE is low; therefore, to expect an MHE patient, who does not have any specific symptoms and lacks insight into their problems, to be adherent on a medication that could cause diarrhea and flatulence is difficult<sup>[3,66]</sup>. Therefore, alternatives to lactulose have also been studied for MHE. Liu *et al* showed that fiber and fiber with probiotics improved psychometric function and importantly the Child class of patients<sup>[21]</sup>. Similar studies have been published using various formulations of probiotics with good improvement in psychometric tests<sup>[67-71]</sup>. Our group has recently completed a randomized control trial of a probiotic yogurt that resulted in a significant reversal of MHE in the yogurt-randomized group compared to the group randomized to no treatment<sup>[72]</sup>. The adherence was excellent and none of the yogurt-treated group developed OHE. Although probiotics are attractive options that spare the patients from the poor palatability of lactulose, difficulties in the availability and the standardization of probiotic organisms remain. However, these preliminary data suggests that dietary intervention may be considered in addition to probiotics for amelioration of MHE.

Therefore, although treatment options for MHE are evolving, it is still important to test patients to offer them the available therapeutic options.

## CONCLUSION

MHE is an epidemic cognitive dysfunction in patients with cirrhosis which is gaining importance in clinical and research spheres due to improved survival in cirrhotic patients.

MHE patients exhibit a specific cognitive impairment that negatively impacts their driving capability and work performance and importantly is not evident to the patients themselves.

A crucial aspect of the clinical care of MHE patients is their driving history, which is often ignored in routine care and can add a vital dimension to the overall disease assessment. Driving history should be an integral part of care in patients with MHE.

The lack of specific signs and symptoms, the preserved communication skills and lack of insight make MHE a difficult condition to diagnose.

Diagnostic strategies for MHE abound, but are usually limited by financial, normative or time constraints. Recent studies into the inhibitory control and critical flicker frequency tests are encouraging since these tests can increase the rates of MHE diagnosis without requiring a psychologist.

Although testing for MHE and subsequent therapy is not standard of care at this time, it is important to con-

sider this in cirrhotics in order to improve their ability to live their life to the fullest.

## REFERENCES

- 1 Ortiz M, Jacas C, Cordoba J. Minimal hepatic encephalopathy: diagnosis, clinical significance and recommendations. *J Hepatol* 2005; **42** Suppl: S45-S53
- 2 Ferenci P, Lockwood A, Mullen K, Tarter R, Weissenborn K, Blei AT. Hepatic encephalopathy—definition, nomenclature, diagnosis, and quantification: final report of the working party at the 11th World Congresses of Gastroenterology, Vienna, 1998. *Hepatology* 2002; **35**: 716-721
- 3 Mullen K, Ferenci P, Bass NM, Leevy CB, E. K. An Algorithm for the Management of Hepatic Encephalopathy. *Seminars in Liver Disease* 2007; **27**: 32-48
- 4 Qadri AM, Ogunwale BO, Mullen KD. Can we ignore minimal hepatic encephalopathy any longer? *Hepatology* 2007; **45**: 547-548
- 5 Talwalkar JA, Kamath PS. Influence of recent advances in medical management on clinical outcomes of cirrhosis. *Mayo Clin Proc* 2005; **80**: 1501-1508
- 6 Noble JA, Caces MF, Steffens RA, Stinson FS. Cirrhosis hospitalization and mortality trends, 1970-87. *Public Health Rep* 1993; **108**: 192-197
- 7 Vergara-Gomez M, Flavia-Olivella M, Gil-Prades M, Dalmau-Obrador B, Cordoba-Cardona J. [Diagnosis and treatment of hepatic encephalopathy in Spain: results of a survey of hepatologists] *Gastroenterol Hepatol* 2006; **29**: 1-6
- 8 Bajaj JS, Etemadian A, Hafeezullah M, Saeian K. Testing for minimal hepatic encephalopathy in the United States: An AASLD survey. *Hepatology* 2007; **45**: 833-834
- 9 Rikkers L, Jenko P, Rudman D, Freides D. Subclinical hepatic encephalopathy: detection, prevalence, and relationship to nitrogen metabolism. *Gastroenterology* 1978; **75**: 462-469
- 10 Saxena N, Bhatia M, Joshi YK, Garg PK, Tandon RK. Auditory P300 event-related potentials and number connection test for evaluation of subclinical hepatic encephalopathy in patients with cirrhosis of the liver: a follow-up study. *J Gastroenterol Hepatol* 2001; **16**: 322-327
- 11 Weissenborn K, Ennen JC, Schomerus H, Ruckert N, Hecker H. Neuropsychological characterization of hepatic encephalopathy. *J Hepatol* 2001; **34**: 768-773
- 12 Ortiz M, Cordoba J, Jacas C, Flavia M, Esteban R, Guardia J. Neuropsychological abnormalities in cirrhosis include learning impairment. *J Hepatol* 2006; **44**: 104-110
- 13 Romero-Gomez M, Cordoba J, Jover R, del Olmo JA, Ramirez M, Rey R, de Madaria E, Montoliu C, Nunez D, Flavia M, Company L, Rodrigo JM, Felipe V. Value of the critical flicker frequency in patients with minimal hepatic encephalopathy. *Hepatology* 2007; **45**: 879-885
- 14 Bajaj JS, Saeian K, Verber MD, Hirschke D, Hoffmann RG, Franco J, Varma RR, Rao SM. Inhibitory control test is a simple method to diagnose minimal hepatic encephalopathy and predict development of overt hepatic encephalopathy. *Am J Gastroenterol* 2007; **102**: 754-760
- 15 Meyer T, Eshelman A, Abouljoud M. Neuropsychological changes in a large sample of liver transplant candidates. *Transplant Proc* 2006; **38**: 3559-3560
- 16 Watanabe A, Sakai T, Sato S, Imai F, Ohto M, Arakawa Y, Toda G, Kobayashi K, Muto Y, Tsujii T, Kawasaki H, Okita K, Tanikawa K, Fujiyama S, Shimada S. Clinical efficacy of lactulose in cirrhotic patients with and without subclinical hepatic encephalopathy. *Hepatology* 1997; **26**: 1410-1414
- 17 Prasad S, Dhiman RK, Duseja A, Chawla YK, Sharma A, Agarwal R. Lactulose improves cognitive functions and health-related quality of life in patients with cirrhosis who have minimal hepatic encephalopathy. *Hepatology* 2007; **45**: 549-559
- 18 Li YY, Nie YQ, Sha WH, Zeng Z, Yang FY, Ping L, Jia L. Prevalence of subclinical hepatic encephalopathy in cirrhotic

- patients in China. *World J Gastroenterol* 2004; **10**: 2397-2401
- 19 **Das A**, Dhiman RK, Saraswat VA, Verma M, Naik SR. Prevalence and natural history of subclinical hepatic encephalopathy in cirrhosis. *J Gastroenterol Hepatol* 2001; **16**: 531-535
  - 20 **Sharma P**, Sharma BC, Puri V, Sarin SK. Critical flicker frequency: diagnostic tool for minimal hepatic encephalopathy. *J Hepatol* 2007; **47**: 67-73
  - 21 **Liu Q**, Duan ZP, Ha DK, Bengmark S, Kurtovic J, Riordan SM. Synbiotic modulation of gut flora: effect on minimal hepatic encephalopathy in patients with cirrhosis. *Hepatology* 2004; **39**: 1441-1449
  - 22 **Kato A**, Kato M, Ishii H, Ichimiya Y, Suzuki K, Kawasaki H, Yamamoto SI, Kumashiro R, Yamamoto K, Kawamura N, Hayashi N, Matsuzaki S, Terano A, Okita K, Watanabe A. Development of quantitative neuropsychological tests for diagnosis of subclinical hepatic encephalopathy in liver cirrhosis patients and establishment of diagnostic criteria-multicenter collaborative study in Japanese. *Hepatol Res* 2004; **30**: 71-78
  - 23 **Weissenborn K**, Giewekemeyer K, Heidenreich S, Bokemeyer M, Berding G, Ahl B. Attention, memory, and cognitive function in hepatic encephalopathy. *Metab Brain Dis* 2005; **20**: 359-367
  - 24 **Weissenborn K**, Heidenreich S, Giewekemeyer K, Ruckert N, Hecker H. Memory function in early hepatic encephalopathy. *J Hepatol* 2003; **39**: 320-325
  - 25 **Joebgies EM**, Heidemann M, Schimke N, Hecker H, Ennen JC, Weissenborn K. Bradykinesia in minimal hepatic encephalopathy is due to disturbances in movement initiation. *J Hepatol* 2003; **38**: 273-280
  - 26 **Bechar M**, Freud M, Kott E, Kott I, Kravvic H, Stern J, Sandbank U, Bornstein B. Hepatic cirrhosis with post-shunt myelopathy. *J Neurol Sci* 1970; **11**: 101-107
  - 27 **Weissenborn K**. Clinical features of hepatic encephalopathy. In: Zakim D, Boyer TD, editors. *Hepatology*. 4th ed. Philadelphia: WB Saunders, 2003: 431-444
  - 28 **Bajaj JS**, Saeian K, Hafeezullah M, Hoffmann RG, Hammeke TA. Patients with minimal hepatic encephalopathy have poor insight into their driving skills. *Clinical Gastroenterology and Hepatology* 2008; **6**: 1135-1139
  - 29 **Schomerus H**, Hamster W. Quality of life in cirrhotics with minimal hepatic encephalopathy. *Metab Brain Dis* 2001; **16**: 37-41
  - 30 **Perry W**, Hilsabeck RC, Hassanein TI. Cognitive dysfunction in chronic hepatitis C: a review. *Dig Dis Sci* 2008; **53**: 307-321
  - 31 **Citro V**, Milan G, Tripodi FS, Gennari A, Sorrentino P, Gallotta G, Postiglione A, Tarantino G. Mental status impairment in patients with West Haven grade zero hepatic encephalopathy: the role of HCV infection. *J Gastroenterol* 2007; **42**: 79-82
  - 32 **Weissenborn K**, Krause J, Bokemeyer M, Hecker H, Schuler A, Ennen JC, Ahl B, Manns MP, Boker KW. Hepatitis C virus infection affects the brain-evidence from psychometric studies and magnetic resonance spectroscopy. *J Hepatol* 2004; **41**: 845-851
  - 33 **Hilsabeck RC**, Hassanein TI, Carlson MD, Ziegler EA, Perry W. Cognitive functioning and psychiatric symptomatology in patients with chronic hepatitis C. *J Int Neuropsychol Soc* 2003; **9**: 847-854
  - 34 **Kalaitzakis E**, Olsson R, Henfridsson P, Hugosson I, Bengtsson M, Jalan R, Bjornsson E. Malnutrition and diabetes mellitus are related to hepatic encephalopathy in patients with liver cirrhosis. *Liver Int* 2007; **27**: 1194-1201
  - 35 **Fontana RJ**, Bieliauskas LA, Lindsay KL, Back-Madruga C, Wright EC, Snow KK, Lok AS, Kronfol Z, Padmanabhan L. Cognitive function does not worsen during pegylated interferon and ribavirin retreatment of chronic hepatitis C. *Hepatology* 2007; **45**: 1154-1163
  - 36 **Mehta SH**, Brancati FL, Sulkowski MS, Strathdee SA, Szklo M, Thomas DL. Prevalence of type 2 diabetes mellitus among persons with hepatitis C virus infection in the United States. *Ann Intern Med* 2000; **133**: 592-599
  - 37 **Sigal SH**, Stanca CM, Kontorinis N, Bodian C, Ryan E. Diabetes mellitus is associated with hepatic encephalopathy in patients with HCV cirrhosis. *Am J Gastroenterol* 2006; **101**: 1490-1496
  - 38 **Cordoba J**, Cabrera J, Lataif L, Penev P, Zee P, Blei AT. High prevalence of sleep disturbance in cirrhosis. *Hepatology* 1998; **27**: 339-345
  - 39 **Engleman HM**, Hirst WS, Douglas NJ. Under reporting of sleepiness and driving impairment in patients with sleep apnoea/hypopnoea syndrome. *J Sleep Res* 1997; **6**: 272-275
  - 40 **Groeneweg M**, Quero JC, De Bruijn I, Hartmann IJ, Essink-bot ML, Hop WC, Schalm SW. Subclinical hepatic encephalopathy impairs daily functioning. *Hepatology* 1998; **28**: 45-49
  - 41 **Steindl PE**, Finn B, Bendok B, Rothke S, Zee PC, Blei AT. Disruption of the diurnal rhythm of plasma melatonin in cirrhosis. *Ann Intern Med* 1995; **123**: 274-277
  - 42 **Spahr L**, Coeytaux A, Giostra E, Hadengue A, Annoni JM. Histamine H1 blocker hydroxyzine improves sleep in patients with cirrhosis and minimal hepatic encephalopathy: a randomized controlled pilot trial. *Am J Gastroenterol* 2007; **102**: 744-753
  - 43 **Montagnese S**, Middleton B, Skene DJ, Morgan MY. Sleep-wake abnormalities do not correlate with neuropsychiatric performance in patients with cirrhosis (abstract). *Hepatology* 2008; **46**: 563A
  - 44 **Marchesini G**, Bianchi G, Amodio P, Salerno F, Merli M, Panella C, Loguercio C, Apolone G, Niero M, Abbiati R. Factors associated with poor health-related quality of life of patients with cirrhosis. *Gastroenterology* 2001; **120**: 170-178
  - 45 **Arguedas MR**, DeLawrence TG, McGuire BM. Influence of hepatic encephalopathy on health-related quality of life in patients with cirrhosis. *Dig Dis Sci* 2003; **48**: 1622-1626
  - 46 **Bao ZJ**, Qiu DK, Ma X, Fan ZP, Zhang GS, Huang YQ, Yu XF, Zeng MD. Assessment of health-related quality of life in Chinese patients with minimal hepatic encephalopathy. *World J Gastroenterol* 2007; **13**: 3003-3008
  - 47 **Ferrer M**, Cordoba J, Garin O, Olive G, Flavia M, Vargas V, Esteban R, Alonso J. Validity of the Spanish version of the Chronic Liver Disease Questionnaire (CLDQ) as a standard outcome for quality of life assessment. *Liver Transpl* 2006; **12**: 95-104
  - 48 **Stewart CA**, Smith GE. Minimal hepatic encephalopathy. *Nat Clin Pract Gastroenterol Hepatol* 2007; **4**: 677-685
  - 49 **Poordad FF**. Review article: the burden of hepatic encephalopathy. *Aliment Pharmacol Ther* 2007; **25** Suppl 1: 3-9
  - 50 **Bustamante J**, Rimola A, Ventura PJ, Navasa M, Cirera I, Reggiardo V, Rodes J. Prognostic significance of hepatic encephalopathy in patients with cirrhosis. *J Hepatol* 1999; **30**: 890-895
  - 51 **Romero-Gomez M**, Boza F, Garcia-Valdecasas MS, Garcia E, Aguilar-Reina J. Subclinical hepatic encephalopathy predicts the development of overt hepatic encephalopathy. *Am J Gastroenterol* 2001; **96**: 2718-2723
  - 52 **Saxena N**, Bhatia M, Joshi YK, Garg PK, Dwivedi SN, Tandon RK. Electrophysiological and neuropsychological tests for the diagnosis of subclinical hepatic encephalopathy and prediction of overt encephalopathy. *Liver* 2002; **22**: 190-197
  - 53 **Romero-Gomez M**, Grande L, Camacho I, Benitez S, Irlés JA, Castro M. Altered response to oral glutamine challenge as prognostic factor for overt episodes in patients with minimal hepatic encephalopathy. *J Hepatol* 2002; **37**: 781-787
  - 54 **Hartmann IJ**, Groeneweg M, Quero JC, Beijeman SJ, de Man RA, Hop WC, Schalm SW. The prognostic significance of subclinical hepatic encephalopathy. *Am J Gastroenterol* 2000; **95**: 2029-2034
  - 55 **Maguire EA**, Burgess N, Donnett JG, Frackowiak RS, Frith CD, O'Keefe J. Knowing where and getting there: a human navigation network. *Science* 1998; **280**: 921-924

- 56 **Evans L.** The dominant role of driver behavior in traffic safety. *Am J Public Health* 1996; **86**: 784-786
- 57 **Watanabe A,** Tuchida T, Yata Y, Kuwabara Y. Evaluation of neuropsychological function in patients with liver cirrhosis with special reference to their driving ability. *Metab Brain Dis* 1995; **10**: 239-248
- 58 **Wein C,** Koch H, Popp B, Oehler G, Schauder P. Minimal hepatic encephalopathy impairs fitness to drive. *Hepatology* 2004; **39**: 739-745
- 59 **Bajaj JS,** Hafeezullah M, Hoffmann RG, Varma RR, Franco J, Binion DG, Hammeke TA, Saeian K. Navigation skill impairment: Another dimension of the driving difficulties in minimal hepatic encephalopathy. *Hepatology* 2008; **47**: 596-604
- 60 **Bajaj JS,** Hafeezullah M, Hoffmann RG, Saeian K. Minimal hepatic encephalopathy: a vehicle for accidents and traffic violations. *Am J Gastroenterol* 2007; **102**: 1903-1909
- 61 **Teh SH,** Nagorney DM, Stevens SR, Offord KP, Therneau TM, Plevak DJ, Talwalkar JA, Kim WR, Kamath PS. Risk factors for mortality after surgery in patients with cirrhosis. *Gastroenterology* 2007; **132**: 1261-1269
- 62 **Bajaj JS,** Ananthakrishnan AN, McGinley E, Hoffmann RG, Brasel KJ. Deleterious impact of cirrhosis on outcomes after motor vehicle crashes using the Nationwide Inpatient Sample. *Am J Gastroenterol* 2008; **103**: 1674-1681
- 63 **Ries ML,** Jabbar BM, Schmitz TW, Trivedi MA, Gleason CE, Carlsson CM, Rowley HA, Asthana S, Johnson SC. Anosognosia in mild cognitive impairment: Relationship to activation of cortical midline structures involved in self-appraisal. *J Int Neuropsychol Soc* 2007; **13**: 450-461
- 64 **Starkstein SE,** Jorge R, Mizrahi R, Adrian J, Robinson RG. Insight and danger in Alzheimer's disease. *Eur J Neurol* 2007; **14**: 455-460
- 65 **Kircheis G,** Wettstein M, Timmermann L, Schnitzler A, Haussinger D. Critical flicker frequency for quantification of low-grade hepatic encephalopathy. *Hepatology* 2002; **35**: 357-366
- 66 **Conn H.** In *Hepatic Encephalopathy: Management with lactulose and related carbohydrates*. Illinois: Medi-Ed Press, 1988
- 67 **Malaguarnera M,** Greco F, Barone G, Gargante MP, Malaguarnera M, Toscano MA. Bifidobacterium longum with fructo-oligosaccharide (FOS) treatment in minimal hepatic encephalopathy: a randomized, double-blind, placebo-controlled study. *Dig Dis Sci* 2007; **52**: 3259-3265
- 68 **Boca M,** Vyskocil M, Mikulecky M, Ebringer L, Kolibas E, Kratochvil'ova H, Buzgova D. [Complex therapy of chronic hepatic encephalopathy supplemented with probiotic: comparison of two studies] *Cas Lek Cesk* 2004; **143**: 324-328
- 69 **Macbeth WA,** Kass EH, McDermott WV Jr. Treatment of hepatic encephalopathy by alteration of intestinal flora with lactobacillus acidophilus. *Lancet* 1965; **1**: 399-403
- 70 **Uribe M,** Dibildox M, Malpica S, Guillermo E, Villalobos A, Nieto L, Vargas F, Garcia Ramos G. Beneficial effect of vegetable protein diet supplemented with psyllium plantago in patients with hepatic encephalopathy and diabetes mellitus. *Gastroenterology* 1985; **88**: 901-907
- 71 **Zhao HY,** Wang HJ, Lu Z, Xu SZ. Intestinal microflora in patients with liver cirrhosis. *Chin J Dig Dis* 2004; **5**: 64-67
- 72 **Bajaj JS,** Saeian K, Christensen KM, Hafeezullah M, Franco J, Varma RR, Pleuss JA, Krakower G, Hoffmann RG, Binion DG. Probiotic yogurt for the treatment of minimal hepatic encephalopathy. *Am J Gastroenterol* 2008; **103**: 1707-1715

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# Extended-therapy duration for chronic hepatitis C, genotype 1: The long and the short of it

Brian L Pearlman

Brian L Pearlman, Atlanta Medical Center, Medical College of Georgia, Emory School of Medicine, 285 Boulevard NE Suite 140 Atlanta, Georgia 30312, United States

Correspondence to: Brian L Pearlman, MD, FACP, Center For Hepatitis C, Atlanta Medical Center, Medical College of Georgia, Emory School of Medicine, 315 Boulevard NE Suite 200, Atlanta, Georgia 30312,

United States. [brianpearlman@hotmail.com](mailto:brianpearlman@hotmail.com)

Telephone: +1-404-265-4644 Fax: +1-404-265-1047

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Research, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan

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## Abstract

With pegylated interferon and ribavirin, more than half of all chronically-infected hepatitis C patients can achieve a sustained virologic response; however, patients with genotype 1 infections and those with other poor prognostic factors have relatively inferior treatment response rates. Since new therapies are still years away from approval, it is incumbent upon providers to maximize the therapeutic efficacy of today's treatment. The later the virus is undetectable in serum during treatment, the less likely it will be eradicated. Patients with a delayed or slow virologic response to therapy (at least a 2-log<sub>10</sub> decrease in baseline hepatitis C RNA yet detectable viremia at 12 wk of therapy and undetectable virus 12 wk subsequently) may, therefore, benefit from an extended therapy course beyond one of standard duration. Although higher rates of treatment discontinuation may plague this approach, 72 wk of treatment for genotype 1-infected slow-responders may improve response rates and diminish relapse rates relative to those of 48 wk. Based on data from both viral kinetic and clinical studies, therapy prolongation in slow responders may be a reasonable strategy to improve response rates in these treatment-refractory patients.

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**Key words:** Hepatitis C virus genotype; Peginterferon alpha; Ribavirin; Slow-responder; Extension

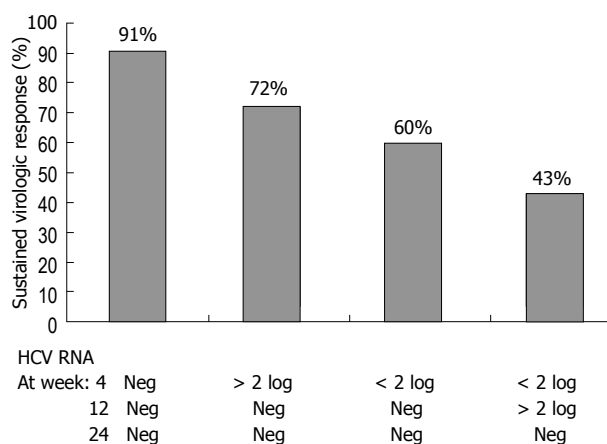
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## INTRODUCTION

One hundred and seventy million persons worldwide are infected with the hepatitis C virus (HCV)<sup>[1]</sup>, and liver-related deaths from the virus are expected to nearly triple by the year 2020<sup>[2]</sup>. Three years prior to the virus' identification in 1986, interferon alpha-2b was first utilized for the treatment of non-A, non-B hepatitis<sup>[3]</sup>. Although sustained virologic response (SVR) rates to interferon monotherapy for 6 mo were only about 8%, treatment extension to 12 mo nearly doubled response rates<sup>[4]</sup>. With the advent of the nucleoside analog ribavirin, used in combination with interferon, rates of SVR more than doubled again<sup>[5,6]</sup>. Whereas 48 wk of standard interferon with ribavirin achieved sustained response rates as high as 43%<sup>[6]</sup>, 48 wk of the newer pegylated interferons plus ribavirin improved rates of SVR to 54%-56% as shown in two multinational, randomized controlled trials<sup>[7,8]</sup>.

Nevertheless, SVR rates are inferior for patients with genotype 1 infection<sup>[7-9]</sup> despite a 48 wk recommended treatment course of peginterferon with weight-based ribavirin<sup>[10,11]</sup>. Although preliminary evidence indicates that small molecule inhibitors in combination with peginterferon and ribavirin may improve response rates even further for genotype 1-infected patients with merely 24 wk of therapy<sup>[12,13]</sup>, the combination of peginterferon and ribavirin alone is likely to remain the recommended treatment regimen for chronic HCV for the next 3-5 years<sup>[14]</sup>. Thus, it is incumbent upon clinicians to maximize their patients' chance of treatment success with existing therapy.

Methods utilized to improve the treatment response in genotype 1-infected patients include the bolstering of patient adherence through aggressive side effect management and increasing the dose of therapy through induction dosing of interferon or through higher doses



**Figure 1** Relationship between hepatitis C viremia and sustained virologic response with pegylated interferon alpha-2a and ribavirin therapy<sup>[19]</sup>. The later the virus becomes undetectable on therapy, the less likely it will be ultimately cleared. Data are from retrospective analysis<sup>[19]</sup> of a registration trial for peginterferon alpha-2a plus ribavirin involving over 1000 chronic, treatment-naïve hepatitis C-infected patients<sup>[8]</sup>. Neg: Undetectable RNA; > 2 log: At least a 2 log<sub>10</sub> decrease in viral RNA compared to pre-treatment value; < 2 log: Less than a 2 log<sub>10</sub> decrease in viral RNA compared to pre-treatment value.

of either interferon or ribavirin. Another strategy is to lengthen duration of treatment in those that are slow-responders to standard doses of peginterferon and ribavirin. The purpose of this survey is to review the justification for therapy prolongation in appropriate patients using viral kinetic data and to summarize the clinical trials supporting this approach.

## VIRAL KINETICS DURING TREATMENT

An initial decrement in HCV RNA level, referred to as phase 1, occurs hours after the administration of interferon; it represents the blocking of viral replication. A subsequent, slower decrease in viremia (phase 2) represents the clearance of HCV-infected hepatocytes and typically occurs days to months after interferon therapy is initiated. The phase 2 decline in virus is the better predictor of ultimate HCV clearance<sup>[15,16]</sup>. Phase 2 decline is significantly slower in genotype 1-infected patients than in those infected with genotypes 2 and 3<sup>[17,18]</sup>. Thus, by measuring virus concentrations of various points along a slope of a phase 2 decline in viremia, treatment outcome may be better predicted, and ultimately modified, based on an individual patient's response to therapy.

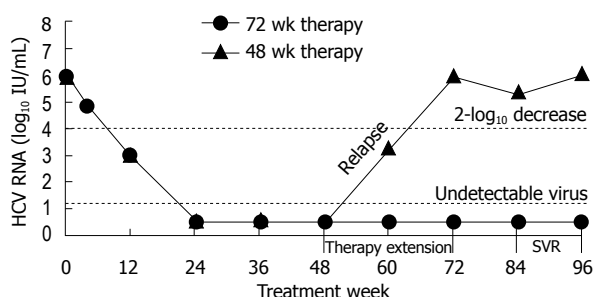
HCV-infected patients who achieve undetectable viremia as early as 4 wk into standard therapy have excellent sustained response rates; these rapid virologic responders achieve SVR about 90% of the time<sup>[19]</sup>. In fact, support exists for truncating therapy duration for genotype 1-infected rapid responders with low baseline HCV viral loads<sup>[20]</sup>. Conversely, patients who show undetectable virus for the first time 24 wk into therapy have less than one-third chance of ultimately achieving SVR<sup>[8]</sup>. Therefore, the earlier the HCV is undetectable in serum during treatment, the greater the likelihood of a successful treatment response (Figure 1).

Similarly, longer durations of viral suppression

on treatment may improve virologic response rates. Investigators have sought to determine if the standard 48 wk treatment duration of patients with genotype 1 infection is adequate. Using data from the peginterferon alpha-2b and ribavirin phase III trial<sup>[7]</sup>, Drusano and Preston developed a prediction model based on the duration of viral suppression on therapy<sup>[21]</sup>. The model was built on the basis of eleven covariables including demographics and virologic characteristics from 771 HCV-infected patients. Among the variables, the durations of viral clearance had the strongest bearing on the likelihood of a SVR. When the model was applied to a validation group of 229 patients, it predicted SVR with a positive predictive value of 97% and a negative predictive value of 91%. To achieve an 80% chance of SVR in genotype 1-infected patients, an undetectable HCV RNA was required for at least 32 wk on therapy, and to achieve a 90% chance of SVR, RNA undetectability was necessary for 36 wk. Since the average time to clear genotype 1 viremia was 30 wk, the authors concluded that the standard 48 wk treatment course for this genotype is inadequate. Problems with this study are its retrospective analysis and the model's requirement for monthly viral loads which may be cost-prohibitive. Furthermore, the model was based on the use of suboptimal ribavirin dosing (800 mg daily) which limits its applicability in genotype 1 infection. However, the study suggests that patients who don't achieve undetectable virus at certain time points may enjoy improved rates of SVR with treatment extension.

A failure to achieve an early virologic response (EVR), defined by an undetectable HCV RNA level or at least a 2-log<sub>10</sub> decrement in RNA from baseline at 12 wk of therapy, has excellent negative predictive value for treatment success<sup>[22,23]</sup>. An analysis of the peginterferon with ribavirin registration trials<sup>[7,8]</sup> suggests that patients who do not achieve a 12 wk EVR have a 3% or lower chance of ultimately achieving SVR<sup>[23]</sup>. Nonetheless, there is a large disparity in treatment response between patients who, at 12 wk, have at least a 2-log<sub>10</sub> decrease in baseline HCV RNA yet still have detectable virus (partial EVR) compared to those who achieve undetectable viremia (complete EVR). In the registration trial for peginterferon alpha-2b with ribavirin, patients in the latter group achieved SVR about four times more frequently than those in the former<sup>[23]</sup>. These former patients are said to be slow or late responders to therapy. Patients have also been characterized as slow responders to therapy if they have detectable virus at 4 wk; either definition necessitates undetectable virus at 24 wk of treatment, since detectable viremia at this time point virtually guarantees treatment failure.

In genotype 1 infection with the standard therapy duration of 48 wk, slow responders have higher relapse rates compared to those that clear virus earlier in treatment<sup>[19]</sup>. High rates of relapse in slow responding patients may indicate that therapy was of insufficient duration; therefore, it has been hypothesized that extending therapy in these patients may improve rates of SVR (Figure 2).



**Figure 2** Virologic responses of slow-responders to 48 wk vs 72 wk of therapy. Treatment extension may improve chance of SVR by lessening the chance of relapse. Slow-responders to standard duration therapy (48 wk) may see relapse after an end-of-treatment response is achieved; those receiving extended duration therapy (72 wk) are not as apt to relapse after treatment is completed.

## EXTENDED DURATION THERAPY WITH STANDARD INTERFERON

Several years before the term slow-responder was popularized, investigators attempted to improve response rates by extending therapy duration utilizing older medications. Using standard interferon monotherapy, authors have demonstrated improved biochemical, histologic and virologic responses with treatment ranging from 60 to 76 wk compared to treatments of shorter durations<sup>[24,25]</sup>. In one of these studies<sup>[24]</sup>, the investigators noted a reduced tendency for patients to relapse after treatment cessation when therapy was extended to 60 wk relative to previously published studies with shorter durations.

The first randomized, controlled trial to show that prolongation of interferon-based therapy combined with ribavirin for 18 mo reduced rates of relapse was performed in the Netherlands and Belgium<sup>[26]</sup>. Three hundred treatment-naïve chronic HCV patients were randomized to 6 or 18 mo of standard-interferon (3 million units thrice weekly) plus ribavirin (1000-1200 mg daily) or to 18 mo of the same dose of standard interferon monotherapy plus placebo. The majority of patients were genotype 1-infected with high viral loads. Although end-of-treatment responses were similar in the two combination therapy arms (55% and 41% in 6 and 18 mo arms, respectively), relapse rates were significantly lower in the 18 mo combination arm (13%) compared to those in the 6 mo treatment arm (38%,  $P = 0.006$ ). It should be noted that the rate of relapse in the group treated with 18 mo of monotherapy was still high at 38%; these results demonstrate that extending the duration of interferon alone is inadequate to decrease relapse rates and attests to ribavirin's effect on relapse diminution. In an intention-to-treat analysis, SVR rates were 16% for 18 mo monotherapy, 34% for 6 mo combination therapy and 43% for 18 mo of combination therapy ( $P < 0.05$ ). Although 25% of patients withdrew from both 18 mo treatment arms prematurely, this withdrawal rate is similar to those from standard interferon plus ribavirin treatment trials of 48 wk duration (21%-27%)<sup>[5,6]</sup>. Prolongation of combination therapy had no significant effect on rates

of relapse or SVR in genotype 2- or 3- infected patients; nevertheless, extension of treatment from 6 to 18 mo had an independent effect on relapse and SVR rates in patients infected with genotype 1 virus (SVR: OR 4; CI: 2-10,  $P = 0.004$ ). Slow responders, those with detectable viremia at 12 wk, enjoyed a decline in relapse rate from 70% to 30% when treatment was prolonged from 24 to 72 wk. The study's major limitation was the absence of a 12 mo treatment arm.

## EXTENDED DURATION THERAPY WITH PEGYLATED INTERFERON

Investigators from Spain and Israel were the first to report successful treatment extension to 72 wk for slow responders to peginterferon and ribavirin<sup>[27]</sup>. Slow responders were defined as those patients with at least a 2 log<sub>10</sub> decline in HCV RNA from baseline, yet detectable viremia at 12 wk after receipt of 1.0 mcg of peginterferon alpha-2b weekly and 800 mg ribavirin daily. Although only 8 patients were treated in this fashion, 7 of the 8 (88%) achieved an SVR, which is a profound improvement relative to a SVR of 21% in slow responders treated for 48 wk in the phase III trial of peginterferon alpha-2b and ribavirin<sup>[23]</sup>. The suboptimal dosing of ribavirin and the paucity of patients studied are the limitations of this analysis.

Furthermore, results from a large, prospective, multicenter trial from Germany support the extension of therapy duration in slow responders to peginterferon and ribavirin<sup>[28]</sup>. 459 treatment-naïve genotype 1-infected patients were randomized to 48 wk *vs* 72 wk of peginterferon alpha-2a, 180 mcg weekly and ribavirin, 800 mg daily. 22% of patients were slow virologic responders to therapy, defined by virus detectability at 12 wk of treatment, but with viral undetectability at 24 wk. Slow responders in the 72 wk group had lower relapse rates than did slow responders treated for 48 wk (40% *vs* 64%, respectively;  $P = 0.021$ ) and had similar end-of-treatment responses; thus, rates of SVR for this subgroup of patients were significantly higher when treated for 72 wk compared those treated for 48 wk (29% *vs* 17%, respectively;  $P = 0.04$ ). Patients with low levels of viremia (less than 6000 IU/mL) at 12 wk of treatment derived the greatest benefit from extended duration therapy. Thus, the investigators concluded that in slow virologic responders to treatment, relapse rates could be reduced and rates of SVR could be augmented by extending therapy to 72 wk. Nonetheless, the slow responders represented only a subgroup of those randomized, and the conclusions were garnered retrospectively. Moreover, the 800 mg ribavirin used in the trial is an inadequate dose for most genotype 1-infected patients<sup>[9]</sup>. It should also be emphasized that, for the majority of patients randomized to 72 wk of therapy, there was no statistical benefit relative to 48 wk of treatment. Overall, SVR rates were 54% and 53%, respectively ( $P = 0.8$ ), and relapse rates were likewise statistically similar. Thus, extended duration

therapy is generally not recommended for all treatment-naïve patients with genotype 1 infection and should be considered only for those with the slow responding phenotype. Finally, although patients in both the standard and extended duration treatment arms had similar rates of medication dose reduction and adverse events, the rate of premature therapy discontinuation was higher in the extended treatment arm compared to that in the standard treatment arm (41% and 24%, respectively).

Another randomized, multicenter clinical trial from Spain, the TeraViC-4 study, prospectively evaluated the effects of extended therapy duration with peginterferon alpha-2a (180 mcg weekly) and ribavirin (800 mg daily)<sup>[29]</sup>. Treatment-naïve patients with detectable HCV RNA levels at week 4 ( $n = 326$ ) were randomized to a therapy duration of 48 wk ( $n = 165$ , 149 of whom were genotype 1-infected) or to 72 wk ( $n = 161$ , 142 of whom were genotype 1-infected). Thus, in this case, a slow responder was defined by absence of a rapid virologic response, and only slow responders were randomized to extended therapy. In TeraViC-4, the 12 wk virologic responses were not reported. Although end-of-treatment responses for patients with genotype 1 infections were statistically similar in both extended and standard treatment arms (62% *vs* 58%, respectively;  $P = 0.53$ ), SVR rates were superior in the extended treatment arm (44% *vs* 28%, respectively;  $P = 0.003$ ), by virtue of a diminution of relapse rate (17% *vs* 53%, respectively;  $P = 0.002$ ). The incidence of adverse events and dose reductions were similar in both groups; yet, treatment discontinuation was more frequent in the extended therapy arm compared to the standard duration arm (36% *vs* 18%, respectively;  $P = 0.0004$ ). In fact, one of the study's limitations was this inordinate number of premature terminations in those randomized to prolonged therapy, largely because of patient preference. Other limitations were the lack of central testing for viral load measurements and the inclusion of patients with other genotypes besides one. Like that of the German study, the primary weakness of this trial may have been the suboptimal dose of ribavirin utilized (800 mg for genotype 1 virus).

In another prospective study from the United States, we randomized genotype 1-infected slow-responders to 48 or 72 wk of peginterferon alpha-2b (1.5 mcg/kg weekly) and weight-based ribavirin (800 to 1400 mg daily)<sup>[30]</sup>. In our trial, slow response was defined by achieving at least a 2 log<sub>10</sub> decrement in HCV RNA from baseline, yet having detectable viremia at 12 wk but undetectable virus at 24 wk (PCR, lower limit of detection 10 IU/mL). One hundred twelve of our treatment-naïve patients were deemed slow-responders who represented about 30% of our patients. This percentage was felt to be high secondary to difficult-to-treat baseline characteristics: 26% had bridging fibrosis or cirrhosis on pre-treatment liver biopsy; 48% were African American; 34% were obese defined by greater than or equal to 30 kg/m<sup>2</sup>; 78% had high baseline viral load (more than 800 000 IU/mL) and 18% had impaired fasting glucose, pre-treatment. Similar to the

aforementioned European trials, we did not observe a higher number of adverse events or therapy reductions in the extended treatment arm relative to the control arm. However, unlike in the other studies, we did not see a greater treatment cessation rate in the prolonged therapy arm; in fact, none of the therapy terminations occurred between weeks 48 and 72. Growth factors were prohibited, yet peginterferon dose reductions for neutropenia were made only if counts were less than 500/mm<sup>3</sup>. Similar to those of the slow-responders in Spain, our randomized patients saw equivalent end-of-treatment response rates in the 72 wk arm *vs* the 48 wk arm (48% *vs* 45%, respectively;  $P = 0.75$ ), yet enjoyed improved relapse rates with therapy prolongation (20% *vs* 59%, respectively;  $P = 0.004$ ). Overall, rates of SVR in slow-responders were superior with treatment extension (38%) compared to those of standard duration therapy (18%,  $P = 0.03$ ). The primary limitation of our analysis is it was conducted in a single medical center.

African Americans, a group with inferior responses to interferon-based therapy<sup>[31-33]</sup>, may likewise benefit from a treatment extension strategy if slowly responsive to treatment. When compared to non-Hispanic whites receiving combination therapy, African Americans showed smaller phase 1 and phase 2 declines in viral load<sup>[34]</sup>; thus, it was unclear if African Americans' viral kinetics on therapy would be analogous to those of white "late" responders. Nearly half of our slow responders were self-identified as African American ( $n = 48$ ) and were randomized to 48 and 72 wk treatment arms in our Atlanta study described above<sup>[30]</sup>. Although overall, African American slow responders had expected inferior SVR rates to those of whites (17% and 39%, respectively), the former group still benefited from treatment extension because of reduced relapse rates. End-of-treatment response rates were 24% *vs* 26% for 48 and 72 wk of treatment, respectively ( $P > 0.05$ ), and SVR rates were 12% *vs* 21%, respectively ( $P = 0.02$ ). Results should be interpreted with caution, however, because this ethnic group's response rates were derived from subgroup analysis.

Preliminary results are available from a multicenter trial in Europe that also utilized extended therapy for slow-responders to peginterferon alpha-2a (180 mcg weekly) with weight-based ribavirin (1000-1200 mg daily)<sup>[35,36]</sup>. Of the 373 patients treated, 11% were slow-responders to therapy, defined by having detectable virus at 12 wk ( $> 50$  IU/mL), yet still at least 2 log<sub>10</sub> below baseline, and thus randomized to complete standard duration therapy of 48 wk or extended treatment to 72 wk. None of these slow-responders had achieved undetectable virus at 4 wk. Over 90% of patients had genotype 1 infections, and the remainder was infected with genotype 4 virus. As in the previously described studies, extended therapy compared to that of standard duration improved SVR rates in slow-responders (69% *vs* 52%, respectively;  $P$  value not available) by virtue of a decrease in relapse rates (18% *vs* 32%, respectively;  $P$  value not available). Although treatment discontinuation data are not available for slow-responders specifically,



**Table 1** Studies of extended therapy for treatment-naïve, genotype 1-infected slow responders to pegylated interferon with ribavirin

Country(ies) in which studies performed	Number of subjects studied	Treatment duration (wk)	Definition of slow response (assay sensitivity)	Pegylated interferon type and weekly dose	Ribavirin daily dose (mg)	End-of-treatment responses	Relapse rates	Sustained virologic response rates	P value (sustained response)	Major study limitation (s)
Spain, Israel <sup>[27]</sup>	8	72	$\geq 2\text{-log}_{10}$ 12 wk decrease <sup>1</sup> (100 IU/mL)	Alpha -2b 1.0 mcg/kg	800	100%	13%	88%	Not applicable	Few subjects suboptimal ribavirin dose
Germany <sup>[28]</sup>	100	48	> 50 IU/mL at wk 12	Alpha 2-a 180 mcg	800	47%	64%	17%	0.04	Retrospective subgroup analysis
	106	72	(50 IU/mL)			49%	40%	29%		suboptimal ribavirin dose
Spain <sup>[29]</sup>	149	48	> 50 IU/mL at wk 4	Alpha 2-a 180 mcg	800	58%	53%	28%	0.003	Suboptimal ribavirin dose
	142	72	(50 IU/mL)			62%	17%	44%		
United States <sup>[30]</sup>	49	48	$\geq 2\text{-log}_{10}$ 12 wk decrease <sup>1</sup> (10 IU/mL)	Alpha 2-b 1.5 mcg/kg	800-1400	45%	59%	18%	0.03	Single center
	52	72				48%	20%	38%		
Europe <sup>[35,36]</sup>	25 <sup>2</sup>	48	$\geq 2\text{-log}_{10}$ 12 wk decrease <sup>1</sup> (50 IU/mL)	Alpha 2-a 180 mcg	1000-1200	Not available	32%	52%	Not available	Few subjects some genotype four infections
	16 <sup>2</sup>	72					18%	69%		
Italy <sup>[37]</sup>	21	48	$\geq 2\text{-log}_{10}$ 12 wk decrease <sup>1</sup> (50 IU/mL)	Alpha -2b 1.5 mcg/kg or Alpha -2a 180 mcg	1000-1200	5% <sup>3</sup>	100% <sup>3</sup>	0% <sup>3</sup>	0.3 <sup>4</sup>	Subgroup analysis
	52	72				19%	60%	8%		
Europe, Canada, Israel <sup>[38]</sup>	63	48	$\geq 2\text{-log}_{10}$ 12 wk decrease <sup>1</sup>	Alpha -2b 1.5 mcg/kg	800-1400	Pending	Pending	Pending	Pending	Pending
	63	72								

<sup>1</sup>Detectable viremia at 12 wk and undetectable viremia at 24 wk required; <sup>2</sup>Less than 10% genotype 4 infections; <sup>3</sup>One of twenty patients had an end-of-treatment response, but relapsed; <sup>4</sup>Not statistically significant.

patients who received extended duration therapy had a greater number of treatment discontinuations (17%) compared to those on standard duration therapy (4%). Limitations of this study are the small numbers of slow-responders analyzed (41 patients) and the mixture of genotype 1- and 4- infected patients.

Finally, a recently published, randomized, multicenter study from Italy assessed the utility of variable therapy duration, including a treatment extension strategy, based on the time to a patient's first undetectable HCV RNA<sup>[37]</sup>. 696 genotype 1-infected treatment-naïve patients were randomized to a standard duration or a variable duration therapy arm in a 2:1 ratio. Patients were treated with either peginterferon alpha-2a (180 mcg weekly) or peginterferon alpha-2b (1.5 mcg/kg weekly), both with weight-based ribavirin (1000-1200 mg daily). Irrespective of first time to viral undetectability in serum (< 50 IU/mL), 237 patients in the standard therapy arm received treatment for 48 wk, unless patients did not achieve EVR at week 12 or had detectable virus at week 24, at which time therapy was discontinued. However, in the variable duration arm, patients were treated for 24, 48 or 72 wk if serum HCV RNA were negative at 4, 8, or 12 wk, respectively. Patients were likewise treated for 72 wk in the variable duration arm if they had at least a 2 log<sub>10</sub> decline in serum RNA from baseline yet detectable viremia at 12 wk of therapy (slow-responders).

Overall, based on the rates of SVR, the standard and variable duration treatment arms were statistically equivalent. Nonetheless, the study proved prospectively

that the longer the virus is undetectable on therapy, the better the chance of achieving a SVR, regardless if a patient had received treatment of a standard or variable duration. In the standard duration arm, 87%, 70% and 38% of patients first attaining undetectable viremia at 4, 8 or 12 wk, respectively, achieved SVR; in the variable duration group, corresponding SVR rates were 77%, 72% and 64%.

More pertinent to this discussion is the fact that the study's slow-responders in the standard treatment duration arm ( $n = 21$ ) did not achieve SVR in a single case (0%), and those slow-responders treated for 72 wk ( $n = 53$ ) saw an SVR of only 7.5%; nonetheless, the results were not statistically significant ( $P = 0.3$ ). Moreover, 10 of the slow-responders treated for 72 wk voluntarily withdrew from the study compared to only 3 patients treated for 48 wk, suggesting that prolonged therapy was not well-tolerated. Most intriguing, patients who achieved undetectable virus for the first time at 12 wk of therapy (having detectable viremia at week 8), enjoyed improved SVR rates when treated for 72 wk compared to 48 wk in 64% and 38% of cases, respectively (difference -25.4, CI: 22.3-28.4). Extended therapy, even in these "complete" early virologic responders, seemed to have improved SVR rates because of a decline in relapse (43% in standard group, and 15% in variable group). The authors deemed this difference "...substantial, (and) may warrant a prospective trial."

The most important limitations of this last study are the large number of patients in the variable treatment duration arm who discontinued therapy because of poor

compliance (49% of all treatment discontinuations), and the relative paucity of patients on which subgroup analysis was performed. For example, only 21 slow-responders were treated for 48 wk.

The Study to Assess Treatment with Pegylated Interferon Alpha-2b and Ribavirin in Treatment-Naïve Patients with Chronic hepatitis C and slow virologic response (SUCCESS) is a multinational study in 133 centers across Europe, Canada and Israel designed to compare response rates of slow-responders to either 48 or 72 wk of therapy<sup>[38]</sup>. Slow-response is again defined by patients with genotype 1 infection who had at least a 2 log<sub>10</sub> reduction, albeit detectable, HCV RNA levels at 12 wk compared to baseline and undetectable virus at week 24. As of late 2006, 126 patients were deemed slow-responders and were randomized into standard and extended therapy duration arms (Table 1). Final results should be available sometime later in 2008 or in early 2009.

## CONCLUSION

Treatment extension has been attempted with varying degrees of success using alternative interferons<sup>[39,40]</sup>, in those with relapse<sup>[40]</sup> or non-response to peginterferon-based therapies<sup>[41]</sup> and in those who are HCV-HIV coinfectd<sup>[42,43]</sup>. Details of these analyses are beyond the scope of this review.

Certainly, not every treatment-naïve patient with genotype-1 infection benefits from therapy prolongation. However, a subgroup with a delayed or slow response to therapy (approximately 15% of patients) may enjoy improved rates of SVR with treatment extension to 72 wk, largely because of a relapse diminution. Data on therapy prolongation in slow-responders are summarized in Table 1. Compared to patients receiving therapy of standard duration, those in extended treatment groups have experienced higher discontinuation rates in most, but not all, studies to date; however, the numbers of adverse events and dose reductions appear to be equivalent. Clearly, if treatment prolongation is utilized, adherence to therapy is paramount. Finally, the use of 72 wk for slow virologic responders to peginterferon and ribavirin may be cost-effective compared to 48 wk, or standard duration therapy<sup>[44]</sup>. Thus, therapy extension in slow-responders seems to be a reasonable strategy to ameliorate response rates in a group with notoriously poor treatment results.

## REFERENCES

- 1 **Global surveillance and control of hepatitis C.** Report of a WHO Consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. *J Viral Hepat* 1999; **6**: 35-47
- 2 **Alter MJ, Kruzon-Moran D, Nainan OV, McQuillan GM, Gao F, Moyer LA, Kaslow RA, Margolis HS.** The prevalence of hepatitis C virus infection in the United States, 1988 through 1994. *N Engl J Med* 1999; **341**: 556-562
- 3 **Hoofnagle JH, Mullen KD, Jones DB, Rustgi V, Di Bisceglie A, Peters M, Waggoner JG, Park Y, Jones EA.** Treatment of chronic non-A, non-B hepatitis with recombinant human alpha interferon. A preliminary report. *N Engl J Med* 1986; **315**: 1575-1578
- 4 **Carithers RL Jr, Emerson SS.** Therapy of hepatitis C: meta-analysis of interferon alfa-2b trials. *Hepatology* 1997; **26**: 83S-88S
- 5 **McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, Goodman ZD, Ling MH, Cort S, Albrecht JK.** Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. Hepatitis Interventional Therapy Group. *N Engl J Med* 1998; **339**: 1485-1492
- 6 **Poynard T, Marcellin P, Lee SS, Niederau C, Minuk GS, Ideo G, Bain V, Heathcote J, Zeuzem S, Trepo C, Albrecht J.** Randomised trial of interferon alpha2b plus ribavirin for 48 weeks or for 24 weeks versus interferon alpha2b plus placebo for 48 weeks for treatment of chronic infection with hepatitis C virus. International Hepatitis Interventional Therapy Group (IHIT). *Lancet* 1998; **352**: 1426-1432
- 7 **Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, Goodman ZD, Koury K, Ling M, Albrecht JK.** Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. *Lancet* 2001; **358**: 958-965
- 8 **Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncalves FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J.** Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975-982
- 9 **Hadziyannis SJ, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, Ramadori G, Bodenheimer H Jr, Bernstein D, Rizzetto M, Zeuzem S, Pockros PJ, Lin A, Ackrill AM.** Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140**: 346-355
- 10 **Dienstag JL, McHutchison JG.** American Gastroenterological Association medical position statement on the management of hepatitis C. *Gastroenterology* 2006; **130**: 225-230
- 11 **Strader DB, Wright T, Thomas DL, Seeff LB.** Diagnosis, management, and treatment of hepatitis C. *Hepatology* 2004; **39**: 1147-1171
- 12 **Jacobson IM, Everson GT, Gordon SC, Kauffman R, McNair L, Muir A, McHutchison JG.** Interim analysis results from a phase 2 study of telaprevir with peginterferon alfa-2a and ribavirin in treatment-naïve subjects with hepatitis C. *Hepatology* 2007; **46**: 315A-316A
- 13 **Hezode C, Ferenci P, Dusheiko GM, Pol S, Goeser T, Bronowicki JP, Gharakhanian S, Devonish D, Kauffman R, Alam J, Pawlotsky JM, Zeuzem S.** PROVE 2: phase II study of VX-950 (Telaprevir) in combination with peginterferon alfa-2a with or without ribavirin in subjects with chronic hepatitis C, first interim analysis. *Hepatology* 2007; **46**: 268A-269A
- 14 **Modi AA, Hoofnagle JH.** New therapies for hepatitis C. *Hepatology* 2007; **46**: 615-617
- 15 **Lam NP, Neumann AU, Gretch DR, Wiley TE, Perelson AS, Layden TJ.** Dose-dependent acute clearance of hepatitis C genotype 1 virus with interferon alfa. *Hepatology* 1997; **26**: 226-231
- 16 **Neumann AU, Lam NP, Dahari H, Gretch DR, Wiley TE, Layden TJ, Perelson AS.** Hepatitis C viral dynamics in vivo and the antiviral efficacy of interferon-alpha therapy. *Science* 1998; **282**: 103-107
- 17 **Neumann AU, Lam NP, Dahari H, Davidian M, Wiley TE, Mika BP, Perelson AS, Layden TJ.** Differences in viral dynamics between genotypes 1 and 2 of hepatitis C virus. *J Infect Dis* 2000; **182**: 28-35
- 18 **Zeuzem S, Herrmann E, Lee JH, Fricke J, Neumann AU, Modi M, Colucci G, Roth WK.** Viral kinetics in patients with chronic hepatitis C treated with standard or peginterferon alpha2a. *Gastroenterology* 2001; **120**: 1438-1447
- 19 **Ferenci P, Fried MW, Shiffman ML, Smith CI, Marinos G, Goncalves FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Chaneac M, Reddy KR.** Predicting

- sustained virological responses in chronic hepatitis C patients treated with peginterferon alfa-2a (40 kD)/ribavirin. *J Hepatol* 2005; **43**: 425-433
- 20 **Davis GL**. Tailoring antiviral therapy in hepatitis C. *Hepatology* 2006; **43**: 909-911
- 21 **Drusano GL**, Preston SL. A 48-week duration of therapy with pegylated interferon alpha 2b plus ribavirin may be too short to maximize long-term response among patients infected with genotype-1 hepatitis C virus. *J Infect Dis* 2004; **189**: 964-970
- 22 **Zeuzem S**, Lee JH, Franke A, Ruster B, Prummer O, Herrmann G, Roth WK. Quantification of the initial decline of serum hepatitis C virus RNA and response to interferon alfa. *Hepatology* 1998; **27**: 1149-1156
- 23 **Davis GL**, Wong JB, McHutchison JG, Manns MP, Harvey J, Albrecht J. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. *Hepatology* 2003; **38**: 645-652
- 24 **Reichard O**, Foberg U, Fryden A, Mattsson L, Norkrans G, Sonnerborg A, Wejstal R, Yun ZB, Weiland O. High sustained response rate and clearance of viremia in chronic hepatitis C after treatment with interferon-alpha 2b for 60 weeks. *Hepatology* 1994; **19**: 280-285
- 25 **Poynard T**, Bedossa P, Chevallier M, Mathurin P, Lemonnier C, Trepo C, Couzigou P, Payen JL, Sajus M, Costa JM. A comparison of three interferon alfa-2b regimens for the long-term treatment of chronic non-A, non-B hepatitis. Multicenter Study Group. *N Engl J Med* 1995; **332**: 1457-1462
- 26 **Brouwer JT**, Nevens F, Bekkering FC, Bourgeois N, Van Vlierberghe H, Weegink CJ, Lefebvre V, Van Hattum J, Henrion J, Delwaide J, Hansen BE, Schalm SW, For The Benelux Study Group On Treatment Of Chronic Hepatitis C. Reduction of relapse rates by 18-month treatment in chronic hepatitis C. A Benelux randomized trial in 300 patients. *J Hepatol* 2004; **40**: 689-695
- 27 **Buti M**, Valdes A, Sanchez-Avila F, Esteban R, Lurie Y. Extending combination therapy with peginterferon alfa-2b plus ribavirin for genotype 1 chronic hepatitis C late responders: a report of 9 cases. *Hepatology* 2003; **37**: 1226-1227
- 28 **Berg T**, von Wagner M, Nasser S, Sarrazin C, Heintges T, Gerlach T, Buggisch P, Goeser T, Rasenack J, Pape GR, Schmidt WE, Kallinowski B, Klinker H, Spengler U, Martus P, Alshuth U, Zeuzem S. Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alfa-2a plus ribavirin. *Gastroenterology* 2006; **130**: 1086-1097
- 29 **Sanchez-Tapias JM**, Diago M, Escartin P, Enriquez J, Romero-Gomez M, Barcena R, Crespo J, Andrade R, Martinez-Bauer E, Perez R, Testillano M, Planas R, Sola R, Garcia-Bengoechea M, Garcia-Samaniego J, Munoz-Sanchez M, Moreno-Otero R. Peginterferon-alfa2a plus ribavirin for 48 versus 72 weeks in patients with detectable hepatitis C virus RNA at week 4 of treatment. *Gastroenterology* 2006; **131**: 451-460
- 30 **Pearlman BL**, Ehleben C, Saifee S. Treatment extension to 72 weeks of peginterferon and ribavirin in hepatitis C genotype 1-infected slow responders. *Hepatology* 2007; **46**: 1688-1694
- 31 **Muir AJ**, Bornstein JD, Killenberg PG. Peginterferon alfa-2b and ribavirin for the treatment of chronic hepatitis C in blacks and non-Hispanic whites. *N Engl J Med* 2004; **350**: 2265-2271
- 32 **Jeffers LJ**, Cassidy W, Howell CD, Hu S, Reddy KR. Peginterferon alfa-2a (40 kd) and ribavirin for black American patients with chronic HCV genotype 1. *Hepatology* 2004; **39**: 1702-1708
- 33 **Conjeevaram HS**, Fried MW, Jeffers LJ, Terrault NA, Wiley-Lucas TE, Afdhal N, Brown RS, Belle SH, Hoofnagle JH, Kleiner DE, Howell CD. Peginterferon and ribavirin treatment in African American and Caucasian American patients with hepatitis C genotype 1. *Gastroenterology* 2006; **131**: 470-477
- 34 **Layden-Almer JE**, Ribeiro RM, Wiley T, Perelson AS, Layden TJ. Viral dynamics and response differences in HCV-infected African American and white patients treated with IFN and ribavirin. *Hepatology* 2003; **37**: 1343-1350
- 35 **Ferenci P**, Laferi H, Scherzer T, Maieron A, Gschwantler M, Brunner H, Hubmann R, Bischof M, Stauder K, Datz C, Steindl-Munda P, Kessler H. Customizing treatment with peginterferon alfa-2a plus ribavirin in patients with HCV genotype 1 or 4 infection. Interim analysis of a prospective, randomized trial. *Hepatology* 2006; **44**: 336A
- 36 **Sanchez-Tapias JM**, Ferenci P, Diago M, Romero-Gomez M, Zeuzem S, Berg T. Which genotype 1 patients may benefit from extended treatment duration with peginterferon alfa-2a plus ribavirin? Proceedings of the 17th Asian Pacific Association Study of Liver Disease conference, Kyoto, Japan. *Hep Intl* 2007; **1**: 242
- 37 **Mangia A**, Minerva N, Bacca D, Cozzolongo R, Ricci GL, Carretta V, Vinelli F, Scotto G, Montalto G, Romano M, Cristofaro G, Mottola L, Spirito F, Andriulli A. Individualized treatment duration for hepatitis C genotype 1 patients: A randomized controlled trial. *Hepatology* 2008; **47**: 43-50
- 38 **Buti M**, Lurie Y, Blokhina N, Teuber G, Holota W, Sumskiene J, Vozianova Z, Wong F, Winkler R, Esteban R. Pegylated interferon alfa-2b plus ribavirin in patients with genotype 1 chronic hepatitis C with a slow virologic response: an early enrollers analysis of the SUCCESS study. *Hepatology* 2006; **44**: 342A
- 39 **Kaiser S**, Holger H, Bissinger L, Bettina L, Sauter B, Gregor M. Extended treatment of 72 versus 48 weeks for chronic hepatitis C patients with genotype 1 and high viral load using daily consensus interferon and ribavirin. *Hepatology* 2006; **44**: 608A
- 40 **Kaiser S**, Lutz B, Sauter B, Bissinger L, Werner C, Hass H, Gregor M. Retreatment of HCV genotype 1 relapse patients to peginterferon/ribavirin therapy with an extended treatment regimen of 72 weeks of consensus interferon/ribavirin versus peginterferon alpha/ribavirin. *Hepatology* 2007; **46**: 819A
- 41 **Jensen DM**, Frelich B, Andreone P, DiBisceglie A, Brandao-Mello CE, Reddy KR, Craxi A, Martin AO, Teuber G, Messinger D, Hooper G, Popescu M, Marcellin P. Pegylated interferon alfa-2a plus ribavirin in prior non-responders to pegylated interferon alfa-2b/ribavirin: final efficacy and safety outcomes of the REPEAT study. *Hepatology* 2007; **46**: 291A-292A
- 42 **Fuster D**, Planas R, Gonzalez J, Force L, Cervantes M, Vilario J, Roget M, Garcia I, Pedrol E, Tor J, Ballesteros AL, Salas A, Sirera G, Videla S, Clotet B, Tural C. Results of a study of prolonging treatment with pegylated interferon-alpha2a plus ribavirin in HIV/HCV-coinfected patients with no early virological response. *Antivir Ther* 2006; **11**: 473-482
- 43 **Nunez M**, Miralles C, Berdun MA, Losada E, Aguirre-bengoa K, Ocampo A, Arazo P, Cervantes M, de Los Santos I, San Joaquin I, Echeverria S, Galindo MJ, Asensi V, Barreiro P, Sola J, Hernandez-Burruezo JJ, Guardiola JM, Romero M, Garcia-Samaniego J, Soriano V. Role of weight-based ribavirin dosing and extended duration of therapy in chronic hepatitis C in HIV-infected patients: the PRESCO trial. *AIDS Res Hum Retroviruses* 2007; **23**: 972-982
- 44 **Nakamura J**, Toyabe SI, Aoyagi Y, Akazawa K. Economic impact of extended treatment with peginterferon alpha-2a and ribavirin for slow hepatitis C virologic responders. *J Viral Hepat* 2008; **15**: 293-299

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## EDITORIAL

# Cystic tumors of the liver: A practical approach

Paolo Del Poggio, Marco Buonocore

Paolo Del Poggio, Marco Buonocore, Hepatology Unit, Treviglio Hospital, Treviglio (Bg) 24047, Italy

**Author contributions:** Del Poggio P and Buonocore M wrote the paper and contributed equally to the work.

**Correspondence to:** Paolo Del Poggio, Hepatology Unit, Treviglio Hospital (Bg), Treviglio (Bg) 24047, Italy. [pdpoggio@ospedale.treviglio.bg.it](mailto:pdpoggio@ospedale.treviglio.bg.it)

Telephone: +39-036-3424494 Fax: +39-036-3424561

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## Abstract

Biliary cyst tumors (cystadenoma and cystadenocarcinoma) are an indication for liver resection. They account for only 5% of all solitary cystic lesions of the liver, but differential diagnosis with multiloculated or complicated biliary cysts, atypical hemangiomas, hamartomas and lymphangiomas may be difficult. The most frequent challenge is to differentiate biliary cyst tumors from hemorrhagic cysts. Computerized tomography (CT) and magnetic resonance imaging (MRI) are often not diagnostic and in these cases fine needle aspiration (FNA) is used to confirm the presence of atypical biliary cells. FNA, however, lacks adequate sensitivity and specificity and should always be used in conjunction with imaging. Pre-operative differentiation of cystadenoma from cystadenocarcinoma is impossible and surgery must be performed if a biliary cyst tumor is suspected. When multiple cystic lesions are observed throughout the liver parenchyma, it is important to exclude liver metastasis, of which colonic cancer is the most common primary site. Multiple biliary hamartomas (von Meyenburg complex) can appear as a mixture of solid and cystic lesions and can be confused with cystic metastasis. Strong and uniform T2 hyperintensity on MRI is usually diagnostic, but occasionally a percutaneous biopsy may be required.

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**Key words:** Biliary cyst tumor; Liver cystic neoplasia; Cystadenoma; Cystadenocarcinoma; Atypical hepatic cysts

**Peer reviewer:** Dr. Nahum Méndez-Sánchez, Departments of Gastroenterology and Liver Unit, Medica Sur Clinic and Foundation, Puente de Piedra 150, Col. Toriello Guerra, Mexico City 14050, Mexico

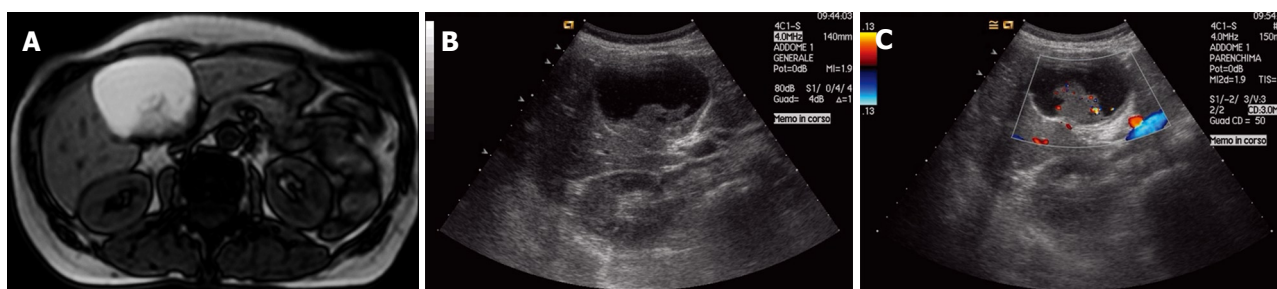
## INTRODUCTION

Although rare, cystic neoplasms of the liver may represent a diagnostic challenge in everyday practice. Cystic tumors may be solitary or multiple and vary from the most benign (e.g. simple cysts, hamartomas) to potentially malignant (cystadenoma) or overtly malignant (cystadenocarcinoma). There are also atypical cystic presentations of normally non-cystic tumors, like cystic hemangiomas or cystic hepatocarcinomas and congenital diseases presenting as diffuse cyst-like involvement of the liver (Caroli's disease and von Meyenburg complex) that must be differentiated from cystic metastasis. The most important issue in the case of solitary cystic tumors is to distinguish biliary cystadenoma and cystadenocarcinoma from other benign conditions that require only observation of the patient.

## SOLITARY CYSTIC TUMORS

Cystadenoma is a biliary cyst tumor arising from biliary epithelium. With its malignant counterpart (cystadenocarcinoma), it accounts for less than 5% of all cystic lesions of the liver; but it is dangerous for its propensity toward local recurrence and malignant change<sup>[1,2]</sup>. These tumors usually present in middle aged women with a mean age of 50 years and have a great variability in size, ranging from 1.5 cm to 30 cm. The majority of patients are asymptomatic, but in the case of large tumors they may present with a palpable mass and cause symptoms<sup>[3]</sup>. Cystadenocarcinoma can arise *de novo* or from a pre-existent cystadenoma, from which it is difficult to differentiate since both have a multiloculated appearance at ultrasound (US), computerized tomography (CT) and magnetic resonance imaging (MRI). Cystadenoma has thinner septa and a less thick and more regular walls than cystadenocarcinoma<sup>[4]</sup>. However, internal papillary projections and foldings with arterial enhancement of the external wall at dynamic CT scan and MRI may be present in both tumors, so that imaging itself cannot reliably differentiate these





**Figure 1** T1 weighted MR image (A) and ultrasound examination (B) showing a large cystic lesion of the left liver lobe with a thick and irregular septum, raising the suspicion of a biliary cystadenoma, and Doppler ultrasound (C) showing vascular signals within the septum disclosing a focus of cystadenocarcinoma confirmed in the surgical specimen.

neoplasms. Internal septa and papillary projections are more often hypovascular in the case of cystadenoma, and the demonstration of vascular signals at color Doppler can be a sign of its malignant transformation (Figure 1). The diagnosis of cystadenocarcinoma is straightforward only when ultrasound, CT scan or MRI shows nodular septa and thick, irregular walls with strong contrast enhancement, but in many cases there are overlapping features. Intracystic hemorrhage and fine punctuate calcifications may present in both conditions and can also be observed in complicated hemorrhagic cysts, their role as diagnostic criterion are, therefore, doubtful<sup>[5]</sup>. Fine needle aspiration can be used to differentiate a biliary cyst from other benign conditions and from a single metastasis, but is totally unreliable in differentiating cystadenoma from cystadenocarcinoma, since small neoplastic foci are undetectable by fine needle aspiration and may be revealed only in the surgical specimen<sup>[6]</sup>.

In conclusion, there is no definite, reliable criterion for differentiating cystadenoma from cystadenocarcinoma and the correct diagnosis is often made only in the surgical specimen.

The majority of biliary cyst tumors do not usually communicate with the bile ducts, although direct luminal communication is occasionally observed. In these cases, dysplastic mucinous epithelium itself may proliferate within the bile ducts and cause obstruction<sup>[7]</sup>. This variant is considered an intraductal papillary neoplasm with prominent cystic dilatation rather than a true biliary cyst neoplasm and must be differentiated from biliary papillomatosis and cholangiocarcinoma of intraductal growth type. CT and MRI are insufficient to show the luminal communication, probably because the communication is too narrow, while intraoperative cholangiography can establish the correct diagnosis. Intraductal spreading of neoplastic cells into the bile duct portends a poorer prognosis of this variant of biliary cyst tumor.

If it is practically impossible to distinguish pre-operatively cystadenoma from cystadenocarcinoma, every effort should be made to differentiate these neoplasms from biliary cysts and other benign tumors without malignant potential. In case of simple cysts, without internal septa or papillary projections,

cystadenoma can be reliably excluded and the patient can only be observed. In case of multiloculated biliary or complicated biliary cyst (hemorrhagic cyst), imaging is often not reliable in ruling out cystadenoma. The most challenging differential diagnosis is hemorrhagic cysts where ultrasound can visualize internal clots as papillary excrescences or nodular and irregular septal images. CT scan is less sensitive than ultrasound in visualizing intracystic blood clots and at times it can only depict homogeneous low density areas inside a huge cyst. In these cases, a discrepancy between US and CT may suggest the correct diagnosis of hemorrhagic cysts. Recently MRI has been shown to be helpful. It was reported that high signals from clot formation could be detected both in T1 and in T2 weighted sequences, and are useful in differentiating it from cystadenocarcinoma, which usually exhibits low signals<sup>[8]</sup>. It should be noted that if a hemorrhagic cyst is suspected, MRI should not be delayed because hemorrhagic signal intensity becomes rapidly low when clots are liquefied<sup>[9]</sup>. History, on the contrary, is totally unreliable to suspect a hemorrhagic cyst since intracystic hemorrhage can occur in the absence of any symptom<sup>[10]</sup>. In the presence of a complicated or multiloculated hepatic cyst, a fine needle aspiration (FNA) of intracystic fluid can be performed to rule out a biliary cyst neoplasm. The presence of atypical cells, mucinous material and elevated levels of CEA and CA19-9 in the cystic fluid has been typically observed both in cystadenoma and in cystadenocarcinoma, but not in hepatic cysts<sup>[11,12]</sup> although CA19-9 is equally expressed in paraffin-embedded tissues from both hepatic cysts and biliary cyst neoplasms<sup>[13]</sup>. To complicate things further, high CA19-9 cyst-fluid levels have been occasionally found in complicated hepatic cysts<sup>[14]</sup> and data on CA19-9 serum levels are scanty and inconclusive<sup>[13,15]</sup>. The macroscopic appearance of the intracystic fluid is likewise useless since in biliary cyst tumors it can be mucinous, but also bile stained or clear, as it occurs when there is abundance of ovary stroma<sup>[16]</sup>. On the whole, FNA alone does not have adequate sensitivity and specificity to confirm or exclude biliary cyst tumors and should be always evaluated together with imaging.

Similar to intracystic bleeding, non suppurative, granulomatous infection of a biliary cyst may simulate cystadenoma and cystadenocarcinoma by the presence

of a thickened wall and a solid component infiltrating the peri-cystic surrounding tissue, thus mimicking a neoplastic lesion<sup>[17]</sup>. On the other hand, suppurated hepatic cysts and echinococcal cysts do not generally pose diagnostic problems. Sonography can easily differentiate the mobile internal debris typical of abscess formation or the multilayered appearance of the echinococcal cystic wall, and only an unexperienced sonographer can confuse the multiloculated appearance of cystadenoma with multiple daughter cysts of echinococcus<sup>[18]</sup>. When in doubt, the presence of anti-echinococcal antibodies is diagnostic.

Mesenchymal hamartoma is an uncommon benign lesion composed of bile ducts, immature mesenchymal cells and hepatocytes and may appear as a multiseptated cyst, causing confusion with biliary cystadenoma. Isolated septal calcification can be observed in both lesions and does not aid in the correct diagnosis<sup>[19]</sup>. Most of the cases are diagnosed in childhood when it presents as a large cystic mass<sup>[20]</sup> and very few cases have been reported in adults<sup>[21]</sup>. FNA can be diagnostic by showing clusters of both epithelial and mesenchymal spindle-shaped cells with pieces of loose connective tissue<sup>[22]</sup>.

Ciliated hepatic foregut cyst is a very rare benign lesion arising from an abnormal budding of the primitive foregut and lined by stratified ciliated columnar cells, similar to the bronchial epithelium. These cysts are often anechogenic, but at times they may show internal echoes and can reach considerable dimensions posing differential diagnostic problems of cystadenoma. The typical subcapsular location in segment IV and the presence of a strong T2 hyper-intensity with T1 signal variability on MRI are quite characteristic. Another helpful clue may be the presence of scattered hyperechogenic spots within the cystic wall with no acoustic shadowing, which are related to cartilaginous remnants<sup>[23]</sup>.

Abdominal lymphangioma may be occasionally located in the liver and appear as a single multiseptated lesion<sup>[24]</sup>. If this diagnosis is suspected, fine needle aspiration is not recommended due to the risk of massive lymphorrhea<sup>[25]</sup> and complete surgical resection should be accomplished, even without a precise pre-operative diagnosis.

Differential diagnosis with cystic hemangioma may also be a problem, especially in the case of a giant hemangioma, with large hypoechogenic central areas simulating the giant cystadenoma. CT and magnetic resonance imaging point out to the correct diagnosis by showing an enhancing rim with globular vessels and centripetal filling, with sparing of the large central lacunar areas<sup>[26]</sup>.

Hepatocellular carcinoma and cholangiocarcinoma may occasionally present as a large hypodense and multiseptated mass at CT scan, simulating a cystic lesion or a biliary cyst tumor. In these cases, arterial phase enhancement on dynamic CT and washing out of the contrast material in the portal phase, are diagnostic<sup>[27]</sup>.

Isolated mucin producing metastasis from melanoma

or colon adenocarcinoma may at times simulate biliary cyst tumors and even benign hepatic cysts. These metastases may be associated to segmental dilatation of the peripheral bile ducts, caused by the presence of mucin casts occluding the bile ducts themselves<sup>[28]</sup>.

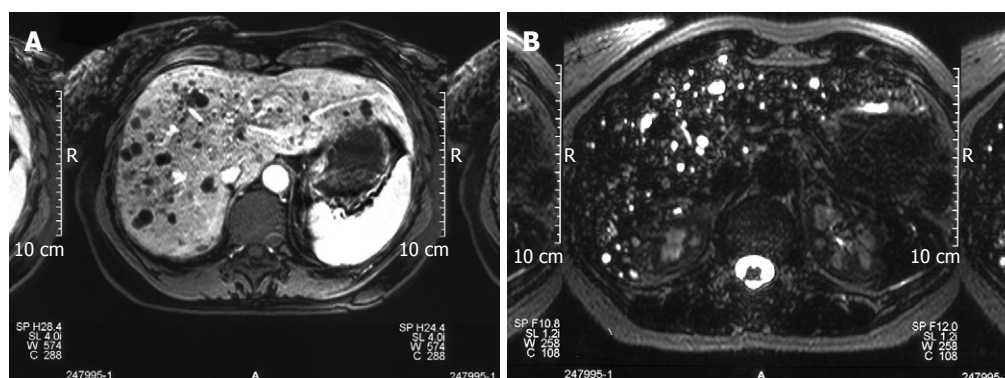
Once a diagnosis of cystadenoma is made, surgery should be performed in any case, because differential diagnosis with cystadenocarcinoma is unreliable and cystadenoma itself has a malignant potential. If the pre-operative diagnostic work up, including cytologic aspiration of the lesion, has not produced a definitive diagnosis and surgery is performed because of a suspected bile cyst tumor, an intraoperative biopsy of the lesion is recommended since an extensive lymph node resection would be required if the tumor is proved to be a cholangiocarcinoma<sup>[14]</sup>. It is important that final decisions regarding indications and type of intervention are jointly discussed by the surgeon and a radiologist expert in liver tumor imaging, particularly in the case of cystic liver tumors, the most frequent mistake is the resection of a complicated biliary cyst incorrectly diagnosed as a cystadenoma.

## MULTIPLE CYSTIC TUMORS

When multiple cystic lesions are observed throughout the liver parenchyma, the most important diagnostic problem is to exclude cystic liver metastases. The primary tumor is usually colonic adenocarcinoma, melanoma, carcinoid, breast or renal or ovarian cancer. Colonic cancer is the common, accounting for about 50% of all hepatic metastases<sup>[27]</sup>. The presence of intra- or peritumoral calcifications may suggest a specific diagnosis, being more frequent in the case of gastrointestinal, ovarian, breast and renal metastases compared to other types of tumors<sup>[29]</sup>. The cystic nature of the metastasis is secondary to the rapid growth and insufficient hepatic arterial supply of the lesion, leading to a large central necrosis simulating a cyst<sup>[30]</sup>.

The differential diagnosis with polycystic liver disease and multiple liver abscesses is usually an easy task on US and CT scan. In case of cystic metastases, the borders of the cystic lesions are heterogeneous and ill-defined, the cystic wall is irregular and the vessels are amputated, but not displaced as in polycystic liver disease. In addition, cystic metastasis has a peripheral enhancing rim on the arterial phase of CT scan and MRI<sup>[27]</sup>, while polycystic liver disease does not show any type of enhancement<sup>[31]</sup>. Another helpful sign may be the presence of peribiliary cysts, such as small cysts with a diameter of less than 10 mm, located within the hilum and adjacent to the hepatic ducts, more frequently on the left. These small cysts are typically observed only in polycystic liver disease and should not be confused with the segmental biliary dilatations occasionally observed in the case of metastasis or macronodular cirrhosis, which are less regular and never adjacent to the main ducts<sup>[32]</sup>.

Multiple hepatic pyogenic microabscesses are easy to differentiate from metastasis by clinical symptoms and



**Figure 2** T1 weighted images showing the left multiple hypo-intense focal lesions of the liver parenchyma simulating metastases (A) and T2 weighted images showing a strong hyper-intensity in Von Meyenburg complex disclosing its cystic nature (B).

the presence of a late faint enhancing peripheral rim on CT scan and MRI<sup>[33]</sup>. This rim is quite different from the early arterial enhancing ring observed in the case of metastasis. An additional sign pointing to liver abscesses is the presence of air densities inside the lesions, which is almost never observed in the case of cystic metastasis.

The differential diagnosis with other types of ductal plate malformations, such as Caroli's disease, Caroli's syndrome and von Meyenburg complex, may at times be more difficult. Caroli's disease is a congenital autosomal recessive malformation characterized by diffuse or segmental cystic dilation of the intrahepatic biliary system. In Caroli's syndrome, periportal congenital fibrosis or multicystic renal diseases are observed in addition to biliary dilations. In both cases, these dilations are less regular than in polycystic liver disease and have intraluminal protrusions or may be associated to segmental dilation of the intrahepatic bile ducts, thus simulating cholangiocarcinoma or multiple cystic metastases. The presence of the "dot signs", such as an intracystic portal branch, surrounded by the dilated bile duct and the demonstration of a communication of the cysts with the biliary system on MR cholangiography, is diagnostic and excludes liver metastasis<sup>[34]</sup>.

Von Meyenburg complex and bile duct hamartoma are small focal developmental lesions composed of innumerable dilated bile ducts mixed with a dense collagenous stroma. The dilated bile duct foci, contrary to those observed in Caroli's disease and Caroli's syndrome do not communicate with the biliary system<sup>[16]</sup>. Depending on the prevalence of fibrous stroma or biliary dilation, these lesions can appear as predominantly solid and cystic or intermediate and may be easily confused with metastases, microabscesses and even biliary cystadenocarcinoma<sup>[35]</sup>. Occasionally, the differential diagnosis may be a real dilemma. Although biliary hamartomas are more uniform in size and measure less than 1 cm in diameter, CT scan and ultrasound are often not specific. MRI, on the contrary, can be very helpful by identifying strong hyper-intensity in biliary hamartomas on heavily T2 weighted images (Figure 2), often with a signal intensity similar to that of the spleen<sup>[36]</sup>. These features, however, are not always sufficient to make a precise diagnosis, especially in intermediate or predominantly solid von Meyenburg complex and a percutaneous or even surgical biopsy is

occasionally required<sup>[37]</sup>. The issue is further complicated by very rare reports on malignant transformation of these hamartomas to cholangiocarcinoma, making this type of cystic malformation the most challenging to differentiate from cystic neoplastic lesions.

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## REFERENCES

- 1 **Wheeler DA**, Edmondson HA. Cystadenoma with mesenchymal stroma (CMS) in the liver and bile ducts. A clinicopathologic study of 17 cases, 4 with malignant change. *Cancer* 1985; **56**: 1434-1445
- 2 **Devaney K**, Goodman ZD, Ishak KG. Hepatobiliary cystadenoma and cystadenocarcinoma. A light microscopic and immunohistochemical study of 70 patients. *Am J Surg Pathol* 1994; **18**: 1078-1091
- 3 **Kim HG**. [Biliary cystic neoplasm: biliary cystadenoma and biliary cystadenocarcinoma] *Korean J Gastroenterol* 2006; **47**: 5-14
- 4 **Teoh AY**, Ng SS, Lee KF, Lai PB. Biliary cystadenoma and other complicated cystic lesions of the liver: diagnostic and therapeutic challenges. *World J Surg* 2006; **30**: 1560-1566
- 5 **Choi BI**, Lim JH, Han MC, Lee DH, Kim SH, Kim YI, Kim CW. Biliary cystadenoma and cystadenocarcinoma: CT and sonographic findings. *Radiology* 1989; **171**: 57-61
- 6 **Del Poggio P**, Jamoletti C, Forloni B, De Benedictis R, Mattiello M, Corti D, Pezzica E. Malignant transformation of biliary cystadenoma: a difficult diagnosis. *Dig Liver Dis* 2000; **32**: 733-736
- 7 **Zen Y**, Fujii T, Itatsu K, Nakamura K, Konishi F, Masuda S, Mitsui T, Asada Y, Miura S, Miyayama S, Uehara T, Katsuyama T, Ohta T, Minato H, Nakanuma Y. Biliary cystic tumors with bile duct communication: a cystic variant of intraductal papillary neoplasm of the bile duct. *Mod Pathol* 2006; **19**: 1243-1254
- 8 **Vilgrain V**, Silberman O, Benhamou JP, Nahum H. MR imaging in intracystic hemorrhage of simple hepatic cysts. *Abdom Imaging* 1993; **18**: 164-167
- 9 **Gomori JM**. Head and neck hemorrhage in Kressel HY editor. *Magnetic Resonance annual* 1987. New York: Raven, 1987: 71-112
- 10 **Kitajima Y**, Okayama Y, Hirai M, Hayashi K, Imai H, Okamoto T, Aoki S, Akita S, Gotoh K, Ohara H, Nomura T, Joh T, Yokoyama Y, Itoh M. Intracystic hemorrhage of a simple liver cyst mimicking a biliary cystadenocarcinoma. *J Gastroenterol* 2003; **38**: 190-193
- 11 **Pinto MM**, Kaye AD. Fine needle aspiration of cystic liver lesions. Cytologic examination and carcinoembryonic

- antigen assay of cyst contents. *Acta Cytol* 1989; **33**: 852-856
- 12 **Horsmans Y**, Laka A, Gigot JF, Geubel AP. Serum and cystic fluid CA 19-9 determinations as a diagnostic help in liver cysts of uncertain nature. *Liver* 1996; **16**: 255-257
  - 13 **Park KH**, Kim JS, Lee JH, Kim HJ, Kim JY, Yeon JE, Park JJ, Byun KS, Bak YT, Lee CH. [Significances of serum level and immunohistochemical stain of CA19-9 in simple hepatic cysts and intrahepatic biliary cystic neoplasms] *Korean J Gastroenterol* 2006; **47**: 52-58
  - 14 **Shimada M**, Takenaka K, Gion T, Fujiwara Y, Taguchi K, Kajiyama K, Shirabe K, Sugimachi K. Treatment strategy for patients with cystic lesions mimicking a liver tumor: a recent 10-year surgical experience in Japan. *Arch Surg* 1998; **133**: 643-646
  - 15 **Kim K**, Choi J, Park Y, Lee W, Kim B. Biliary cystadenoma of the liver. *J Hepatobiliary Pancreat Surg* 1998; **5**: 348-352
  - 16 **Precetti S**, Gandon Y, Vilgrain V. [Imaging of cystic liver diseases] *J Radiol* 2007; **88**: 1061-1072
  - 17 **Kawashita Y**, Kamohara Y, Furui J, Fujita F, Miyamoto S, Takatsuki M, Abe K, Hayashi T, Ohno Y, Kanematsu T. Destructive granuloma derived from a liver cyst: a case report. *World J Gastroenterol* 2006; **12**: 1798-1801
  - 18 **Lewall DB**, McCorkell SJ. Hepatic echinococcal cysts: sonographic appearance and classification. *Radiology* 1985; **155**: 773-775
  - 19 **Konez O**, Goyal M, Vyas PK, Boinapally SB. Mesenchymal hamartoma of the liver. *Comput Med Imaging Graph* 2001; **25**: 61-65
  - 20 **Papastratis G**, Margaris H, Zografos GN, Korkolis D, Mannika Z. Mesenchymal hamartoma of the liver in an adult: a review of the literature. *Int J Clin Pract* 2000; **54**: 552-554
  - 21 **Brkic T**, Hrstic I, Vucelic B, Jakic-Razumovic J, Skegro M, Romic B, Cukovic-Cavka S, Pulanic R, Ostojic R. Benign mesenchymal liver hamartoma in an adult male: a case report and review of the literature. *Acta Med Austriaca* 2003; **30**: 134-137
  - 22 **al-Rikabi AC**, Buckai A, al-Sumayer S, al-Damegh S, al-Bassam AR. Fine needle aspiration cytology of mesenchymal hamartoma of the liver. A case report. *Acta Cytol* 2000; **44**: 449-453
  - 23 **Del Poggio P**, Jamoletti C, Mattiello M, Corti D, Pezzica E. Images in Hepatology. Ciliated hepatic foregut cyst. *J Hepatol* 2003; **39**: 1090
  - 24 **Allen JG**, Riall TS, Cameron JL, Askin FB, Hruban RH, Campbell KA. Abdominal lymphangiomas in adults. *J Gastrointest Surg* 2006; **10**: 746-751
  - 25 **Damascelli B**, Spagnoli I, Garbagnati F, Ceglia E, Milella M, Masciadri N. Massive lymphorrhoea after fine needle biopsy of the cystic haemolymphangioma of the liver. *Eur J Radiol* 1984; **4**: 107-109
  - 26 **Coumbaras M**, Wendum D, Monnier-Cholley L, Dahan H, Tubiana JM, Arrive L. CT and MR imaging features of pathologically proven atypical giant hemangiomas of the liver. *AJR Am J Roentgenol* 2002; **179**: 1457-1463
  - 27 **Alobaidi M**, Shirkhoda A. Malignant cystic and necrotic liver lesions: a pattern approach to discrimination. *Curr Probl Diagn Radiol* 2004; **33**: 254-268
  - 28 **Tokai H**, Kawashita Y, Eguchi S, Kamohara Y, Takatsuki M, Okudaira S, Tajima Y, Hayashi T, Kanematsu T. A case of mucin producing liver metastases with intrabiliary extension. *World J Gastroenterol* 2006; **12**: 4918-4921
  - 29 **Sica GT**, Ji H, Ros PR. CT and MR imaging of hepatic metastases. *AJR Am J Roentgenol* 2000; **174**: 691-698
  - 30 **Mortele KJ**, Ros PR. Cystic focal liver lesions in the adult: differential CT and MR imaging features. *Radiographics* 2001; **21**: 895-910
  - 31 **Gupta S**, Seith A, Dhiman RK, Chawla YK, Sud K, Kohli HS, Sakhuja V, Suri S. CT of liver cysts in patients with autosomal dominant polycystic kidney disease. *Acta Radiol* 1999; **40**: 444-448
  - 32 **Terayama N**, Matsui O, Hoshiba K, Kadoya M, Yoshikawa J, Gabata T, Takashima T, Terada T, Nakanuma Y, Shinozaki K. Peribiliary cysts in liver cirrhosis: US, CT, and MR findings. *J Comput Assist Tomogr* 1995; **19**: 419-423
  - 33 **Mortele KJ**, Segatto E, Ros PR. The infected liver: radiologic-pathologic correlation. *Radiographics* 2004; **24**: 937-955
  - 34 **Brancatelli G**, Federle MP, Vilgrain V, Vullierme MP, Marin D, Lagalla R. Fibropolycystic liver disease: CT and MR imaging findings. *Radiographics* 2005; **25**: 659-670
  - 35 **Karahan OI**, Kahrman G, Soyuer I, Ok E. Hepatic von Meyenburg complex simulating biliary cystadenocarcinoma. *Clin Imaging* 2007; **31**: 50-53
  - 36 **Maher MM**, Dervan P, Keogh B, Murray JG. Bile duct hamartomas (von Meyenburg complexes): value of MR imaging in diagnosis. *Abdom Imaging* 1999; **24**: 171-173
  - 37 **Eisenberg D**, Hurwitz L, Yu AC. CT and sonography of multiple bile-duct hamartomas simulating malignant liver disease (case report). *AJR Am J Roentgenol* 1986; **147**: 279-280

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REVIEW

## Is there a place for serum laminin determination in patients with liver disease and cancer?

Heitor Rosa, Edison Roberto Parise

Heitor Rosa, Unit of Gastroenterology and Hepatology, Federal University of Goiás School of Medicine, Goiânia, Goiás 74093-080, Brazil

Edison Roberto Parise, Unity of Hepatology, Federal University of São Paulo (UNIFESP), São Paulo 04024-002, Brazil

Author contributions: Rosa H and Parise ER contributed equally to this work.

Correspondence to: Heitor Rosa, Professor, MD, PhD, Chief, Unit of Gastroenterology and Hepatology, Federal University of Goiás School of Medicine, Rua 126 n. 21, Setor Sul, Goiânia, Goiás 74093-080, Brazil. [hrosa@cultura.com.br](mailto:hrosa@cultura.com.br)

Telephone: +55-62-32816128 Fax: +55-62-32096248

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### INTRODUCTION

Laminin was initially identified by TIMPL and MARTIN in 1979<sup>[1]</sup>, from a murine fibrosarcoma, the Engelbreth-Holm-Swan (EHS) tumor. Its molecule is a large complex (approximately 850 kilodaltons) made up of three polypeptidic chains called  $\alpha 1$  (with approximately 440 kDa),  $\beta 1$  e  $\gamma 1$  (each one with approximately 200 kDa). These chains are intertwined by disulphide bridges, forming a characteristic cross-shaped asymmetrical structure<sup>[1-3]</sup>.

Laminin is one of the main glycoproteins of the basement membrane and participates in a series of such biological phenomena as adhesion, migration, cellular differentiation and growth, inflammatory response and the maintenance of the cytoskeleton upon its binding to several components of the matrix, such as collagen type IV, heparan-sulphate and entacin<sup>[3-7]</sup>.

Laminin receptors are also found on the surface of a wide range of cells, such as platelets, muscle cells, neutrophils, endothelial cells and hepatocytes<sup>[3,6]</sup>. Recently, the existence of a class of transmembrane receptors for laminin known as integrins has been demonstrated. These integrins are involved in the mechanisms of cell-cell, cell-matrix and, more recently, pathogen-cell adhesion<sup>[3,7,8]</sup>. Laminin binding proteins have been described in a number of such pathogenic agents as *Staphylococcus aureus*, *Escherichia coli*, *Helicobacter pylori* (*H pylori*), and *Candida albicans*.

### LAMININ IN THE LIVER

In normal liver, laminin is found around the vessels and biliary ducts, where basement membranes are identified. Little or only a slight reaction for antibodies against laminin can be observed in the hepatic sinusoids<sup>[9,10]</sup>. In this organ, glycoprotein is also involved in intracellular activities, such as the normal differentiation of the biliary ducts, genetic expressions for albumin messenger RNA in hepatocyte, and regeneration with normal

### Abstract

Laminin is a glycoprotein which has an important role in the mechanism of fibrogenesis and is, thus, related to hepatic fibrosis in addition to presenting increased levels in several types of neoplasias. However, its determination is not routinely considered in the study of hepatic fibrosis. In this review, the authors critically comment on the role of this glycoprotein compared to other markers of fibrosis through non-invasive procedures (Fibroscan). They also consider its clinical investigational potential and believe that the continuation of these investigations might contribute to a better understanding of the fibrogenic mechanism, which could in turn either lead to the identification of patients at risk of developing fibrosis non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) or at least be used as an indicator for hepatic biopsy in such patients. Finally, the authors believe that serum laminin determination might contribute to the diagnosis of epithelial tumor metastasis and peritoneal carcinomatosis.

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**Key words:** Laminin; Hepatic fibrosis; Cancer; Cirrhosis; Fibrosis markers

**Peer reviewers:** Dr. Maribel Rodriguez-Torres, Fundacion De Investigacion De Diego, ave. De diego 359 suite 302, Santurce 00909, Puerto Rico; Dr. Devanshi Seth, Drug Health Services & Centenary Institute, Royal Prince Alfred Hospital, Missenden Road, Camperdown NSW 2050, Australia

lobular organization following partial hepatectomy<sup>[11-14]</sup>. Laminin is thought to be synthesized by hepatocytes and sinusoidal cells<sup>[14,15]</sup>. Among all cellular types in the sinusoids, special attention should be given to stellate cells or lipocytes, which produce the largest amount of serum laminin.

With the development of hepatic cirrhosis, laminin and collagen deposition occurs both along the fibers of septal fibrosis and subendothelial sinusoids or Disse's space. At the latter site, laminin deposition, together with collagen deposition, determine the formation of a true basement membrane along sinusoids. This phenomenon is called capillarization of Disse's space<sup>[16]</sup>. Besides the increased production of laminin in the liver an additional effect due to a lack of degradation of this protein by liver endothelial cells should also be taken into consideration. As demonstrated by Smedsrod *et al*<sup>[17]</sup> in an experimental model, apart from an increase in tissue deposition or turnover, there would be a decrease in the liver's ability to degrade this protein. With the development of anti-laminin antibodies, directed against the laminin P1 portion, increased levels of this circulating protein were observed in the more advanced stages of fibrosis in patients with hepatic disease<sup>[18-22]</sup> and as expected these serum levels have a positive correlation with portal pressure<sup>[21-26]</sup>. Kropf *et al* have proposed laminin serum concentration as a sensitive screening test for hepatic fibrotic disease and portal hypertension, if the test is carried out together with hyaluronic acid determination<sup>[21,22]</sup>. Laminin as an isolated parameter was found to be highly sensitive, but with low specificity to detect portal pressure above 5 mmHg.

We have assessed laminin serum levels in patients with alcoholic liver cirrhosis and with preserved hepatic function in an attempt to evaluate its predictive value for the risk of variceal bleeding, which is assessed through a portal pressure level equal to or higher than 12 mmHg<sup>[26]</sup>. In these patients, serum laminin levels were significantly correlated with portal pressure levels ( $r = 0.70$ ). Such correlation enabled us to find a cut-off level for serum laminin that could correspond more closely to a portal pressure of 12 mmHg, accepted as a threshold for esophageal rupture in those patients. As it was found by others, these laminin levels presented very low specificity and negative predictive values to identify those patients with increased portal hypertension. In fact, patients presenting laminin serum concentration of less than 2.20 U/mL have almost 50% chance of having or not a portal pressure of 12 mmHg or higher.

This low specificity of serum laminin determination in portal hypertension could be related to the fact that laminin levels reflect only structural changes and do not take into account changes in the systemic and portal blood flow, which contribute significantly to portal pressure.

On the other hand, due to the great distribution of laminin in the body's basement membranes, and its limited participation in the liver's extracellular matrix, some issues need to be further investigated and clarified as for the origin of this protein. A study of concentrations of

serum laminin in different vascular territories showed that its levels in supra-hepatic veins were higher compared to those found in the renal and femoral veins of patients with fibrosis or hepatic cirrhosis<sup>[27]</sup>, which would be indicative of its hepatic synthesis. Similar findings were also demonstrated in control and carbon tetrachloride treated animals; there was also a significant correlation found between laminin serum levels and the degree of hepatic fibrosis<sup>[28]</sup> and an important increase was observed in the concentration of this glycoprotein in the supra-hepatic vein when compared to its amount in portal blood<sup>[29]</sup>. However, the hepatic contribution was much smaller in cirrhotic animals than in those with fibrosis as the sole condition. This fact could point towards decreased hepatic extraction of the protein or an increase in laminin synthesis in other organs of the splanchnic circulation, secondary to the venous congestion of this system. The latter possibility seems to be reinforced by studies with patients infected by *Schistosoma mansoni*. In the hepatosplenic form of the disease portal hypertension is due to periportal fibrosis, which determines pre-sinusoidal portal hypertension with large splenomegaly. Because these patients do not present hepatic cirrhosis, collagenization and capillarization of with Disse's space are not usually found. In these patients, initial studies revealed a significant increase in the levels of circulating laminin, when compared to patients presenting hepatointestinal form and the control group<sup>[30-32]</sup>. This increase correlated with the levels of portal pressure measured *via* the splenic vein<sup>[30, 31]</sup>. But, when these patients were submitted to splenectomy, a significantly decrease in the levels of type IV collagen and laminin in the serum of these patients was observed<sup>[32]</sup>. Since an increased synthesis of basement membrane in the spleen of such patients has been reported, the reduced levels of laminin after splenectomy strongly suggest an important participation of an extra-hepatic source for the serum levels of laminin in these patients<sup>[33,34]</sup>.

Thus, not only might circulating levels of laminin reflect the hepatic processes of synthesis and degradation, but also the increase of the synthesis of basement membranes, as a result of the congestion observed in other splanchnic organs.

Hence, the use of serum laminin as a marker of portal hypertension for clinical use suffers from other extra-hepatic factors which might influence its blood concentration. In addition, recent studies with Fibroscan have found sensitivity and specificity for the diagnosis of portal hypertension in cirrhotic patients far higher than those found through the determination of serum laminin<sup>[35,36]</sup>.

Due to this relationship between laminin tissue deposition and advanced fibrosis, serum levels of laminin have been used by several authors as a non-invasive parameter to assess liver fibrosis in alcoholic patients as well as in those presenting with viral hepatitis and hemochromatosis<sup>[37]</sup>. Such determination, however, was progressively discontinued as it did not demonstrate to be superior to those of other such components of the extracellular matrix as TIMPs and hyaluronic acid. However,

in recent studies laminin determination has been included in a set of test together with PIIINP, hydroxyproline, prothrombin activity, and AST/ALT in the diagnosis of advanced fibrosis in chronic hepatitis C<sup>[35,38-41]</sup>.

In non-alcoholic fatty liver disease (NAFLD), however, laminin serum levels should be further investigated. In this condition, the fibrogenic stimulus in the perisinusoidal region occurs earlier, with the detection of pericellular and perisinusoidal fibrosis in the early stages of fibrosis<sup>[39]</sup>.

We have more recently been able to assess serum laminin values in NAFLD, and to measure collagen type IV and hyaluronic acid<sup>[42]</sup>. Ballooning and hepatic fibrosis in these patients is associated with the progression of the disease<sup>[43]</sup>. In this preliminary study, we analyzed the discriminative ability of serum laminin, type IV collagen and hyaluronan and hepatic enzymes levels to predict the presence of fibrosis in 30 overweight patients divided into two groups according to the absence or presence of fibrosis upon liver biopsy. All the three biochemical markers of fibrosis were able to differentiate between these two groups, but laminin presented the best correlation ( $r_s = 0.65$ ) with hepatic fibrosis and the best diagnostic performance, with 87% accuracy. When compared with the BAAT criteria proposed by Ratzliff *et al*<sup>[44]</sup>, laminin values presented a better diagnostic accuracy for the diagnosis of septal fibrosis (83%  $\times$  70%) and for the presence of any fibrosis.

Although laminin was not evaluated, in a study with 112 patients with NAFLD, 70 of whom with at least grade 1 fibrosis, Sakugawa *et al*<sup>[38]</sup> were able to confirm our findings that hyaluronic acid and type IV collagen were useful in discriminating the patients with fibrosis from those with steatosis only. The subtle differences in diagnostic accuracy performance for these biochemical markers of liver fibrosis found in our study and that by Sakugawa *et al*<sup>[38]</sup> might be attributable to the fact that their study included a higher number of patients with any given degree of fibrosis (62%  $\times$  37%) or advanced fibrosis (37%  $\times$  10%). On the other hand, Lydatakis *et al*<sup>[36]</sup> showed that HA determination was more useful in the diagnosis of fibrosis than serum laminin and type IV collagen. No correlation was observed among laminin level and the grade of hepatic fibrosis, possibly due to the method and patients selection. It's important to take into account the small number of studies on fibrosis markers by indirect method.

In studies by Sakugawa *et al*<sup>[38]</sup> HA serum levels have been well demonstrated to significantly increase in cirrhotic patients when compared to the other degrees of fibrosis. In this manner, the determination of serum laminin values can not only play a useful role in the identification of NAFLD patients with a certain degree of fibrosis, but also in the distinction between patients with simple steatosis and those with non-alcoholic steatohepatitis (NASH) and a certain degree of fibrosis. Finally, the determination of serum laminin values might become a selection parameter of patients for the indication of fibrosis. A study bearing this purpose is currently being conducted in our laboratories.

## SERUM LAMININ IN NEOPLASTIC DISEASES

Not only have serum laminin levels been studied in patients with liver diseases, but also in patients with cancer, especially in cases where tumor proliferation and invasion are found. Serum values tend to increase significantly with the emergence of metastases, irrespective of tumor lineage or the organ originating the neoplasm<sup>[45-48]</sup>.

Hence, serum laminin could be regarded as a tumoral marker in cases of alterations in the basement membrane, proliferation and tumoral invasion<sup>[48]</sup>. In fact serum laminin concentration is increased in metastatic cancer of different origins as melanoma, gastric adenocarcinoma, hepatocellular carcinoma, colorectal cancer, epithelial ovarian tumor<sup>[49-52]</sup>.

Grounded on these observations and the findings by Byers *et al*<sup>[53]</sup> and Chu *et al*<sup>[54]</sup>, who observed increased concentrations of laminin in the ascites of metastatic breast tumors, we decided to study the discriminative ability for this glycoprotein in serum and in ascites to (differentiate) discriminate between ascites due to peritoneal carcinomatosis and hepatic cirrhotics<sup>[55]</sup>. By using polyclonal antibodies against laminin isolated from human placenta, a significant increase in serum and ascitic laminin levels was observed in patients with peritoneal carcinomatosis when compared to patients with hepatic cirrhosis with or without hepatocellular carcinoma.

Although immunohistochemical studies have shown important laminin deposition in cases of neoplastic transformation of hepatocytes<sup>[56,57]</sup> and despite the considerable representation of the group of patients with HCC once they presented advanced disease with large tumor masses with high serum alpha-fetoprotein levels, blood and ascites laminin values did not distinguish these patients from those with liver cirrhosis without tumor complication. In benign and malignant ascites, serum laminin values were higher and showed excellent correlation with its value in the ascitic fluid ( $r = 0.93$ ,  $P < 0.0001$ ). Thus, these findings indicated that serum laminin levels can also be a marker of neoplastic ascites. Indeed serum laminin showed high discriminative ability for the diagnosis of malignant ascites, with 75% sensitivity, 100% specificity and 91% accuracy<sup>[43]</sup>.

So, considering the potential of laminin for clinical investigation, it seems to us that more studies are needed in order to clarify if there are still a place for serum laminin determination in the diagnosis of hepatic fibrosis in NAFLD and in the diagnosis of epithelial tumors metastasis and peritoneal carcinomatosis.

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## REFERENCES

- 1 Timpl R, Rohde H, Robey PG, Rennard SI, Foidart JM,

- Martin GR. Laminin--a glycoprotein from basement membranes. *J Biol Chem* 1979; **254**: 9933-9937
- 2 **Burgeson RE**, Chiquet M, Deutzmann R, Ekblom P, Engel J, Kleinman H, Martin GR, Meneguzzi G, Paulsson M, Sanes J. A new nomenclature for the laminins. *Matrix Biol* 1994; **14**: 209-211
  - 3 **Aumailley M**, Smyth N. The role of laminins in basement membrane function. *J Anat* 1998; **193** (Pt 1): 1-21
  - 4 **Kleinman HK**, Cannon FB, Laurie GW, Hassell JR, Aumailley M, Terranova VP, Martin GR, DuBois-Dalcq M. Biological activities of laminin. *J Cell Biochem* 1985; **27**: 317-325
  - 5 **Kershenobich Stalnikowitz D**, Weissbrod AB. Liver fibrosis and inflammation. A review. *Ann Hepatol* 2003; **2**: 159-163
  - 6 **Mecham RP**. Receptors for laminin on mammalian cells. *FASEB J* 1991; **5**: 2538-2546
  - 7 **Haas TA**, Plow EF. Integrin-ligand interactions: a year in review. *Curr Opin Cell Biol* 1994; **6**: 656-662
  - 8 **Valkonen KH**, Ringner M, Ljungh A, Wadstrom T. High-affinity binding of laminin by *Helicobacter pylori*: evidence for a lectin-like interaction. *FEMS Immunol Med Microbiol* 1993; **7**: 29-37
  - 9 **Martinez-Hernandez A**. The hepatic extracellular matrix. I. Electron immunohistochemical studies in normal rat liver. *Lab Invest* 1984; **51**: 57-74
  - 10 **Parise ER**, Summerfield JA, Hahn E, Wiedmann KH, Doenhoff MJ. Basement membrane proteins and type III procollagen in murine schistosomiasis. *Trans R Soc Trop Med Hyg* 1985; **79**: 663-670
  - 11 **Shah KD**, Gerber MA. Development of intrahepatic bile ducts in humans. Possible role of laminin. *Arch Pathol Lab Med* 1990; **114**: 597-600
  - 12 **Caron JM**. Induction of albumin gene transcription in hepatocytes by extracellular matrix proteins. *Mol Cell Biol* 1990; **10**: 1239-1243
  - 13 **Martinez-Hernandez A**, Delgado FM, Amenta PS. The extracellular matrix in hepatic regeneration. Localization of collagen types I, III, IV, laminin, and fibronectin. *Lab Invest* 1991; **64**: 157-166
  - 14 **Voss B**, Rauterberg J. Investigation on the biosynthesis of connective tissue components by cultured mouse liver macrophages and mouse peritoneal macrophages. In: Sinusoidal liver cells. Amsterdam: Elsevier, 1982; 201-208
  - 15 **Gressner AM**, Bachem MG. Cellular sources of noncollagenous matrix proteins: role of fat-storing cells in fibrogenesis. *Semin Liver Dis* 1990; **10**: 30-46
  - 16 **Schaffner F**, Popper H. Capillarization of hepatic sinusoids in man. *Gastroenterology* 1963; **44**: 239-242
  - 17 **Smedsrod B**, Paulsson M, Johansson S. Uptake and degradation in vivo and in vitro of laminin and nidogen by rat liver cells. *Biochem J* 1989; **261**: 37-42
  - 18 **Hahn E**, Wick G, Pencev D, Timpl R. Distribution of basement membrane proteins in normal and fibrotic human liver: collagen type IV, laminin, and fibronectin. *Gut* 1980; **21**: 63-71
  - 19 **Schneider M**, Voss B, Hogemann B, Eberhardt G, Gerlach U. Evaluation of serum laminin P1, procollagen-III peptides, and N-acetyl-beta-glucosaminidase for monitoring the activity of liver fibrosis. *Hepatogastroenterology* 1989; **36**: 506-510
  - 20 **Niemela O**, Risteli J, Blake JE, Risteli L, Compton KV, Orrego H. Markers of fibrogenesis and basement membrane formation in alcoholic liver disease. Relation to severity, presence of hepatitis, and alcohol intake. *Gastroenterology* 1990; **98**: 1612-1619
  - 21 **Korner T**, Kropf J, Gressner AM. Serum laminin and hyaluronan in liver cirrhosis: markers of progression with high prognostic value. *J Hepatol* 1996; **25**: 684-688
  - 22 **Kropf J**, Gressner AM, Tittor W. Logistic-regression model for assessing portal hypertension by measuring hyaluronic acid (hyaluronan) and laminin in serum. *Clin Chem* 1991; **37**: 30-35
  - 23 **Annoni G**, Colombo M, Cantaluppi MC, Khlai B, Lampertico P, Rojkind M. Serum type III procollagen peptide and laminin (Lam-P1) detect alcoholic hepatitis in chronic alcohol abusers. *Hepatology* 1989; **9**: 693-697
  - 24 **Gressner AM**, Tittor W. Serum laminin--its concentration increases with portal hypertension in cirrhotic liver disease. *Klin Wochenschr* 1986; **64**: 1240-1248
  - 25 **Mal F**, Hartmann DJ, Trinchet JC, Lacombe F, Ville G, Beaugrand M. [Serum laminin and portal pressure in alcoholic cirrhosis. A study of 39 patients] *Gastroenterol Clin Biol* 1988; **12**: 841-844
  - 26 **Kondo M**, Miszputen SJ, Leite-mor MM, Parise ER. The predictive value of serum laminin for the risk of variceal bleeding related to portal pressure levels. *Hepatogastroenterology* 1995; **42**: 542-545
  - 27 **Gressner AM**, Tittor W, Negwer A. Serum concentrations of N-terminal propeptide of type III procollagen and laminin in the outflow of fibrotic livers compared with liver-distal regions. *Hepatogastroenterology* 1986; **33**: 191-195
  - 28 **Neves LB**, Catarino RM, Silva MR, Parise ER. [Increased serum levels of laminin in the experimental cirrhosis induced by carbon tetrachloride] *Arq Gastroenterol* 2003; **40**: 173-176
  - 29 **Neves LB**. Estudo da laminina sérica e de sua deposição no fígado de ratos com fibrose hepática induzida pelo tetracloreto de carbono. Thesis UNIFESP, 2000
  - 30 **Parise ER**, Rosa H. Serum laminin in hepatic schistosomiasis. *Trans R Soc Trop Med Hyg* 1992; **86**: 179-181
  - 31 **Parise ER**, Leite-Mor MM, Rosa H. Serum laminin in hepatosplenic human schistosomiasis. *Mem Inst Oswaldo Cruz* 1992; **87** Suppl 4: 127-128
  - 32 **Grimaud JA**, Borojevic R. Chronic human schistosomiasis mansoni. Pathology of the Disse's space. *Lab Invest* 1977; **36**: 268-273
  - 33 **Wyszomirska RMAF**. Determinação sérica dos marcadores de fibrose hepática em portadores de esquistossomose mansônica: avaliação do colágeno tipo IV e laminina. Thesis. 1999 University of Campinas 1999. Sao Paulo, **118**: 1117-1123
  - 34 **Borojevic R**, Grimaud JA. Collagen fibers in enlarged basement membranes in human schistosomal liver and spleen. *Cell Mol Biol Incl Cyto Enzymol* 1980; **26**: 247-250
  - 35 **Li ZX**, He Y, Wu J, Liang DM, Zhang BL, Yang H, Wang LL, Ma Y, Wei KL. Noninvasive evaluation of hepatic fibrosis in children with infant hepatitis syndrome. *World J Gastroenterol* 2006; **12**: 7155-7160
  - 36 **Lydatakis H**, Hager IP, Kostadelou E, Mpousmpoulas S, Pappas S, Diamantis I. Non-invasive markers to predict the liver fibrosis in non-alcoholic fatty liver disease. *Liver Int* 2006; **26**: 864-871
  - 37 **Lebensztejn DM**, Skiba E, Sobaniec-Lotowska ME, Kaczmarek M. Serum hyaluronan and laminin level in children with chronic hepatitis B during long-term lamivudine treatment. *Hepatogastroenterology* 2007; **54**: 834-838
  - 38 **Sakugawa H**, Nakayoshi T, Kobashigawa K, Yamashiro T, Maeshiro T, Miyagi S, Shiroma J, Toyama A, Nakayoshi T, Kinjo F, Saito A. Clinical usefulness of biochemical markers of liver fibrosis in patients with nonalcoholic fatty liver disease. *World J Gastroenterol* 2005; **11**: 255-259
  - 39 **Attallah AM**, Toson EA, Shiha GE, Omran MM, Abdel-Aziz MM, El-Dosoky I. Evaluation of serum procollagen aminoterminal propeptide III, laminin, and hydroxyproline as predictors of severe fibrosis in patients with chronic hepatitis C. *J Immunoassay Immunochem* 2007; **28**: 199-211
  - 40 **Katayama M**, Funakoshi A, Sumii T, Sanzen N, Sekiguchi K. Laminin gamma2-chain fragment circulating level increases in patients with metastatic pancreatic ductal cell adenocarcinomas. *Cancer Lett* 2005; **225**: 167-176
  - 41 **Gressner OA**, Weiskirchen R, Gressner AM. Biomarkers of liver fibrosis: clinical translation of molecular pathogenesis or based on liver-dependent malfunction tests. *Clin Chim Acta* 2007; **381**: 107-113



- 42 **Santos VN**, Leite-Mor MM, Kondo M, Martins JR, Nader H, Lanzoni VP, Parise ER. Serum laminin, type IV collagen and hyaluronan as fibrosis markers in non-alcoholic fatty liver disease. *Braz J Med Biol Res* 2005; **38**: 747-753
- 43 **Matteoni CA**, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. *Gastroenterology* 1999; **116**: 1413-1419
- 44 **Ratziu V**, Giral P, Charlotte F, Bruckert E, Thibault V, Theodorou I, Khalil L, Turpin G, Opolon P, Poynard T. Liver fibrosis in overweight patients. *Gastroenterology* 2000; **118**: 1117-1123
- 45 **Liotta LA**, Rao CN, Wewer UM. Biochemical interactions of tumor cells with the basement membrane. *Annu Rev Biochem* 1986; **55**: 1037-1057
- 46 **AbouFarha KM**, Menheere PP, Nieman FH, Arends JW, Janknegt RA. Value of serum laminin P1 as a diagnostic and monitoring parameter in transitional cell carcinoma of the bladder. *Urol Int* 1992; **49**: 130-136
- 47 **Nakano T**, Iwahashi N, Maeda J, Hada T, Higashino K. Serum laminin P1 in small cell lung cancer: a valuable indicator of distant metastasis? *Br J Cancer* 1992; **65**: 608-612
- 48 **Rochlitz C**, Hasslacher C, Brocks DG, Herrmann R. Serum concentration of laminin, and course of the disease in patients with various malignancies. *J Clin Oncol* 1987; **5**: 1424-1429
- 49 **Saito N**, Kameoka S. Serum laminin is an independent prognostic factor in colorectal cancer. *Int J Colorectal Dis* 2005; **20**: 238-244
- 50 **Gao ZL**, Zhang C, Du GY, Lu ZJ. Clinical significance of changes in tumor markers, extracellular matrix, MMP-9 and VEGF in patients with gastric carcinoma. *Hepatogastroenterology* 2007; **54**: 1591-1595
- 51 **Qin LX**, Tang ZY. Recent progress in predictive biomarkers for metastatic recurrence of human hepatocellular carcinoma: a review of the literature. *J Cancer Res Clin Oncol* 2004; **130**: 497-513
- 52 **Burchardt ER**, Hein R, Bosserhoff AK. Laminin, hyaluronan, tenascin-C and type VI collagen levels in sera from patients with malignant melanoma. *Clin Exp Dermatol* 2003; **28**: 515-520
- 53 **Byers LJ**, Osborne JL, Carson LF, Carter JR, Haney AF, Weinberg JB, Ramakrishnan S. Increased levels of laminin in ascitic fluid of patients with ovarian cancer. *Cancer Lett* 1995; **88**: 67-72
- 54 **Chu Y**, Yang Y, Lin M, Wang Z. Detection of laminin in serum and ascites from patients with epithelial ovarian tumor. *J Huazhong Univ Sci Technol Med Sci* 2002; **22**: 58-59, 68
- 55 **Catarino RM**, Lopes JD, Forones NM, Parise ER. Laminin concentration in ascites of patients with hepatic cirrhosis and peritoneal carcinomatosis. *Braz J Med Biol Res* 2005; **38**: 271-276
- 56 **Su Q**, Fu Y, Liu YF, Zhang W, Liu J, Wang CM. Laminin induces the expression of cytokeratin 19 in hepatocellular carcinoma cells growing in culture. *World J Gastroenterol* 2003; **9**: 921-929
- 57 **Yoshida K**, Tadaoka Y, Manabe T. Expression of laminin in hepatocellular carcinoma: an adjunct for its histological diagnosis. *Jpn J Clin Oncol* 1996; **26**: 70-76

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## Adipokines and ghrelin in gastric cancer cachexia

Mustafa Kerem, Zafer Ferahkose, Utku Tonguc Yilmaz, Hatice Pasaoglu, Ebru Ofluoglu, Abdulkadir Bedirli, Bulent Salman, Tevfik Tolga Sahin, Murat Akin

Mustafa Kerem, Zafer Ferahkose, Utku Tonguc Yilmaz, Abdulkadir Bedirli, Bulent Salman, Tevfik Tolga Sahin, Murat Akin, Department of General Surgery, Medical Faculty, Gazi University, Besevler 06510, Ankara, Turkey  
Hatice Pasaoglu, Ebru Ofluoglu, Department of Biochemistry, Medical Faculty, Gazi University, Besevler 06510, Ankara, Turkey

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**Correspondence to:** Mustafa Kerem, MD, Department of General Surgery, Faculty of Medicine, Gazi University, Besevler 06510, Ankara,

Turkey. [keremm1@yahoo.com](mailto:keremm1@yahoo.com); [mkerem@gazi.edu.tr](mailto:mkerem@gazi.edu.tr)

Telephone: +90-312-2025727 Fax: +90-312-2124647

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was found between resistin and cancer cachexia. Also, because of the correlation between these parameters and GPS, these parameters might be used as a predictor factor.

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### Abstract

**AIM:** To investigate the roles of the adipocytokines, ghrelin and leptin in gastric cancer cachexia.

**METHODS:** Resistin, ghrelin, leptin, adiponectin, insulin and insulin-like growth factor (IGF- I ), were measured in 30 healthy subjects, and 60 gastric cancer patients of which 30 suffered from cancer-induced cachexia and 30 served as a control group. The relationships between hormones, body mass index (BMI) loss ratio, age, gender, and Glasgow Prognostic Score (GPS) were investigated.

**RESULTS:** Cachexia patients had higher tumor stage and GPS when compared with non-cachexia patients ( $P < 0.05$ ). Ghrelin, resistin, leptin, adiponectin and IGF- I , showed a significant correlation with BMI loss ratio and GPS ( $P < 0.05$ ). A strong correlation was seen between GPS and BMI loss ( $R = -0.570$ ,  $P < 0.0001$ ). Multivariate analysis indicated that BMI loss was significantly independent as a predictor of ghrelin, resistin, leptin and IGF- I ( $P < 0.05$ ). Existence of an important significant relationship between resistin and insulin resistance was also noted.

**CONCLUSION:** These results showed that serum ghrelin, leptin, adiponectin, and IGF- I play important roles in cachexia-related gastric cancers. No relationship

### INTRODUCTION

Cachexia, characterized by marked weight loss, anorexia, asthenia and anemia, is often associated with the presence and growth of a tumor and leads to malnutrition secondary to the induction of anorexia or decreased food intake<sup>[1]</sup>. As major mediators of metabolism; growth hormone (GH) and insulin-like growth factor- I (IGF- I ) have attracted many researchers in the field of cachexia associated gastrointestinal cancer<sup>[2,3]</sup>. Recent evidence suggests that an intricate interplay between multiple hypothalamic effector pathways and afferent hormonal signals of diverse systemic origin (resistin, leptin and adiponectin from adipocytes, ghrelin and polypeptides from the gastrointestinal tract, and insulin from the pancreas) is important in the regulation of energy intake and expenditure<sup>[4]</sup>.

Ghrelin was discovered as the peptide hormone that stimulates the release of GH from the anterior pituitary and has crucial roles in the regulation of food intake and energy homeostasis in both humans and rodents<sup>[5]</sup>. It is produced primarily by the mammalian gastric enteroendocrine cells of the oxyntic mucosa, likely the

X/A-like cells<sup>[6]</sup>. It has been reported that there was an increase in the levels of total ghrelin in cachectic lung<sup>[7]</sup>, breast and colon<sup>[8]</sup> cancer patients. Ghrelin infusion has recently been shown to increase appetite in subjects with cancer-induced cachexia<sup>[9]</sup>. Data on the association between ghrelin levels and gastric cancer cachexia are contradictory.

Leptin is a member of a group of adipocyte-secreted proteins, collectively known as the adipocytokines. Leptin acts in the central nervous system, particularly in the hypothalamus, to suppress food intake and stimulate energy expenditure<sup>[4]</sup>. Leptin levels were reported to be low in gastrointestinal<sup>[10]</sup> and pancreatic cancers<sup>[11]</sup>, and high in breast and gynecologic cancer patients<sup>[12]</sup>.

Resistin which is an 108-amino acid, 12.5-kDa peptide hormone member of the cysteine-rich secreted protein family, is also referred to as resistin-like molecules or "found in inflammatory zone" molecules. Resistin has mainly been studied in mice, in which there is compelling evidence linking the protein to insulin resistance, obesity, and type 2 diabetes mellitus<sup>[13]</sup>. There is only 55% amino acid homology between human and murine resistin, and findings have been inconclusive regarding a potential role of resistin in human insulin regulation, obesity and type 2 diabetes mellitus<sup>[14]</sup>. There is no information about the role of resistin in cancer cachexia.

Adiponectin is a member of the adipocytokine family. It is induced during adipocyte differentiation, and its secretion is stimulated by insulin and IGF- I<sup>[4]</sup>. A negative correlation between obesity and circulating adiponectin has been well established and adiponectin concentrations increase concomitantly with weight loss<sup>[15,16]</sup>. Although low adiponectin levels were reported among patients with weight-loss in advanced lung cancer<sup>[16]</sup>, there was no correlation between adiponectin levels and cachexia in breast and colon cancer patients<sup>[8]</sup>. A more recent study demonstrated that an impaired response of adiponectin, ghrelin, and leptin may play a role in the pathogenesis of cancer cachexia with breast and colon cancer<sup>[8]</sup>. However, the role of adiponectin in gastric cancer patients is not clearly understood.

There is increasing evidence that the presence of a systemic inflammatory response, as evidenced by elevated concentrations of C-reactive protein (CRP), is a prognostic factor independent of stage, performance status and weight loss in patients with advanced cancer. Recently, Forrest *et al*<sup>[17,18]</sup> have shown that an elevated CRP and hypoalbuminemia may be combined to form a score, the Glasgow Prognostic score (GPS), which has prognostic value in patients with inoperable non-small-cell lung cancer.

The aim of the present study was to investigate: (1) the nature of the relationship between serum levels of resistin, leptin, adiponectin, ghrelin, and cancer related cachexia; (2) the relationship of these three hormones, insulin, IGF- I and insulin-resistance; and (3) to evaluate the relation between the hormones and GPS.

## MATERIALS AND METHODS

The protocol was approved by the Gazi University

Medical Faculty Ethics Committee and was conducted between October 2005 and December 2006. All clinical investigations described in this paper were conducted within the guidelines mentioned in The Declaration of Helsinki. Patients with a histopathological diagnosis of gastric adenocarcinoma were included in the study, while patients with gastric lymphoma and malignant stromal tumors were excluded from the study for homogenization. The contributors were informed of the nature of the study and informed consent was obtained.

### Exclusion criteria

Patients were excluded if there was evidence of drug or alcohol abuse defined as any use of reactional drugs or more than two drinks per day; presence of congestive heart failure (ejection fraction < 35% on a echocardiogram or signs such as edema, dyspnea, or jugular venous distension); severe liver disease; severe chronic obstructive pulmonary disease; diabetes with hemoglobin A1c levels greater than 7%; fasting plasma glucose greater than 160 mg/dL or random glucose levels greater than 200 mg/dL; presence of thyroid diseases or renal failure; active infection (temperature > 38°C or other signs or symptoms of infection); history of neuroendocrine tumor; use of glucocorticoids, progesterone, testosterone or other orexigenic agents; history of eating disorders or dysphagia; treatment by chemotherapy, radiotherapy, or a major operation within the last 6 mo prior to hospitalization; malignancy with an obstructing lesion in the cardia or antrum together or if patients refused consent.

Control group patients were chosen from healthy people over 40 years of age, because the majority of the patients were over 50 and most of the parameters studied in the study were affected by age.

### Demographic findings and anthropometrical measurements

Clinical parameters obtained in the study included age, gender, BMI, cancer localization and staging, cachexia, performance status, and GPS. All pathology reports were evaluated and data on tumor histology were recorded. The extent of tumor spread was recorded using the American Joint Cancer Committee TNM Classification and Stage System. Patient height and weight were measured and BMI was calculated as body weight divided by height squared (kg/m<sup>2</sup>). Patients were defined as cachectic, based on > 10% reduction in BMI within 6 mo prior to admission, as calculated from reported weight differences given by these subjects<sup>[1]</sup>. Performance status was evaluated by using the World Health Organization (WHO) performance status.

### Analytical methods

Blood samples were drawn from each subject between 8 and 9 AM, after an overnight bed rest for measurement of the hormones, CRP, and fasting sugar, as well as a complete blood count and chemistry. All samples were stored at -80°C until analytical measurements were

performed, except for glucose, which was determined immediately after blood was drawn.

### Biochemical parameters

Routine laboratory measurements of hemoglobin, albumin and CRP were conducted. Serum glucose was measured using the glucose oxidase method. Homeostasis model assessment of insulin resistance (HOMA-IR) was calculated from fasting insulin and glucose levels [fasting glucose (millimoles per litre X fasting insulin microunits per millilitre)/22.5] as previously described<sup>[19]</sup>.

### Prealbumin

Prealbumin concentrations in the serum were determined using a commercial solid phase sandwich enzyme linked immuno-sorbent assay (ELISA) kit from Immundiagnostic® kit (Bensheim, Germany).

### Hormone determination

Resistin, leptin, adiponectin, IGF- I, and insulin concentrations in serum were determined using a commercially available ELISA kit from RayBiotech® (Norcross, GA, USA). The intra- and interassay coefficients of variation were less than 7%-10% for all parameters.

### Active ghrelin levels

For human ghrelin assessment, we used a RIA kit (Linco Research®, Missouri, USA) which incorporates an antibody that is specific for active ghrelin. The sensitivity was 100 pg/mL (in a 100 µL sample size) with a range of 100 to 10000 pg/mL. The intra- and inter assay coefficients of variation were 5.63% and 16%, respectively.

### Statistical analysis

All calculations and statistical tests were performed using SPSS (version 13.00 software, SPSS, Inc., Chicago, IL, USA). Descriptive data were expressed as mean  $\pm$  SD. Categorical parameters were expressed as percentage. The study variables were compared between the study groups using Student *t*-tests or ANOVA for continuous variables and Fisher exact test for categorical variables. For multiple comparisons, the Tukey test was used. Pearson or Kendall's tau-b correlation coefficient was used to determine the relationship between continuous variables. Multiple linear regression analysis was performed to ascertain independent effects of BM, after adjustment for age, gender, and differentiation of tumor on adiponectin, ghrelin, leptin, and IGF- I levels. All significance tests were two-tailed.

## RESULTS

### Subjects characteristics and demographic finding

Sixty patients (30 cachectic and 30 non-cachectic), who satisfied study criteria, were selected from 76 gastric cancer patients admitted to our unit between the study dates.

The patient demographic characteristics are presented in Table 1. There was no significant difference between the ages of cachectic ( $63.6 \pm 13.8$ ), non-cachectic ( $55.6 \pm 13.3$ ), and control patients ( $56.4 \pm 3.0$ ). There was no difference with respect to gender within the three groups. While 60% of non-cachectic patients had stage 1 and 2 cancers, this rate was only 10% in cachectic patients. The majority of cachectic patients were stage 3 (40%) or 4 (50%) and in non-cachectic patients, this ratio was 25% and 15%, respectively ( $P = 0.007$ ). None of the non-cachectic patients were inoperable. On the other hand, there were 2 inoperable patients (5%) in the cachectic group ( $P < 0.05$ ). There was no difference between the two groups according to tumor location and differentiation ( $P = 0.369$ ). While the WHO score for gastric cancer patients was significantly worse than the control group ( $P < 0.001$ ), there was no significant difference between the WHO scores of cachectic and non-cachectic groups ( $P = 0.108$ ). Baseline biochemical findings and changes in BMI and weights are listed in Table 2. Changes in BMI and weight were significantly higher in cachectic patients than the non-cachectic patients ( $P < 0.001$ ). Hb levels of cachectic patients were significantly lower than the levels of control and non cachectic patients ( $P < 0.001$ ). There were no significant differences in the levels of TSH, AST, creatinine, WBC or lymphocytes in the three groups.

### Ghrelin

Mean ghrelin levels were significantly elevated in cachectic patients compared with non-cachectic cancer patients and healthy control subjects ( $2305 \pm 818$  ng/mL *vs*  $1980 \pm 913$  ng/mL, and  $1332 \pm 620$  ng/mL, respectively,  $P = 0.013$ ; Table 3). No significant difference in ghrelin levels between non-cachexia and healthy control groups was observed.

A significant negative correlation was found between the serum ghrelin levels and BMI loss in the previous 6 mo ( $R = -0.439$ ,  $P = 0.008$ , Figure 1A). There was also a positive significant correlation between the serum ghrelin levels and age ( $R = 0.467$ ,  $P = 0.039$ , Table 4), and GPS ( $R = 0.327$ ,  $P = 0.002$ ).

### Resistin

Mean resistin levels were significantly elevated in cachectic patients compared with non-cachectic cancer patients and healthy control subjects ( $P = 0.013$ ). It was found that resistin levels in non-cachectic gastric cancer patients were significantly higher than the control group ( $P = 0.042$ , Table 3). A significant negative correlation was found between the serum ghrelin levels and BMI loss in the previous 6 mo ( $R = -0.574$ ,  $P < 0.001$ , Figure 1B), and GPS ( $R = 0.387$ ,  $P < 0.01$ , Table 4). There was also a significant correlation between the serum resistin levels and insulin ( $R = 0.348$ ,  $P = 0.016$ ), glucose ( $R = 0.418$ ,  $P = 0.0018$ ), and HOMA-IR ( $R = 0.518$ ,  $P = 0.0001$ , Table 4).

### Adiponectin

The average serum adiponectin levels were  $36.5 \pm 15.0$  µg/mL in patients with cachectic gastric cancer,



Table 1 Clinical characteristics of the subjects ( $n = 30$ )

	Healthy controls	Patients with gastric cancer		P value
		Non-cachexia	Cachexia	
Age (mean $\pm$ SD)	56.4 $\pm$ 3.0	55.8 $\pm$ 13.3	63.6 $\pm$ 13.8	0.001 <sup>1</sup>
Gender (%)				0.344 <sup>2,3</sup>
Male	20 (67)	25 (84)	23 (77)	
Female	10 (33)	5 (16)	7 (23)	
Tumor stage (%)				0.007 <sup>2</sup>
I	-	6 (20)	0	
II	-	12 (40)	3 (10)	
III	-	8 (25)	12 (40)	
IV	-	4 (15)	15 (50)	
Tumor differentiation				0.347 <sup>2</sup>
Differentiated		26 (87)	23 (77)	
Undifferentiated		4 (13)	7 (23)	
Operability				0.351 <sup>2</sup>
Operable/No operable	-	30/0	28/2	
Localization (%)				0.369 <sup>2</sup>
Cardia	-	3 (10)	2 (6)	
Corpus	-	6 (20)	10 (34)	
Fundus	-	3 (10)	0	
Antrum	-	18 (60)	18 (60)	
WHO performance status (%)				0.108 <sup>2</sup> , < 0.001 <sup>3</sup>
0	30 (100)	9 (30)	3 (10)	
1	0	14 (45)	11 (36)	
2	0	6 (20)	6 (20)	
3	0	0	7 (24)	
4	0	1 (5)	3 (10)	
GPS				< 0.001 <sup>2,3</sup>
0	30 (100)	18 (60)	1 (5)	
1	0	12 (40)	17 (55)	
2	0	0	12 (40)	

<sup>1</sup>Patients with gastric cancer vs healthy control (ANOVA); <sup>2</sup>Cachexia vs non-cachexia ( $\chi^2$ ); <sup>3</sup>Cachexia and non-cachexia vs healthy control ( $\chi^2$ ).

Table 2 Changes in BMI and body weight and baseline biochemical findings ( $n = 30$ , mean  $\pm$  SD)

	Healthy controls ( $n = 30$ )	Patients with gastric cancer		P value (between groups) (ANOVA)
		Non-cachexia ( $n = 30$ )	Cachexia ( $n = 30$ )	
Initial BMI (kg/m <sup>2</sup> )	25.3 $\pm$ 2.3	27 $\pm$ 5.1	26.3 $\pm$ 2.5	0.985
Final BMI (kg/m <sup>2</sup> )	26.7 $\pm$ 2.8	24.8 $\pm$ 5.0	20.3 $\pm$ 2.5 <sup>a</sup>	0.002
Change BMI (%)	1.26 $\pm$ 2.6	-4.0 $\pm$ 2.4 <sup>c</sup>	-5.7 $\pm$ 5.2 <sup>a</sup>	< 0.001
Initial weight (kg)	75.4 $\pm$ 11.0	73.8 $\pm$ 13.3	70.0 $\pm$ 12.3	0.436
Final weight (kg)	76.2 $\pm$ 10.2	70.8 $\pm$ 16.3 <sup>c</sup>	53.6 $\pm$ 13.0 <sup>a</sup>	0.001
Change weight (kg)	0.15 $\pm$ 0.76	-2.9 $\pm$ 1.5 <sup>c</sup>	-10.4 $\pm$ 3.3 <sup>a</sup>	< 0.001
Hb (g/L)	12.8 $\pm$ 2.8	12.6 $\pm$ 1.9	11.1 $\pm$ 1.3 <sup>a</sup>	< 0.001
TSH (mIU/mL)	1.96 $\pm$ 0.28	1.98 $\pm$ 0.85	2.11 $\pm$ 0.11	0.492
AST (IU/L)	23.3 $\pm$ 8.3	28.7 $\pm$ 8.2	26.5 $\pm$ 5.1	0.088
Creatinine (mg/dL)	0.81 $\pm$ 0.36	1.03 $\pm$ 0.27	0.97 $\pm$ 0.28	0.085
WBC ( $\times 10^9$ )	6.9 $\pm$ 1.2	7.1 $\pm$ 2.1	7.0 $\pm$ 2.2	0.230
Lymphocytes (%)	22.3 $\pm$ 4.2	24.3 $\pm$ 3.8	23.98 $\pm$ 4.8	0.156

<sup>a</sup> $P < 0.05$  vs healthy controls and non cachexia; <sup>c</sup> $P < 0.05$  vs healthy controls.

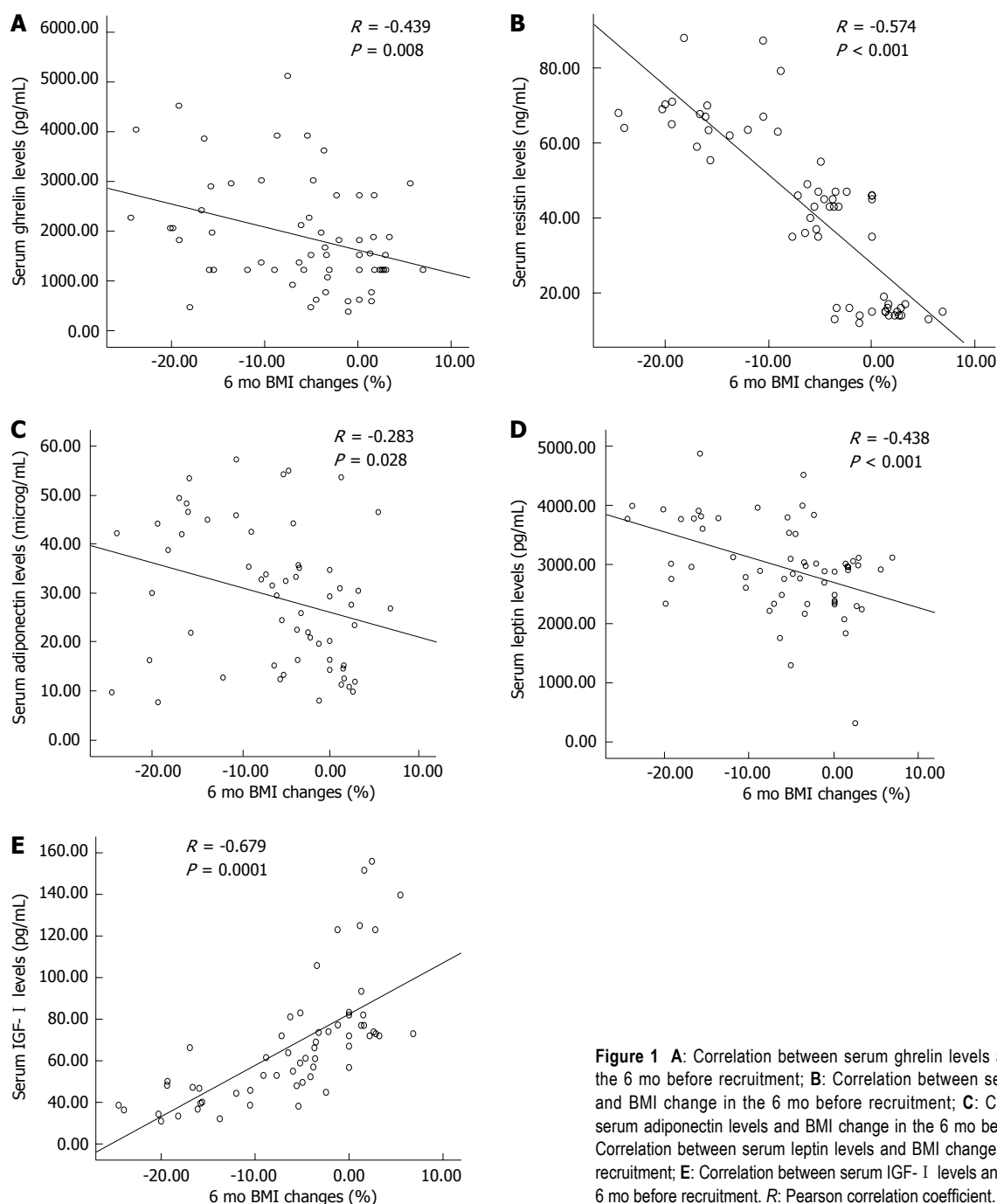
22.6  $\pm$  12.4  $\mu$ g/mL in healthy controls, and 27.8  $\pm$  11.9  $\mu$ g/mL in the non-cachexia group. Thus, there was a significant difference between the cachexia group and healthy and non-cachexia groups ( $P = 0.006$ , Table 3). However, there was no significant difference between the serum adiponectin levels of healthy and non-cachectic controls.

The association between adiponectin levels and BMI loss in the last 6 mo is plotted in Figure 1C. A significant

negative correlation was found between the serum adiponectin levels and BMI changes in the previous 6 mo ( $R = -0.283$ ,  $P = 0.028$ ). Adiponectin showed a strong positive correlation with GPS ( $R = 0.241$ ,  $P = 0.008$ ).

### Leptin

Cachectic gastric cancer patients had significantly higher serum leptin levels than healthy controls and non-cachectic gastric cancer patients (3 405  $\pm$  640 pg/mL



**Figure 1** A: Correlation between serum ghrelin levels and BMI change in the 6 mo before recruitment; B: Correlation between serum resistin levels and BMI change in the 6 mo before recruitment; C: Correlation between serum adiponectin levels and BMI change in the 6 mo before recruitment; D: Correlation between serum leptin levels and BMI change in the 6 mo before recruitment; E: Correlation between serum IGF-1 levels and BMI change in the 6 mo before recruitment. R: Pearson correlation coefficient.

vs  $2623 \pm 665$  pg/mL and  $2810 \pm 818$  pg/mL, respectively,  $P = 0.003$ , Table 3). No significant differences in serum leptin levels between non-cachexia and healthy control groups were observed. A significant negative correlation was found between leptin levels and BMI loss in the previous 6 mo ( $R = -0.438$ ,  $P < 0.001$ , Figure 1D). Leptin also showed a strong positive correlation with GPS ( $R = 0.303$ ,  $P = 0.003$ ).

#### Albumin, prealbumin, CRP, and GPS

Albumin and prealbumin, markers of nutritional status, were lower in gastric cancer patients when compared with healthy and non-cachectic subjects ( $P = 0.03$ ,  $P = 0.001$ , respectively). The mean CRP levels were significantly higher in cachexia patients than the levels

of healthy and non-cachexia controls ( $P < 0.001$ ). CRP levels also increased significantly in non-cachectic and healthy subjects ( $P < 0.05$ , Table 3). The average GPS was significantly higher in cachexia patients than non-cachectic cancer patients and healthy controls ( $P < 0.001$ ). BMI loss in last 6 mo showed a strong negative correlation with GPS ( $R = -0.758$ ,  $P < 0.0001$ ) and CRP ( $R = -0.570$ ,  $P < 0.0001$ ), however, a positive correlation with prealbumin ( $R = 0.302$ ,  $P = 0.019$ ).

#### Glucose, insulin, and HOMA-IR

Fasting glucose levels increased borderline significantly in cachectic patients compared with the healthy and non-cachectic patients ( $P = 0.05$ ). Insulin levels were increased in non-cachectic gastric cancer patients when

**Table 3** All biochemical parameters in blood in whole subjects are shown

	Healthy controls ( <i>n</i> = 30)	Patients with gastric cancer		<i>P</i> -value (between groups) (ANOVA)
		Non-cachexia ( <i>n</i> = 30)	Cachexia ( <i>n</i> = 30)	
Albumin (gr/dL)	4.2 ± 0.2	4.0 ± 0.5	2.9 ± 0.2 <sup>a</sup>	0.03
CRP (mg/L)	5.95 ± 0.8	9.72 ± 4.3 <sup>c</sup>	12.9 ± 2.2 <sup>a</sup>	< 0.001
Prealbumin (ng/mL)	52.5 ± 8.3	49.2 ± 6.4	41.0 ± 12.3 <sup>a</sup>	0.001
Fasting glucose (mg/dL)	102.7 ± 8.3	105.7 ± 13.7	114.3 ± 13.8 <sup>a</sup>	0.005
Insulin (μIU/mL)	21.8 ± 9.0	24.4 ± 6.3	18.4 ± 6.0 <sup>c</sup>	0.04
HOMA-IR	5.58 ± 2.55	6.58 ± 1.89	5.05 ± 1.68	0.07
IGF-1 (pg/mL)	95.0 ± 30.1	63.1 ± 13.1 <sup>c</sup>	43.8 ± 9.5 <sup>a</sup>	< 0.001
Resistin (ng/mL)	18.1	43.4 <sup>c</sup>	66.7 <sup>a</sup>	< 0.001
Ghrelin (ng/mL)	1332 ± 620	1980 ± 913	2305 ± 818 <sup>a</sup>	< 0.001
Adiponectin (μg/mL)	22.6 ± 12.4	27.8 ± 11.9	36.5 ± 15.0 <sup>a</sup>	0.045
Leptin (pg/mL)	2810 ± 818	2623 ± 665	3405 ± 640	0.003

<sup>a</sup>*P* < 0.05 vs healthy controls and non cachexia; <sup>c</sup>*P* < 0.05 vs non-cachexia; <sup>c</sup>*P* < 0.05 vs healthy controls.

**Table 4** Bivariate correlation analysis between different hormones and age, GPS and other parameters

		Resistin	Ghrelin	Leptin	Adiponectin	IGF- I
Age	<i>R</i>	0.115	0.267 <sup>a</sup>	0.253	-0.092	-0.331 <sup>a</sup>
	<i>P</i>	0.687	0.039 <sup>a</sup>	0.051	0.486	0.010 <sup>a</sup>
GPS	<i>R</i>	0.387 <sup>a</sup>	0.327 <sup>a</sup>	0.303 <sup>a</sup>	0.241 <sup>a</sup>	0.363 <sup>a</sup>
	<i>P</i>	0.0017 <sup>a</sup>	0.002 <sup>a</sup>	0.003 <sup>a</sup>	0.019 <sup>a</sup>	0.004 <sup>a</sup>
Insulin	<i>R</i>	0.348 <sup>a</sup>	-0.126	-0.225	0.197	0.06
	<i>P</i>	0.016 <sup>a</sup>	0.339	0.084	0.132	0.905
Glucose	<i>R</i>	0.418 <sup>a</sup>	0.194	-0.324 <sup>a</sup>	0.172	-0.358 <sup>a</sup>
	<i>P</i>	0.0018 <sup>a</sup>	0.138	0.012 <sup>a</sup>	0.190	0.005 <sup>a</sup>
HOMA-IR	<i>R</i>	0.518 <sup>a</sup>	-0.100	-0.181	-0.094	0.073
	<i>P</i>	0.0001 <sup>a</sup>	0.449	0.166	0.475	0.581
Prealbumin	<i>R</i>	-0.218	-0.223	-0.097	-0.341 <sup>a</sup>	0.287 <sup>a</sup>
	<i>P</i>	0.082	0.087	0.461	0.008 <sup>a</sup>	0.026 <sup>a</sup>

*P*: Pearson correlation coefficient; *R*: Kendall's tau-b correlation coefficient. Significantly correlations and <sup>a</sup>*P* < 0.05.

compared with cachectics (*P* = 0.04); however, there was no significant difference between non-cachectic cancer patients and healthy subjects (*P* > 0.05). After adjusting for the presence of diabetes mellitus, HOMA-IR values were not significantly different between groups. Only glucose showed an inverse significant correlation with BMI loss in 6 mo (*R* = -0.324, *P* = 0.012).

### IGF- I

IGF- I levels significantly decreased in cachectic subjects compared with healthy and non-cachectic subjects (43.8 ± 9.5 pg/mL vs 95.0 ± 30.1 pg/mL and 63.1 ± 13.1 pg/mL; *P* < 0.05). Moreover, there was a significant difference in IGF- I levels between healthy and non-cachectic cancer patients (63.1 ± 13.1 pg/mL vs 43.8 ± 9.5 pg/mL; *P* < 0.05, Figure 1E). IGF- I also showed a positive correlation with BMI loss in the previous 6 mo (*R* = -0.679, *P* < 0.0001, Figure 1E) and GPS (*R* = 0.363, *P* = 0.004); negative correlations with age (*R* = -0.331, *P* = 0.01) and fasting glucose (*R* = -0.358, *P* = 0.005, Table 4) were observed.

### Multivariate analysis results

Multiple regression analysis was used to evaluate the role of BMI loss as a continuous variable, along with

**Table 5** Multiple regression analysis with age, BMI change, gender, and tumor differentiation as predictors of ghrelin, leptin, adiponectin, and IGF- I in gastric cancer patients

		Resistin	Ghrelin	Leptin	Adiponectin	IGF- I
Age	<i>β</i>	0.148	0.168	0.019	-0.323	0.115
	<i>P</i>	0.402	0.305	0.899	0.053	0.377
Gender	<i>β</i>	0.126	0.286	-0.339 <sup>a</sup>	0.057	0.073
	<i>P</i>	0.387	0.085	0.032 <sup>a</sup>	0.726	0.377
BMI change	<i>β</i>	-0.583 <sup>a</sup>	-0.318 <sup>a</sup>	-0.418 <sup>a</sup>	-0.202	0.699 <sup>a</sup>
	<i>P</i>	0.001 <sup>a</sup>	0.009 <sup>a</sup>	0.008 <sup>a</sup>	0.21	< 0.0001 <sup>a</sup>

*β*: Standardized coefficient. Significant regression analysis and <sup>a</sup>*P* < 0.05 are shown.

age, gender, and BMI change to predict ghrelin, resistin, leptin, adiponectin, and IGF- I levels. The results of the regression model indicated that age was not a significant predictor of hormone levels. Gender was a negative independent significant predictor for leptin, and BMI was found to be a negative independent significant predictor for all parameters, except adiponectin (Table 5).

## DISCUSSION

In this prospective study, BMI loss in gastric cancer patients negatively correlated with serum active ghrelin, resistin, adiponectin and leptin levels, but positively correlated with the level of serum IGF- I. It was also noted that there was a correlation between resistin which was found to be high in cachectic patients, and insulin resistance, insulin and blood glucose.

Ghrelin an anabolic hormone, has several roles in metabolism, appetite, nutrition, weight gain, gastric motility, and gastric emptying. In addition, it has an important role in the regulation of synthesis of GH, IGF- I, insulin, and leptin<sup>[4-6]</sup>. Total ghrelin levels in cachectic patients with colon, breast and lung cancer were significantly higher than the levels in non-cachectic patients<sup>[7,8]</sup>. Garcia *et al*<sup>[20]</sup> showed that the ratio of active to total ghrelin levels increased in cancer-induced cachexia. In the same study, it was stated that the increase in active ghrelin levels could have been explained by ghrelin resistance. As the ratio of active/total ghrelin levels were

in favor of active ghrelin in cancer cachexia, we evaluated active ghrelin levels. Our study showed that active ghrelin levels were higher in healthy subjects and non-cachectic cancer patients, especially females. Although we were not able to confirm the ghrelin resistance mentioned by Garcia *et al*<sup>[20]</sup>, we saw the indirect effects. Under normal conditions, endogenous ghrelin increases GH secretion and indirectly increases IGF- I by stimulation of its own receptors. However in our study, the existence of a negative correlation between decreased IGF- I and ghrelin may show that efficiency decreases while the levels of active ghrelin increase. These findings support the presence of ghrelin resistance. It is also possible that ghrelin levels could increase to compensate for the increased metabolic rate and energy need, which was hypothesized by Nagaya *et al*<sup>[21]</sup> Several experimental studies have shown that ghrelin has an important role in the regulation of insulin *via* controlling pancreatic endocrine functions<sup>[22,23]</sup>. No significant relationship between ghrelin and insulin, fasting glucose level and HOMA-IR were found in the present study.

Resistin is a member of the newly discovered family of cysteine-rich secretory proteins, called resistin-like proteins. The role of resistin in pathogenesis of insulin resistance remains questionable, with conflicting data in animal models and negative findings in clinical observation<sup>[13,14]</sup>. The role of insulin resistance in cancer cachexia occurrence is not fully understood<sup>[1,2]</sup>. From this point of view, it came to mind whether resistin can have effect in gastric cancer cachexia occurrence. There are no clinical studies of serum resistin levels in cancer cachexia. It has been shown that serum resistin levels are high in lymphoma patients<sup>[24]</sup>, but the resistin-tumor cachexia relationship was not investigated in this study. From this point of view, our study is the first study in the literature. We found that serum resistin levels in cachectic gastric cancer patients were significantly higher than the noncachectic patients and healthy controls. Resistin showed negative correlation with BMI loss. The effect of resistin on cachexia is probably due to insulin resistance and ineffective usage of glucose. The existence of a correlation between serum resistin levels and insulin, insulin resistance and blood glucose levels supports this idea<sup>[25]</sup>. The role of leptin in modulating the immune response and inflammation has become increasingly evident and has been reviewed recently<sup>[26,27]</sup>. Complex interactions among the nervous, endocrine and immune systems affect the leptin loop and the potential role of these mediators in cancer-related cachexia-anorexia syndrome<sup>[8,11,12]</sup>. Wallace *et al*<sup>[10]</sup> showed that serum levels of leptin did not differ between normal subjects and patients with gastrointestinal cancer. Other studies have shown that there is a relationship between cachexia and leptin levels in pancreatic, lung, breast, and colon cancer patients<sup>[8,11,12]</sup>. Wolf *et al*<sup>[8]</sup> showed that changes in leptin levels in cancer cachexia were significantly higher. Our results were similar to this last study. It is known that leptin receptors are found in  $\beta$  islet cells of the pancreas and inhibit insulin secretion<sup>[28,29]</sup>. However, our study revealed that there

was a reverse correlation between leptin and fasting glucose levels, whereas no relation was found between leptin, insulin resistance, and insulin levels.

It also has been showed that insulin resistance and low serum IGF- I levels are important factors for cancer cachexia. These hormones are strongly anabolic and increase muscle protein synthesis<sup>[11,2]</sup>. IGF- I concentrations increase with growth hormone and testosterone administration, thereby accounting for some of the effects of these hormones on muscle bulk and strength. Low IGF- I concentrations in malnourished humans suggest a role for IGF- I in the pathogenesis of cachexia<sup>[30]</sup>. These findings showed that IGF- I was one of the most important factors in the gastric cancer cachexia.

GPS was found to be an important parameter for determining the prognosis of advanced cancers<sup>[17,18]</sup>. In our study, besides the strong correlation between GPS and BMI loss, significant correlations were found between the GPS and the levels of hormones and cytokines which could be very important for clinical evaluation. GPS which is calculated using routine measurements of albumin and CRP can help us in evaluation and management of the cancer cachexia in clinical practice.

Adiponectin, which has an obvious anti-inflammatory effect, is inversely related to weight gain<sup>[4]</sup>. Serum adiponectin levels were low in cases with increased insulin resistance like obesity, type 2 diabetics, and non-alcoholic fatty liver diseases<sup>[30]</sup>. However, serum adiponectin levels decreased in patients who lost weight voluntarily. High adiponectin levels are risk factors for endometrial and breast cancer, whereas low levels are risk factors for gastric cancers<sup>[31,32]</sup>. No relationship was found between adiponectin and cachexia in breast and colon cancer. In our study, a significant positive correlation was found between adiponectin and BMI loss, but multivariate analysis did not show BMI loss as predictive for adiponectin. Adiponectin, which is predominantly secreted from adipose tissues, might have increased due to lipolysis which occurred with muscle loss in the catabolic state. No correlation was found between adiponectin, whose close relationship with insulin resistance was known, and insulin, blood glucose levels, HOMA-IR, or IGF- I<sup>[4]</sup>.

As a result, cachexia in gastric cancer is a complex process in which ghrelin, resistin, leptin, adiponectin, and IGF- I function. It is of note that these hormones have important roles in occurrence of cachexia in gastric cancer patients. This study will be one of the corner stones of the further studies about prevention and treatment of cachexia.

## COMMENTS

### Background

Adipocytokines are the peptide hormones secreted from adipocytes and have special roles in regulation of metabolism, glucose metabolism, and inflammation. Ghrelin which is secreted from especially from the gastric fundus has roles in regulation of blood glucose level, appetite and secretion of growth hormone. In this study, the role of adiponectin, ghrelin, and insulin like growth factor in gastric cancer patients with cachexia is evaluated.



## Research frontiers

Significant correlation between cachexia and ghrelin, leptin adiponectin and IGF- I can inform more sophisticated studies about the mechanism of cachexia. Despite sufficient nutritional support, cachexia is always an expected morbidity in cancer patients. This study will help future studies about the pathophysiology of the cachexia.

## Innovations and breakthroughs

Although there have been several studies concerning nutritional support and measurement of cachexia in cancer patients, there have not been many studies about the pathophysiology. It is important to understand the behaviour of cancer with cachexia and also to support the patient. Several factors contributing to cancer biology demand attention. However, this study is original for being the first evaluating the correlation between cancer cachexia and resistin. The relationship between adipocytokines and colon, lung and breast cachexia have been studied before. This study has investigated the relationship between gastric cancer cachexia and adipocytokines.

## Applications

To date, the treatment of cancer cachexia has gone no further than nutritional and fluid-electrolyte support. Alternative treatment modalities may be identified if the role of hormones and peptides in cachexia is well-understood. For example, administration of recombinant ghrelin induces growth hormone secretion and appetite.

## Terminology

Adipocytokines are secreted from adipocytes. They are peptide hormones that have systemic effect. For example: resistin, adiponectin, leptin, etc. Cachexia means loss of lipid, carbohydrate and protein in a short time.

## Peer review

This paper investigated resistin, ghrelin, leptin, adiponectin, insulin, IGF- I in gastric cancer subjects with and without cachexia and healthy controls. Ghrelin, resistin, adiponectin significantly differed between subjects with and without cachexia. The study was well described and appropriately presented. It's an important study.

## REFERENCES

- 1 Tisdale MJ. Cachexia in cancer patients. *Nat Rev Cancer* 2002; **2**: 862-871
- 2 Yoshikawa T, Noguchi Y, Doi C, Makino T, Nomura K. Insulin resistance in patients with cancer: relationships with tumor site, tumor stage, body-weight loss, acute-phase response, and energy expenditure. *Nutrition* 2001; **17**: 590-593
- 3 Huang Q, Nai YJ, Jiang ZW, Li JS. Change of the growth hormone-insulin-like growth factor-I axis in patients with gastrointestinal cancer: related to tumour type and nutritional status. *Br J Nutr* 2005; **93**: 853-858
- 4 Meier U, Gressner AM. Endocrine regulation of energy metabolism: review of pathobiochemical and clinical chemical aspects of leptin, ghrelin, adiponectin, and resistin. *Clin Chem* 2004; **50**: 1511-1525
- 5 Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature* 1999; **402**: 656-660
- 6 Gualillo O, Lago F, Gomez-Reino J, Casanueva FF, Dieguez C. Ghrelin, a widespread hormone: insights into molecular and cellular regulation of its expression and mechanism of action. *FEBS Lett* 2003; **552**: 105-109
- 7 Shimizu Y, Nagaya N, Isobe T, Imazu M, Okumura H, Hosoda H, Kojima M, Kangawa K, Kohno N. Increased plasma ghrelin level in lung cancer cachexia. *Clin Cancer Res* 2003; **9**: 774-778
- 8 Wolf I, Sadetzki S, Kanety H, Kundel Y, Pariente C, Epstein N, Oberman B, Catane R, Kaufman B, Shimon I. Adiponectin, ghrelin, and leptin in cancer cachexia in breast and colon cancer patients. *Cancer* 2006; **106**: 966-973
- 9 Neary NM, Small CJ, Wren AM, Lee JL, Druce MR, Palmieri C, Frost GS, Ghatei MA, Coombes RC, Bloom SR. Ghrelin increases energy intake in cancer patients with impaired appetite: acute, randomized, placebo-controlled trial. *J Clin Endocrinol Metab* 2004; **89**: 2832-2836
- 10 Wallace AM, Kelly A, Sattar N, McArdle CS, McMillan DC. Circulating concentrations of "free" leptin in relation to fat mass and appetite in gastrointestinal cancer patients. *Nutr Cancer* 2002; **44**: 157-160
- 11 Brown DR, Berkowitz DE, Breslow MJ. Weight loss is not associated with hyperleptinemia in humans with pancreatic cancer. *J Clin Endocrinol Metab* 2001; **86**: 162-166
- 12 Bolukbas FF, Kilic H, Bolukbas C, Gumus M, Horoz M, Turhal NS, Kavakli B. Serum leptin concentration and advanced gastrointestinal cancers: a case controlled study. *BMC Cancer* 2004; **4**: 29
- 13 Steppan CM, Bailey ST, Bhat S, Brown EJ, Banerjee RR, Wright CM, Patel HR, Ahima RS, Lazar MA. The hormone resistin links obesity to diabetes. *Nature* 2001; **409**: 307-312
- 14 Kusminski CM, McTernan PG, Kumar S. Role of resistin in obesity, insulin resistance and Type II diabetes. *Clin Sci (Lond)* 2005; **109**: 243-256
- 15 Faraj M, Havel PJ, Phelis S, Blank D, Sniderman AD, Cianflone K. Plasma acylation-stimulating protein, adiponectin, leptin, and ghrelin before and after weight loss induced by gastric bypass surgery in morbidly obese subjects. *J Clin Endocrinol Metab* 2003; **88**: 1594-1602
- 16 Jamieson NB, Brown DJ, Michael Wallace A, McMillan DC. Adiponectin and the systemic inflammatory response in weight-losing patients with non-small cell lung cancer. *Cytokine* 2004; **27**: 90-92
- 17 Forrest LM, McMillan DC, McArdle CS, Angerson WJ, Dunlop DJ. Comparison of an inflammation-based prognostic score (GPS) with performance status (ECOG) in patients receiving platinum-based chemotherapy for inoperable non-small-cell lung cancer. *Br J Cancer* 2004; **90**: 1704-1706
- 18 Forrest LM, McMillan DC, McArdle CS, Angerson WJ, Dunlop DJ. Evaluation of cumulative prognostic scores based on the systemic inflammatory response in patients with inoperable non-small-cell lung cancer. *Br J Cancer* 2003; **89**: 1028-1030
- 19 Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF, Turner RC. Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 1985; **28**: 412-419
- 20 Garcia JM, Garcia-Touza M, Hijazi RA, Taffet G, Epner D, Mann D, Smith RG, Cunningham GR, Marcelli M. Active ghrelin levels and active to total ghrelin ratio in cancer-induced cachexia. *J Clin Endocrinol Metab* 2005; **90**: 2920-2926
- 21 Nagaya N, Uematsu M, Kojima M, Date Y, Nakazato M, Okumura H, Hosoda H, Shimizu W, Yamagishi M, Oya H, Koh H, Yutani C, Kangawa K. Elevated circulating level of ghrelin in cachexia associated with chronic heart failure: relationships between ghrelin and anabolic/catabolic factors. *Circulation* 2001; **104**: 2034-2038
- 22 Prado CL, Pugh-Bernard AE, Elghazi L, Sosa-Pineda B, Sussel L. Ghrelin cells replace insulin-producing beta cells in two mouse models of pancreas development. *Proc Natl Acad Sci USA* 2004; **101**: 2924-2929
- 23 Wierup N, Yang S, McEvelly RJ, Mulder H, Sundler F. Ghrelin is expressed in a novel endocrine cell type in developing rat islets and inhibits insulin secretion from INS-1 (832/13) cells. *J Histochem Cytochem* 2004; **52**: 301-310
- 24 Pamuk GE, Demir M, Harmandar F, Yesil Y, Turgut B, Vural O. Leptin and resistin levels in serum of patients with hematologic malignancies: correlation with clinical characteristics. *Exp Oncol* 2006; **28**: 241-244
- 25 Burcelin R. Leptin and resistin: master enemy adipokines unified in brain to control glucose homeostasis. *Endocrinology* 2008; **149**: 443-444
- 26 La Cava A, Matarese G. The weight of leptin in immunity. *Nat Rev Immunol* 2004; **4**: 371-379
- 27 Kieffer TJ, Heller RS, Habener JF. Leptin receptors expressed on pancreatic beta-cells. *Biochem Biophys Res Commun* 1996; **224**: 522-527
- 28 Ahren B, Havel PJ. Leptin inhibits insulin secretion induced

- by cellular cAMP in a pancreatic B cell line (INS-1 cells). *Am J Physiol* 1999; **277**: R959-R966
- 29 **Arita Y**, Kihara S, Ouchi N, Takahashi M, Maeda K, Miyagawa J, Hotta K, Shimomura I, Nakamura T, Miyaoka K, Kuriyama H, Nishida M, Yamashita S, Okubo K, Matsubara K, Muraguchi M, Ohmoto Y, Funahashi T, Matsuzawa Y. Paradoxical decrease of an adipose-specific protein, adiponectin, in obesity. *Biochem Biophys Res Commun* 1999; **257**: 79-83
- 30 **Dal Maso L**, Augustin LS, Karalis A, Talamini R, Franceschi S, Trichopoulos D, Mantzoros CS, La Vecchia C. Circulating adiponectin and endometrial cancer risk. *J Clin Endocrinol Metab* 2004; **89**: 1160-1163
- 31 **Miyoshi Y**, Funahashi T, Kihara S, Taguchi T, Tamaki Y, Matsuzawa Y, Noguchi S. Association of serum adiponectin levels with breast cancer risk. *Clin Cancer Res* 2003; **9**: 5699-5704
- 32 **Ishikawa M**, Kitayama J, Kazama S, Hiramatsu T, Hatano K, Nagawa H. Plasma adiponectin and gastric cancer. *Clin Cancer Res* 2005; **11**: 466-472

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## LIVER CANCER

# Secretory Transactivating Transcription-apoptin fusion protein induces apoptosis in hepatocellular carcinoma HepG2 cells

Su-Xia Han, Jin-Lu Ma, Yi Lv, Chen Huang, Hai-Hua Liang, Kang-Min Duan

Su-Xia Han, Hai-Hua Liang, Kang-Min Duan, College of Life Sciences, Northwest University, Xi'an 710069, Shaanxi Province, China

Su-Xia Han, Jin-Lu Ma, Yi Lv, the First Affiliated Hospital; College of Medicine, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China

Chen Huang, College of Medicine, Xi'an Jiaotong University, Xi'an 710061, Shaanxi Province, China

Author contributions: Duan KM, Han SX, Ma JL, and Lv Y designed the research; Han SX, Ma JL, Lv Y, and Huang C performed the research; Han SX, Ma JL and Liang HH analyzed the data; and Han SX, Ma JL and Duan KM wrote the paper.

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Correspondence to: Kang-Min Duan, College of Life Sciences, Northwest University, 229 Taibai Rd. North, Xi'an 710069, Shaanxi Province, China. [kduan@ucalgary.ca](mailto:kduan@ucalgary.ca)

Telephone: +86-29-88302132 Fax: +86-29-88305288

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in HepG2 cells, but not in HUVECs.

**CONCLUSION:** The data demonstrated that SP-TAT-apoptin induces apoptosis only in malignant cells, and its secretory property might greatly increase its potency once it is delivered *in vivo* for cancer therapy.

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**Key words:** Apoptin; Apoptosis; Hepatoma; Human immunodeficiency Virus-Transactivating Transcription protein; Secretory

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## Abstract

**AIM:** To determine whether SP-TAT-apoptin induces apoptosis and also maintains its tumor cell specificity.

**METHODS:** In this study, we designed a secretory protein by adding a secretory signal peptide (SP) to the N terminus of Transactivating Transcription (TAT)-apoptin (SP-TAT-apoptin), to test the hypothesis that it gains an additive bystander effect as an anti-cancer therapy. We used an artificial human secretory SP whose amino acid sequence and corresponding cDNA sequence were generated by the SP hidden Markov model.

**RESULTS:** In human liver carcinoma HepG2 cells, SP-TAT-apoptin expression showed a diffuse pattern in the early phase after transfection. After 48 h, however, it translocated into the nuclear compartment and caused massive apoptotic cell death, as determined by 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and annexin-V binding assay. SP-TAT-apoptin did not, however, cause any cell death in non-malignant human umbilical vein endothelial cells (HUVECs). Most importantly, the conditioned medium from Chinese hamster ovary (CHO) cells transfected with SP-TAT-apoptin also induced significant cell death

## INTRODUCTION

Apoptin or viral protein 3 (VP3), a protein of 13.6 kDa derived from the chicken anemia virus (CAV), represents a new anti-cancer tool with great potential<sup>[1,2]</sup>. It appears to have innate tumor-specific, *p53*-independent<sup>[3,4]</sup>, Bcl-2-enhanced proapoptotic activity<sup>[4,5]</sup>, and hence is of considerable interest for efficient targeting and specific elimination of cancer cells<sup>[3,4,6-8]</sup>. The antitumor activity of apoptin appears to be linked to its ability to localize in the nuclei of transformed cells, but not in those of primary or non-transformed cells<sup>[9]</sup>. Therefore, apoptin has been explored to achieve efficient targeting and specific elimination of cancer cells.

To use apoptin in cancer therapy, efficient delivery to or expression of apoptin in cancer cells is required. The Human Immunodeficiency Virus (HIV) Transactivating Transcription (TAT)-derived protein transduction peptide is a small basic peptide that has been successfully shown to deliver a large variety of materials, from small particles to proteins, peptides and nucleic acids, across

the cell membrane<sup>[10-12]</sup>. The region that conveys the cell-penetrating properties appears to be confined to a small (11 amino acids) stretch of basic amino acids (aa 47-57, YGRKKRRQRRR)<sup>[13]</sup>. This TAT transduction domain has been successfully used to deliver apoptin to cancer cells<sup>[14]</sup>.

In this study, we designed a secretory TAT-apoptin fusion protein by adding a secretory signal to the N-terminal of the recombinant molecule to gain an additive by-stander effect as an anti-cancer therapy. Secreted TAT-apoptin from transformed cells enters un-transformed cancer cells and causes apoptosis. We employed an artificial human secretory signal peptide (SP) whose amino acid sequence and corresponding cDNA sequences were generated by an SP hidden Markov model (SP-HMM)<sup>[15]</sup>. We demonstrated expression of the secretory fusion protein (SP-TAT-apoptin) and induction of apoptosis by the secreted protein in HepG2 cells.

## MATERIALS AND METHODS

### Generation and cloning of SP-TAT-apoptin

The human secretory SP was designed and optimized by an HMM that has been used to predict, identify and generate secretory SP sequences<sup>[15]</sup>. PCR was used to amplify the apoptin gene and to incorporate the TAT transduction domain and SP sequence upstream. The primers were designed based on the published sequences in GenBank (NC\_001427), and synthesized by Shanghai Sangon Biological Engineering Technology & Services (Shanghai, China). The first pair of designed primers were: 5'-AAGAATGAACGCTCTGCAGG AAGATACTCC-3' (sense) and 5'-CTGCAGTCTTA TACGCCITTTTGCGG-3' (antisense), with a product size of 406 bp. The sense primer contains the TAT transduction domain sequence. The second pair of primers, which incorporated the secretory signal sequence into the TAT-apoptin fusion protein, were: 5'-GCTGCTGCTGCTGCTGCTGTGGCCCATGGTG TGGGCCTATGGCAGG-3' (sense) and the same antisense primer as the first pair, with a product size of 466 bp. The templates used for generating recombinant TAT-apoptin and TAT-GFP in the first round PCR were the *apoptin* and *gfp* genes carried on pCDNA3.1-apoptin plasmid<sup>[16]</sup> and pEGFP plasmid, respectively. The conditions for both rounds of PCR were as follows: 30 cycles of 94°C for 40 s, 56°C for 40 s, and 72°C for 1 min. The PCR products obtained were TOPO<sup>®</sup> cloned into the pLenti6/V5-D-TOPO<sup>®</sup> vector (Invitrogen, USA) resulting pLenti6/V5-D-TOPO/SP-TAT-apoptin and pLenti6/V5-D-TOPO/SP-TAT-EGFP. The plasmids were transformed into Stbl3<sup>™</sup> *Escherichia coli* (*E. coli*) (Invitrogen) by electroporation. The SP-TAT-apoptin cDNA cloned in pLenti6/V5-D- TOPO<sup>®</sup> vector was confirmed by restriction enzyme digestion and by DNA sequencing.

### Cell lines and cell culture

HepG2 human hepatoma cells, human umbilical vein

endothelial cells (HUVECs) and Chinese hamster ovary (CHO) cells were purchased from Keygen Company (Nanjing, China). All cells were maintained and grown at 37°C in DMEM (Hyclone, USA), supplemented with 1% penicillin–streptomycin, and 10% fetal bovine serum in an incubator with CO<sub>2</sub> controlled at 5%.

**Conditioned medium:** The conditioned medium from Chinese hamster ovary (CHO) cells transfected with SP-TAT-apoptin. (CHO cells were cultured in a six-well plate. The cells were transfected with the pLenti6/V5-D-TOPO/SP-TAT-apoptin plasmid using the Lipofectamine<sup>™</sup> 2000 protocol according to manufacturer's instructions (Invitrogen). After 6 h incubation, the cells were washed with fresh culture medium and cultured for an additional 24 h. The culture supernatants were then collected and added, respectively, to the monolayers of HepG2 cells and HUVECs grown in 24-well plates.)

**Stbl3<sup>™</sup> *Escherichia coli*:** Stbl3<sup>™</sup> *E. coli* for transformation as this strain is particularly well-suited for use in cloning unstable DNA such as lentiviral DNA containing direct repeats.

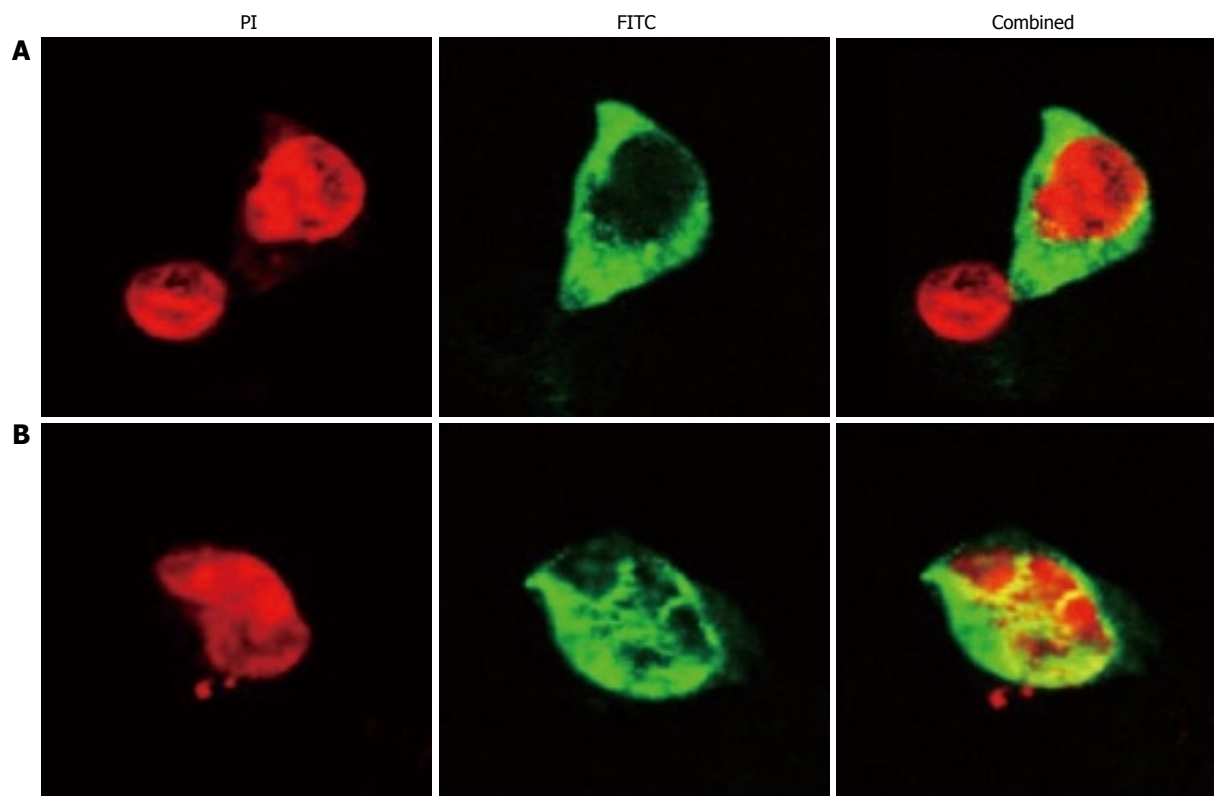
### Reverse transcriptase-PCR (RT-PCR)

Cells were rinsed twice with PBS and total RNA was isolated from cells using a Simply P Total RNA Extraction Kit (Bioer, Japan) according to the manufacturer's instructions. One microgram of total RNA was reverse transcribed to first-strand cDNA with Superscript II reverse transcriptase (Invitrogen) at 46°C for 45 min. Synthesized first-strand cDNA was then subjected to PCR analysis using gene-specific primers. The primers used were: CMV forward 5'-CGCAAATGGGCGGTAGGCGTG-3' and V5(C-term) reverse 5'-ACCGAGGAGAGGGTTAGGGAT-3', with a product size of 700 bp. The PCR conditions were as follows: 30 cycles at 94°C for 40 s, 56°C for 40 s, and 72°C for 1 min for SP-TAT-apoptin; 25 cycles at 94°C for 30 s, 60°C for 45 s, and 72°C for 1 min for  $\beta$ -actin control. PCR products were run on 1.5% agarose gels containing ethidium bromide and photographed using a Syngene Gene Genius imaging system (Syngene, USA).

### Transient transfection and fluorescence microscopy

Cells were cultivated in 24-well culture plates. In each well, the cells were grown at 50%-80% confluency and transfected with 400 ng plasmid DNA pre-incubated with 1.4  $\mu$ L Lipofectamine<sup>™</sup> 2000 (Invitrogen), according to the manufacturer's instructions. Coverslips were placed at the bottom of the wells to allow the cells grow on the slides. Apoptin expression was detected with anti-V5-FITC antibody (Invitrogen) as green fluorescence, and the cell nuclei were stained by propidium iodide (PI) as red fluorescence. The cells were incubated with anti-V5-FITC antibody in the dark for 1 h and washed twice with PBS before staining with PI. Fluorescence images were recorded on a confocal imaging system equipped with krypton–argon laser (Leica SP2 Confocal System, Germany).





**Figure 1** SP-TAT-apoptin expression in HepG2 cells ( $\times 1000$ ). Cells transfected with plenti6/V5-D-TOPO/SP-TAT-apoptin plasmid and fixed at 24 h (A) and 48 h (B) post-transfection. Recombinant apoptin detected by anti-V5-FITC antibody is shown in green and cell nuclei stained by PI in red. Apoptin protein showed a diffuse pattern in the cytoplasm at 24 h post-transfection, and in the nucleus at 48 h.

### Flow cytometry

The loss of cell membrane asymmetry in apoptotic cells was determined by using an Annexin-V FITC Apoptosis Assay Kit (Keygen, China). Apoptin-expressing cells were stained with annexin-V FITC as green fluorescent cells, and the nuclei of the apoptotic cells were stained by PI with red fluorescence. After staining, the cell suspensions were analyzed on a Cytometric FC500 flow cytometer, and  $10^5$  events were collected for each sample. Viable cells were defined as annexin-V FITC and PI double-negative events.

### Cell viability assay

Cell viability was also determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay which measures mitochondrial respiratory function<sup>[17,18]</sup>. Exponentially growing cells were plated in 96-well flat bottom plates (Corning, USA) and allowed to adhere for 24 h. At various times post-transfection with the recombinant plasmid, cells were incubated with MTT dye (1 mg/mL) for 2 h and solubilized with 20  $\mu$ L 10% SDS.  $A_{560}$  was then measured.

### DNA laddering assay

DNA fragmentation was detected using an Apoptotic DNA Laddering Kit (Keygen) according to the manufacturer's instructions. DNA was extracted, separated by 1.5% agarose gel electrophoresis, followed by ethidium bromide staining to visualize the ladder DNA.

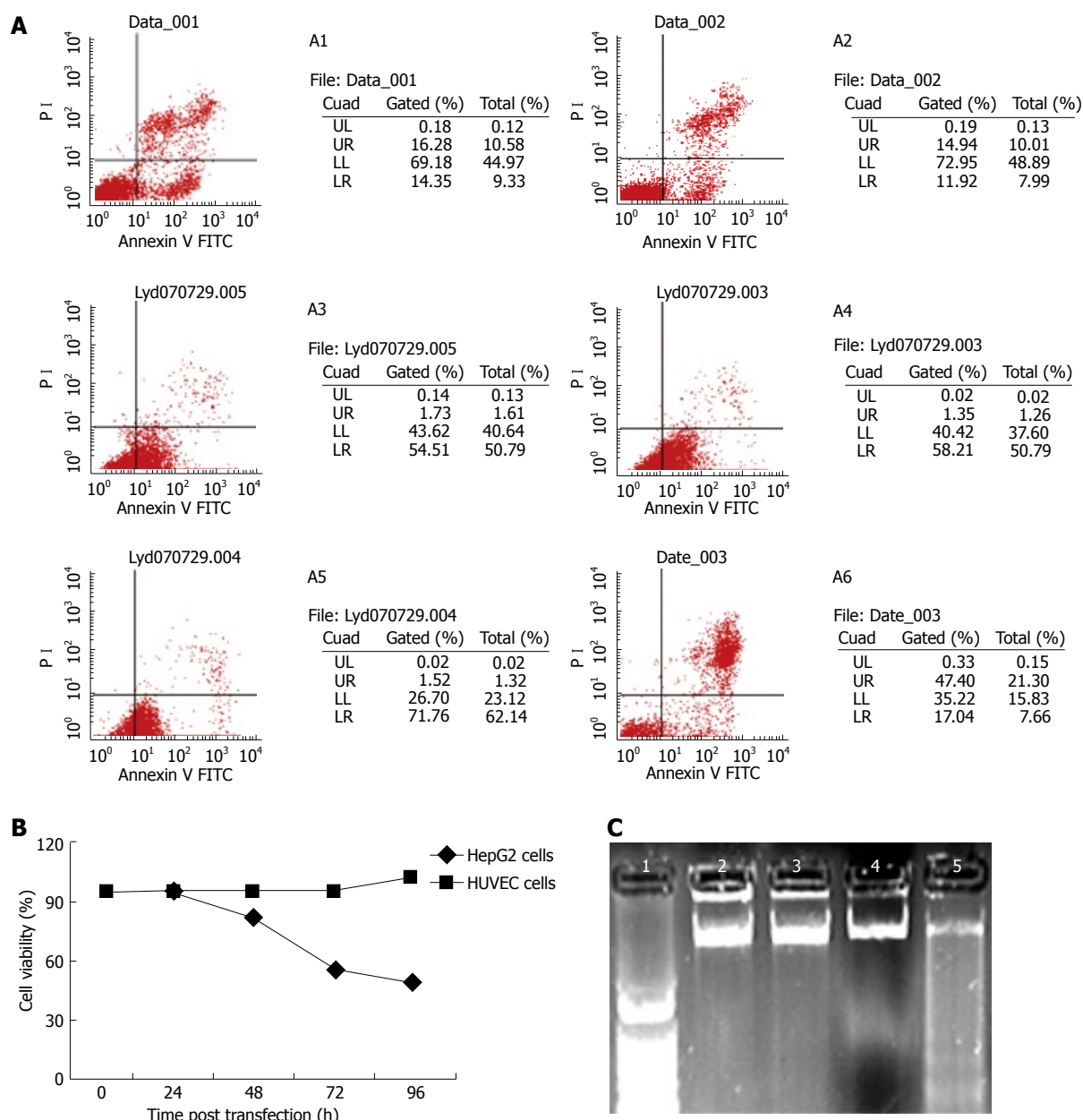
### Immunocytochemical assay and DAPI staining

At various times post-transfection, cells grown in six-well plates were harvested and treated by trypsinization and resuspended in PBS. The cells were then spread on a slide, fixed by 100% methanol for 5 min at room temperature, stained by the Apoptotic/Necrotic Cell Detection Kit (Keygen), and embedded in resin, after permeabilization, for long-term storage.

Cytotoxicity of SP-TAT was also tested by 2,4-diamidino-2-phenylindole (DAPI) staining. Transfected cells grown on coverslips that were placed on the bottom of a 24-well plate were washed with PBS and fixed, and apoptotic cells were differentiated by staining with mounting medium containing DAPI, and visualized using an Olympus AX70 fluorescence microscope.

### Protein secretion and activity test

CHO cells were cultured in a six-well plate. The cells were transfected with the pLenti6/V5-D-TOPO/SP-TAT-apoptin plasmid using the Lipofectamine<sup>TM</sup> 2000 protocol according to manufacturer's instructions (Invitrogen). After 6 h incubation, the cells were washed with fresh culture medium and cultured for an additional 24 h. The culture supernatants were then collected and added respectively to the monolayers of HepG2 cells and HUVECs grown in 24-well plates. These cells had also been washed with PBS before adding the supernatants. At various times post co-culture, these cells were fixed and stained, and apoptin localization and apoptosis were analyzed.



**Figure 2** SP-TAT-apoptin-induced cell death. **A:** Cell viability measured by flow cytometry. **A1 and A2:** HUVECs at 48 and 72 h post-transfection; **A3-A6:** HepG2 cells at 24, 48, 72 and 96 h. HepG2 cells were susceptible to SP-TAT-apoptin-induced apoptosis in a time-dependent manner; **B:** Cell viability determined by MTT dye reduction assay; **C:** DNA fragmentation in HepG2 cells demonstrated by agarose gel electrophoresis. Lane 1: 1 kb DNA marker; Lanes 2-5: DNA from cells at 24, 48, 60 and 72 h post-transfection, respectively.

### Statistical analysis

ANOVA was performed for multiple group comparison. In conjunction with ANOVA, post hoc pairwise comparisons were performed by Bonferroni's test, with  $P < 0.05$  regarded as statistically significant.

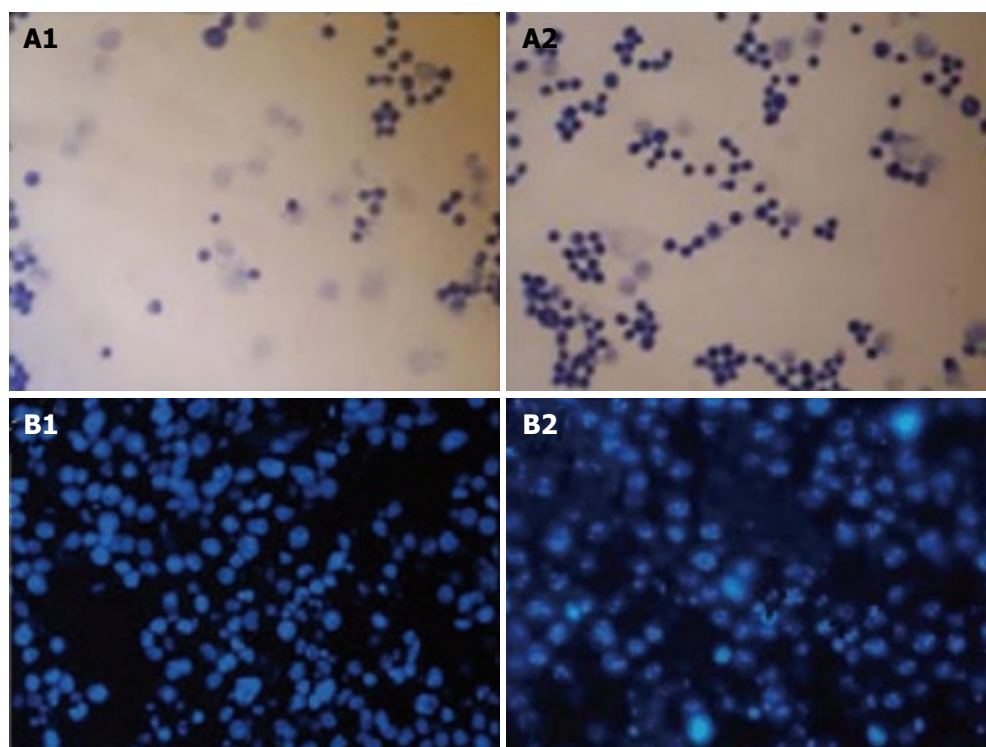
## RESULTS

### Generation of SP-TAT-apoptin fusion construct and expression of SP-TAT-apoptin

The human secretory SP was constructed and optimized virtually by the HMM, which has been used to describe, predict, identify, and generate secretory SP sequences<sup>[13]</sup>. It was inserted at the N terminus of recombinant TAT-

apoptin to generate SP-TAT-apoptin fusion protein, and it contained a positively charged N region, a hydrophobic central region, and a C region that contained a cleavage site. Two rounds of PCR were carried out to amplify the apoptin gene and to fuse TAT and the synthetic SP into the construct to create recombinant secretory-TAT-apoptin.

To determine whether the SP-TAT-apoptin cDNA construct generated was expressed *in vivo*, the HepG2 cell line was transfected with the plenti6/V5-D-TOPO/SP-TAT-apoptin plasmid. Analysis by RT-PCR revealed that SP-TAT-apoptin was expressed 24 h after transfection (data not shown). The expression of SP-TAT-apoptin in HepG2 cells was confirmed by immunofluorescence microscopy (Figure 1).



**Figure 3** Cytotoxicity of SP-TAT-apoptin compared to SP-TAT. **A:** Micrographs of HepG2 cells transfected with SP-TAT-apoptin construct stained by Apoptotic/Necrotic Cell Detection Kit. An inverted microscope ( $\times 400$ ) was used. The nuclei of apoptotic cells were stained deep blue. **A1** and **A2:** HepG2 cells at 24 and 72 h post-transfection; **B:** HepG2 cells stained with DAPI and observed by fluorescence microscopy ( $\times 400$ ). **B1:** HepG2 cells 72 h after transfection with plenti6/V5-D-TOPO/SP-TAT-GFP plasmid; **B2:** HepG2 cells 72 h after transfection with plenti6/V5-D-TOPO/SP-TAT-apoptin. Arrow indicates apoptotic cells.

### Induction of apoptosis by SP-TAT-apoptin

To determine whether SP-TAT-apoptin induces apoptosis and also maintains its tumor cell specificity, HUVECs and HepG2 cells were transfected with the plenti6/V5-D-TOPO/SP-TAT-apoptin plasmid. SP-TAT-apoptin-induced apoptosis was investigated in these two cell lines. Three different assays were used to gauge apoptosis. In the first assay, cell viability was measured by co-staining with annexin-V FITC and PI, followed by flow cytometry. This assay was based on the loss of plasma membrane asymmetry (integrity) as a result of apoptosis. Viable cells were defined as annexin-V FITC and PI double-negative events. As shown in Figure 2A, only HepG2 cells were susceptible to SP-TAT-apoptin-induced apoptosis in a time-dependent manner. These results also confirm previous reports that apoptin has the ability to induce apoptosis specifically in tumor cells<sup>[3,4,7,19,20]</sup>. In the second assay, cells that were exponentially grown were inoculated in 96-well flat-bottom plates and allowed to adhere for 24 h. After transfection, cell viability was determined at various times by MTT dye reduction assay. Expression of the recombinant protein slightly decreased the viability of HepG2 cells at 24 h post-transfection, and the same was true in HUVECs (Figure 2B). At 48 h post-transfection, the viability of HUVECs was only slightly decreased compared to that at 24 h. However, the viability of HepG2 cells was significantly decreased at 48 h post-transfection. The presence of recombinant apoptin caused a decrease in HepG2 cell viability to

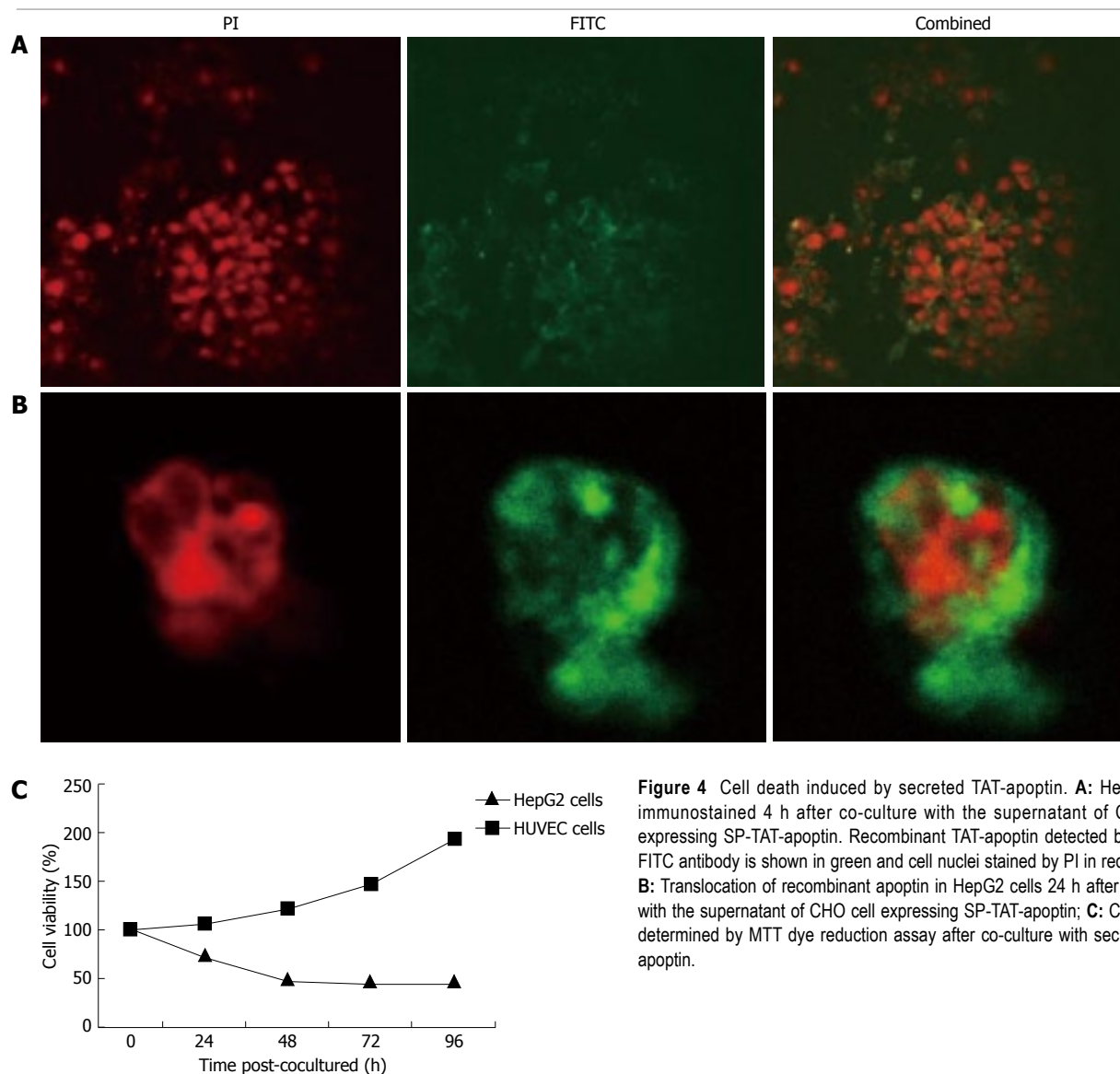
$< 60\%$  at 72 h and  $< 50\%$  at 96 h post-transfection (Figure 2B). In the third assay, genomic DNA fragmentation in HepG2 cells and HUVECs was investigated. As shown in Figure 2C, the apoptin fusion protein brought about significant DNA fragmentation in HepG2 cells at 72 h post-transfection. In contrast, detectable apoptotic DNA laddering in HUVECs was not seen during the experiment (data not shown).

### Lack of cytotoxicity of SP-TAT

In order to determine the cytotoxicity of SP-TAT, plenti6/V5-D-TOPO/SP-TAT-apoptin plasmid and plenti6/V5-D-TOPO/SP-TAT-GFP plasmids were transfected into HepG2 cells separately. Robust apoptosis of HepG2 cells was observed, as demonstrated by microscopy at different times after transfection, while in contrast, expression of SP-TAT-GFP did not induce noticeable apoptosis in these cells (Figure 3). Therefore, SP-TAT did not seem to exhibit cytotoxicity in HepG2 cells and apoptosis induced by SP-TAT-apoptin was due to apoptin.

### Secretion of SP-TAT-apoptin and the effect of secreted TAT-apoptin on HepG2 cells

The presence of synthesized SP enables TAT-apoptin to be secreted outside the transfected cells and re-enter adjacent un-transfected HepG2 cells, potentially increasing the efficacy of apoptin when used as cancer therapy. To test its feasibility, the recombinant construct was used to transfect CHO cells and the culture supernatant was collected. HepG2 cells and HUVECs



**Figure 4** Cell death induced by secreted TAT-apoptin. **A:** HepG2 cells immunostained 4 h after co-culture with the supernatant of CHO cells expressing SP-TAT-apoptin. Recombinant TAT-apoptin detected by anti-V5-FITC antibody is shown in green and cell nuclei stained by PI in red ( $\times 1000$ ); **B:** Translocation of recombinant apoptin in HepG2 cells 24 h after co-culture with the supernatant of CHO cell expressing SP-TAT-apoptin; **C:** Cell viability determined by MTT dye reduction assay after co-culture with secreted TAT-apoptin.

were then co-cultured with this supernatant. At 24 h post co-culture, TAT-apoptin had translocated from the cytoplasm to the nucleus (Figure 4A). At various times post co-culture, cell viability was also determined by MTT dye reduction assay. As shown in Figure 4B, the recombinant protein TAT-apoptin slightly decreased cell viability of HepG2 cells at 24 h post co-culture. In contrast, the viability of HUVECs was actually increased at 24 h, which continued during the course of the experiment. At 48 h post co-culture, viability of HepG2 cells was significantly decreased compared to that of HUVECs. The recombinant apoptin protein decreased HepG2 cell viability to  $< 50\%$  at 48 h post-transfection.

## DISCUSSION

New therapeutic approaches that facilitate selective targeting of cancer cells while sparing normal cells have emerged in recent years. Apoptin represents a new anti-cancer tool in such new approaches with great potential<sup>[21-25]</sup>. Two routes can be taken using apoptin or its encoding cDNA, i.e. as protein or gene therapy. In any case, efficient systems are required to facilitate

the delivery of apoptin to cancer cells or expression of apoptin within these cells<sup>[26-30]</sup>. The HIV TAT transduction domain has been successfully used to deliver apoptin into cancer cells<sup>[14]</sup>, and no apoptosis of normal cells (HUVECs) was observed with this TAT-apoptin fusion protein. In this study, we generated a cDNA construct of SP-TAT-apoptin. Cancer cells transfected with this construct expressed recombinant apoptin and apoptosis was induced. By incorporating a synthetic SP we also expected apoptin to be secreted from the transfected cells as TAT-apoptin fusion protein and re-enter adjacent untransfected HepG2 cells, which enabled the construct to act as both a protein and gene therapeutic agent, and increased the potency of apoptin in cancer therapy.

SP-TAT-apoptin was expressed in HUVECs and HepG2 cells, and the protein was initially located in the cytoplasm. At 48 h post-transfection, the protein was located in the nucleus of HepG2 cells, which indicated that SP-TAT-apoptin was capable of translocating to the nucleus. SP-TAT-apoptin was also functionally active and efficiently induced HepG2 cell apoptosis, in a time-dependent manner. In HUVECs, SP-TAT-



apoptin remained in the cytoplasm and no induction of apoptosis above the background level was observed. Meanwhile, no apoptosis was observed in cells in which SP-TAT-GFP was expressed, which indicates that SP-TAT alone is not cytotoxic for HepG2 cells. Therefore, SP-TAT-apoptin retained the characteristic expression pattern of apoptin and induced apoptosis in cancer cells.

Having a synthetic SP, recombinant apoptin was able to be secreted from transfected cells and re-enter adjacent untransfected HepG2 cells. The recombinant protein was detected in the cytoplasm in HepG2 cells and HUVECs shortly after co-culture of the cells with the cell-free supernatant of the transfected CHO cells. This indicated that the secreted TAT-apoptin fusion protein contained in the CHO cell culture medium was able to enter these cells. The fusion protein was later found in the nucleus of HepG2 cells and induced HepG2 apoptosis. The new secretory characteristic increased the possibility of apoptin being used in cancer gene therapy. However, there are still a large number of unanswered questions regarding the mechanisms and therapeutic usage of apoptin, and further studies are certainly required.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Apoptin is a protein encoded by Constant Angular Velocity (CAV) and it can cause apoptotic cell death. It has been shown to possess a striking specificity for cancer cells. Apoptin, therefore, has great potential for efficient targeting and specific elimination of cancer cells.

### Research frontiers

Human Immunodeficiency Virus (HIV)-Transactivating Transcription (TAT)-fused apoptin has been shown to possess a striking specificity for cancer cells. However, the cancer killing activity is limited in cells transfected with the apoptin expression construct, which spares the untransfected cancer cells. A secretory TAT-apoptin fusion protein with a secretory signal has an additive by-stander effect as an anti-cancer therapy. Secreted TAT-apoptin from transformed cells enters un-transformed cancer cells and causes apoptosis.

### Innovations and breakthroughs

The new secretory characteristic increased the possibility of apoptin being used in cancer gene therapy. However, there are still a large number of unanswered questions regarding the mechanisms and therapeutic usage of apoptin, and further studies are certainly required.

### Terminology

Apoptin or VP3 is a protein of 13.6 kDa derived from CAV, represents a new anti-cancer tool with great potentials. It appears to have innate tumor-specific, p53-independent, Bcl-2-enhanced pro-apoptotic activity.

### Peer review

The authors investigated the role of secretory TAT-apoptin fusion protein in HepG2 cells. They conclude that such a protein induces apoptosis in HCC cell lines, but not in non-cancer cell line HUVEC. This is a very interesting study, which may be applicable for the treatment of human liver cancer in the future.

## REFERENCES

- 1 Noteborn MH. Chicken anemia virus induced apoptosis: underlying molecular mechanisms. *Vet Microbiol* 2004; **98**:

- 89-94
- 2 Olijslagers SJ, Zhang YH, Backendorf C, Noteborn MH. Additive cytotoxic effect of apoptin and chemotherapeutic agents paclitaxel and etoposide on human tumour cells. *Basic Clin Pharmacol Toxicol* 2007; **100**: 127-131
- 3 Janssen K, Hofmann TG, Jans DA, Hay RT, Schulze-Osthoff K, Fischer U. Apoptin is modified by SUMO conjugation and targeted to promyelocytic leukemia protein nuclear bodies. *Oncogene* 2007; **26**: 1557-1566
- 4 Burek M, Maddika S, Burek CJ, Daniel PT, Schulze-Osthoff K, Los M. Apoptin-induced cell death is modulated by Bcl-2 family members and is Apaf-1 dependent. *Oncogene* 2006; **25**: 2213-2222
- 5 Russo A, Terrasi M, Agnese V, Santini D, Bazan V. Apoptosis: a relevant tool for anticancer therapy. *Ann Oncol* 2006; **17** Suppl 7: vii115-vii123
- 6 Liu X, Elojeimy S, El-Zawahry AM, Holman DH, Bielawska A, Bielawski J, Rubinchik S, Guo GW, Dong JY, Keane T, Hannun YA, Tavassoli M, Norris JS. Modulation of ceramide metabolism enhances viral protein apoptin's cytotoxicity in prostate cancer. *Mol Ther* 2006; **14**: 637-646
- 7 Maddika S, Mendoza FJ, Hauff K, Zamzow CR, Paranjthy T, Los M. Cancer-selective therapy of the future: apoptin and its mechanism of action. *Cancer Biol Ther* 2006; **5**: 10-19
- 8 Peng DJ, Sun J, Wang YZ, Tian J, Zhang YH, Noteborn MH, Qu S. Inhibition of hepatocarcinoma by systemic delivery of Apoptin gene via the hepatic asialoglycoprotein receptor. *Cancer Gene Ther* 2007; **14**: 66-73
- 9 Wang QM, Fan GC, Chen JZ, Chen HP, He FC. A putative NES mediates cytoplasmic localization of Apoptin in normal cells. *Acta Biochim Biophys Sin (Shanghai)* 2004; **36**: 817-823
- 10 Hashida H, Miyamoto M, Cho Y, Hida Y, Kato K, Kurokawa T, Okushiba S, Kondo S, Dosaka-Akita H, Katoh H. Fusion of HIV-1 Tat protein transduction domain to poly-lysine as a new DNA delivery tool. *Br J Cancer* 2004; **90**: 1252-1258
- 11 Kubo E, Fatma N, Akagi Y, Beier DR, Singh SP, Singh DP. TAT-mediated PRDX6 protein transduction protects against eye lens epithelial cell death and delays lens opacity. *Am J Physiol Cell Physiol* 2008; **294**: C842-C855
- 12 Song HY, Lee JA, Ju SM, Yoo KY, Won MH, Kwon HJ, Eum WS, Jang SH, Choi SY, Park J. Topical transduction of superoxide dismutase mediated by HIV-1 Tat protein transduction domain ameliorates 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced inflammation in mice. *Biochem Pharmacol* 2008; **75**: 1348-1357
- 13 Ziegler A, Seelig J. Interaction of the protein transduction domain of HIV-1 TAT with heparan sulfate: binding mechanism and thermodynamic parameters. *Biophys J* 2004; **86**: 254-263
- 14 Guelen L, Paterson H, Gaken J, Meyers M, Farzaneh F, Tavassoli M. TAT-apoptin is efficiently delivered and induces apoptosis in cancer cells. *Oncogene* 2004; **23**: 1153-1165
- 15 Barash S, Wang W, Shi Y. Human secretory signal peptide description by hidden Markov model and generation of a strong artificial signal peptide for secreted protein expression. *Biochem Biophys Res Commun* 2002; **294**: 835-842
- 16 Sun GJ, Tong X, Sun ZX. Gene clone and activity assay of apoptin. *Junshi Yixue Kexueyuan Yuankan* 2001; **2**: 85-87
- 17 Hayon T, Dvilansky A, Shpilberg O, Nathan I. Appraisal of the MTT-based assay as a useful tool for predicting drug chemosensitivity in leukemia. *Leuk Lymphoma* 2003; **44**: 1957-1962
- 18 Kubota T. (Cancer chemosensitivity test-from laboratory to clinic) *Hum Cell* 1995; **8**: 189-194
- 19 Lee YH, Cheng CM, Chang YF, Wang TY, Yuo CY. Apoptin T108 phosphorylation is not required for its tumor-specific nuclear localization but partially affects its apoptotic activity. *Biochem Biophys Res Commun* 2007; **354**: 391-395
- 20 Olijslagers SJ, Zhang YH, Backendorf C, Noteborn MH.

- Additive cytotoxic effect of apoptin and chemotherapeutic agents paclitaxel and etoposide on human tumour cells. *Basic Clin Pharmacol Toxicol* 2007; **100**: 127-131
- 21 **Danen-Van Oorschot AA**, Zhang YH, Leliveld SR, Rohn JL, Seelen MC, Bolk MW, Van Zon A, Erkeland SJ, Abrahams JP, Mumberg D, Noteborn MH. Importance of nuclear localization of apoptin for tumor-specific induction of apoptosis. *J Biol Chem* 2003; **278**: 27729-27736
- 22 **Alvisi G**, Poon IK, Jans DA. Tumor-specific nuclear targeting: promises for anti-cancer therapy? *Drug Resist Updat* 2006; **9**: 40-50
- 23 **Poon IK**, Oro C, Dias MM, Zhang J, Jans DA. Apoptin nuclear accumulation is modulated by a CRM1-recognized nuclear export signal that is active in normal but not in tumor cells. *Cancer Res* 2005; **65**: 7059-7064
- 24 **He X**, Zhang Q, Liu Y, He P. Apoptin Induces Chromatin Condensation in Normal Cells. *Virus Genes* 2005; **31**: 49-55
- 25 **Gdynia G**, Lehmann-Koch J, Sieber S, Tagscherer KE, Fassl A, Zentgraf H, Matsuzawa S, Reed JC, Roth W. BLOC1S2 interacts with the HIPPI protein and sensitizes NCH89 glioblastoma cells to apoptosis. *Apoptosis* 2008; **13**: 437-447
- 26 **Maddika S**, Wiehce E, Ande SR, Poon IK, Fischer U, Wesselborg S, Jans DA, Schulze-Osthoff K, Los M. Interaction with PI3-kinase contributes to the cytotoxic activity of apoptin. *Oncogene* 2008; **27**: 3060-3065
- 27 **Maddika S**, Bay GH, Krocak TJ, Ande SR, Maddika S, Wiehce E, Gibson SB, Los M. Akt is transferred to the nucleus of cells treated with apoptin, and it participates in apoptin-induced cell death. *Cell Prolif* 2007; **40**: 835-848
- 28 **Backendorf C**, Visser AE, de Boer AG, Zimmerman R, Visser M, Voskamp P, Zhang YH, Noteborn M. Apoptin: therapeutic potential of an early sensor of carcinogenic transformation. *Annu Rev Pharmacol Toxicol* 2008; **48**: 143-169
- 29 **Schoop RA**, Kooistra K, Baatenburg De Jong RJ, Noteborn MH. Bcl-xL inhibits p53- but not apoptin-induced apoptosis in head and neck squamous cell carcinoma cell line. *Int J Cancer* 2004; **109**: 38-42
- 30 **Maddika S**, Mendoza FJ, Hauff K, Zamzow CR, Paranjothy T, Los M. Cancer-selective therapy of the future: apoptin and its mechanism of action. *Cancer Biol Ther* 2006; **5**: 10-19

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CLINICAL RESEARCH

## Partial overlap of anti-mycobacterial, and anti-*Saccharomyces cerevisiae* mannan antibodies in Crohn's disease

Stefan Müller, Thomas Schaffer, Alain M Schoepfer, Annamarie Hilty, Thomas Bodmer, Frank Seibold

Stefan Müller, Thomas Schaffer, Alain M Schoepfer, Frank Seibold, Department of Clinical Research, Division of Gastroenterology, University of Bern, Bern CH-3010, Switzerland

Stefan Müller, Thomas Schaffer, Annamarie Hilty, Thomas Bodmer, Institute for Infectious Diseases, University of Bern, Bern CH-3010, Switzerland

**Author contributions:** Müller S and Schaffer T contributed equally to this work; Müller S contributed to the study design, planned experiments, carried through the animal part of the study and ELISAs with affinity purified sera, and wrote the paper; Schaffer T screened patients' sera by ELISA, performed neutralization experiments and affinity purifications; Schoepfer AM provided clinical data and performed statistic analyses; Hilty A, and Bodmer T prepared and supervised mycobacterial cultures; and Seibold F had the original idea and designed the study.

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**Correspondence to:** Frank Seibold, Department of Gastroenterology, Inselspital Bern, University Hospital, Freiburgstrasse 10, Bern CH-3010, Switzerland. [frank.seibold@insel.ch](mailto:frank.seibold@insel.ch)

Telephone: +41-31-6328025 Fax: +41-31-63297 65

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of CD patients but only 0%-6% of controls were seropositive against different mycobacterial antigens. Anti-mycobacterial IgG correlated with ASCA ( $r = 0.37-0.64$ ;  $P = 0.003-P < 0.001$ ). ASCA-positivity and deficiency for mannan-binding lectin synergistically associated with anti-mycobacterial IgG. In some patients, anti-mycobacterial antibodies represent cross-reactive ASCA. Vice-versa, the predominant fraction of ASCA did not cross-react with mycobacteria. Finally, fistulizing disease associated with antibodies against *M avium*, *M smegmatis* and MAP ( $P = 0.024$ ,  $0.004$  and  $0.045$ , respectively).

**CONCLUSION:** Similar to ASCA, seroreactivity against mycobacteria may define CD patients with complicated disease and a predisposition for immune responses against ubiquitous antigens. While in some patients anti-mycobacterial antibodies strongly cross-react with yeast mannan; these cross-reactive antibodies only represent a minor fraction of total ASCA. Thus, mycobacterial infection unlikely plays a role in ASCA induction.

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**Key words:** Crohn's disease; Anti-mycobacterial antibodies; Anti-*Saccharomyces cerevisiae* antibodies; Cross-reactivity; Mannan; Lipoarabinomannan

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### Abstract

**AIM:** To test whether humoral immune reaction against mycobacteria may play a role in anti-*Saccharomyces cerevisiae* antibodies (ASCA) generation in Crohn's disease (CD) and/or whether it correlates with clinical subtypes.

**METHODS:** The dominant ASCA epitope was detected by *Galanthus nivalis* lectin (GNL)-binding assay. ASCA and IgG against mycobacterial lysates [*M avium*, *M smegmatis*, *M chelonae*, *M bovis* BCG, *M avium ssp. paratuberculosis* (MAP)] or purified lipoarabinomannans (LAM) were detected by ELISA. ASCA and anti-mycobacterial antibodies were affinity purified to assess cross-reactivities. Anti-mycobacterial IgG were induced by BCG-infection of mice.

**RESULTS:** GNL bound to different extents to mycobacterial lysates, abundantly to purified mannose-capped (Man) LAM from *M tuberculosis*, but not to uncapped LAM from *M smegmatis*. Fifteen to 45%

### INTRODUCTION

Crohn's disease (CD) is a multifactorial disease that affects genetically susceptible hosts. The exact pathogenesis is still largely unknown. However, it is generally accepted that the disease, once established,

is driven by antigens of the intestinal flora, reflecting a loss of tolerance against commensal microorganisms<sup>[1,2]</sup>. The hypothesis that genetic predisposition, together with unfavorable environmental and commensal triggers cause CD with its various phenotypes contradicts the highly controversial idea of a single infectious origin of the disease<sup>[3]</sup>.

A number of serological markers have been detected that have a certain degree of specificity and sensitivity for CD<sup>[4,5]</sup>. Of the most intriguing antibodies are those directed against outer cell wall mannans of the baker's yeast *Saccharomyces cerevisiae* (anti-*Saccharomyces cerevisiae* antibodies, ASCA)<sup>[6-9]</sup>. These antibodies are found in more than 50% of CD patients, but rarely in healthy controls or patients with ulcerative colitis (UC)<sup>[8]</sup>. Yeasts are ubiquitous and ingested on a daily basis. Why an organism that, with a few reported exceptions of virulent mutants<sup>[10]</sup>, is not adapted to live or even grow in the human body elicits a strong IgG response in CD patients has not yet been conclusively answered. A recent report presented experimental data supporting the idea that the facultative opportunistic pathogen *Candida albicans* may be the inducer of ASCA<sup>[11]</sup>. However, our recent study showed that ASCA and anti-*C. albicans* antibodies correlate to a lower degree than ASCA with antibodies to mannans from other ubiquitous yeasts<sup>[12]</sup>. Thus, whether *C. albicans* infection may indeed represent the dominant trigger for ASCA cannot be definitively answered so far and there may be other cross-reactivities that play a role in ASCA induction. Potential candidates are mycobacteria since their cell wall contains lipoarabinomannans with similar mannose side chains as the cell wall mannans of yeast. The exact epitope recognized by ASCA has been demonstrated to be an  $\alpha$ -1,3 mannose-( $\alpha$ -1,2 mannose)<sub>n</sub> with  $n = 2$  or 3 by two independent studies<sup>[9,13]</sup>. Similar or equal oligo-mannose motives are found in other yeasts, as well as in the mannosylated side chains of mycobacterial lipoarabinomannans (LAM)<sup>[14,15]</sup>. Part of this motif, the terminal  $\alpha$ -1,3 linked mannose, can be detected by the *Galanthus nivalis* lectin (GNL)<sup>[16,17]</sup> and has been shown to be present in the lipo(arabino)mannan of *M. chelonae*<sup>[18]</sup>. Two other publications have demonstrated presence of the GNL-reactive motif in some mycobacterial species, including *M. bovis*, *M. avium* and *Mycobacterium avium* ssp. paratuberculosis (MAP)<sup>[19,20]</sup>. Hence, we were interested whether ASCA-positive CD patients may also more frequently contain antibodies against distinct mycobacterial strains and specifically against LAM, and whether these antibodies would be of cross-reactive nature.

In the case of MAP studies have shown a very high (77%-87%) prevalence of seroreactivity against the MAP antigens p35 and p36 in CD<sup>[21,22]</sup>. While this is intriguing, the interest in the possible relationship between CD and MAP mainly comes from the fact that Johne's disease in cattle which is caused by MAP infection in some aspects resembles CD.

The acute phase reactant mannose-binding lectin (MBL) specifically binds to mannose residues and is an

important first line of defense innate immune effector molecule<sup>[23-26]</sup>. We have previously shown that deficiency for MBL associates with the ASCA-positive subgroup of CD patients<sup>[27,28]</sup>. Thus, it was of interest, if in CD, deficiency for MBL might associate with elevated levels of anti-mycobacterial IgG as well. Finally, we correlated our findings regarding anti-mycobacterial antibodies with different clinical CD phenotypes.

## MATERIALS AND METHODS

### Patients and sera

Sera from 105 patients with CD, 45 patients with UC and 35 healthy controls were obtained with informed consent and with the approval of the local ethical authorities. Diagnosis of CD and UC was established by endoscopic, histological and clinical criteria. CD: 54 women and 51 men, mean age 40 years (19-73); UC: 22 women and 23 men, mean age 38 years (20-65); healthy controls: 21 women and 14 men, mean age 37 years (18-67). Disease activity was graded for CD according to the CD activity index (CAI), or for UC according to the Mayo-score<sup>[29,30]</sup>. At the time of blood collection, CD patients were treated as follows: no medical treatment ( $n = 29$ ), 5-ASA ( $n = 4$ ), steroids ( $n = 8$ ), antibiotics ( $n = 3$ ), infliximab ( $n = 4$ ), or immunomodulators such as 6-mercaptopurine or azathioprine (6-MP/aza,  $n = 47$ ), or methotrexate ( $n = 20$ ). Some patients had combined medication: infliximab + methotrexate ( $n = 1$ ), steroids + methotrexate ( $n = 4$ ), steroids + 6-MP/aza ( $n = 3$ ), infliximab + 6-MP/aza ( $n = 2$ ). CAI ranged between 75 and 380 (mean: 138 points). UC patients were treated as follows: no medical treatment ( $n = 9$ ), 5-ASA ( $n = 15$ ), steroids ( $n = 6$ ), immunomodulators 6-MP/aza ( $n = 20$ ), or tacrolimus ( $n = 2$ ). Some of these patients had combined medication: 5-ASA + 6-MP/aza ( $n = 4$ ), steroids + 6-MP/aza ( $n = 3$ ). CD patients were grouped according to the Montreal classification<sup>[31]</sup> into a UC-like (purely inflammatory, non-stenosing, non-fistulizing), a stenosing, or a fistulizing phenotype. The latter two are summarized as complicated disease phenotype.

### Mycobacteria, yeast and mannan

Mycobacterial strains used in this study were *M. avium*, *M. smegmatis*, *M. chelonae*, MAP (all strains are patients' isolates and property of the Institute of Infectious Diseases, University of Bern), and BCG (commercial vaccination strain). Strains were grown in Middlebrook 7H9 medium (Difco™ BBL™, BD Biosciences, San Jose, CA, USA) with 10% OADC enrichment (Difco™ BBL™, BD Biosciences), 1 mg/mL casein peptone (Merck, Glattbrugg, Switzerland) and 0.5% glycerin, and were collected during logarithmic growth phase. For MAP cultures, growth medium was supplemented with 2 mg/mL Mycobactin J (Synbiotics Europe SAS, Munich, Germany). Bacteria were heat-inactivated for 1 h at 80°C and then lysed in the presence of trypsin inhibitor and PMSF (Sigma, Buchs, Switzerland) by needle sonication (2 × 1 min. with 100 watts, on ice). The lysate was cleared by centrifugation and protein concentration determined.



Mannose-capped lipoarabinomannan (ManLAM) from *M. tuberculosis* and (non-mannose-capped) phosphomyo-inositol-capped LAM (PILAM, subsequently termed LAM) were kindly provided by J. Belisle, Colorado State University. *S. cerevisiae* Vita Gold was obtained from Deutsche Hefewerke, Nürnberg, Germany. The yeast was grown in 5% yeast peptone D-glucose (YPD, Sigma) and harvested during logarithmic growth. Mannan was extracted according to Kocourek and Balou<sup>[32]</sup>. Briefly, cells were resuspended in 20 mmol/L Na-citrate buffer (pH 7.0) and autoclaved for 3 h at 125°C. The supernatant was cleared by centrifugation and the pellet resuspended in Na-citrate buffer (1.5 × initial volume). After centrifugation, the supernatants were pooled and cleared from residual debris by centrifugation. The mannans were complexed with equal volumes of Fehling's solutions (Fehling I: 6.93 g CuSO<sub>4</sub> · 5 H<sub>2</sub>O/100 mL in H<sub>2</sub>O; Fehling II: 34.6 g C<sub>4</sub>H<sub>4</sub>KNaO<sub>6</sub> · 4H<sub>2</sub>O + 12 g NaOH/100 mL in H<sub>2</sub>O) and pelleted by centrifugation. The pellet was resolved in 3 mol/L HCl before precipitating the mannans off the CuSO<sub>4</sub> complex with a methanol/acetic acid 8:1 v/v solution while stirring. The precipitate was pelleted and washed repeatedly with methanol/acetic acid 8:1 v/v until the supernatant was colorless and clear and finally washed twice with methanol alone and dried in a desiccator at 4°C.

## ELISA

ELISA was performed in Nunc-Immuno™ Maxisorp 96-well plates (Nunc, Wiesbaden, Germany). For mycobacteria-specific ELISA, 50 µL of mycobacterial lysates, purified LAM or ManLAM at 5 µg/mL PBS was added per well and dried over night at 37°C. The next day plates were blocked with 1% skim milk in PBS. For ASCA, tetanus toxoid (TT) or galanthus nivalis lectin (GNL)-binding ELISA, plates were coated with the respective antigen at 0.25 µg/mL carbonate-bicarbonate (25 mmol/L Na<sub>2</sub>CO<sub>3</sub>, 25 mmol/L NaHCO<sub>3</sub>) coating buffer pH 9.6, overnight at 4°C. On the next day, plates were blocked with PBS + 0.5% BSA (PBS-BSA). Plates were washed and patients' sera added 1/500 (mycobacteria) or 1/1000 (ASCA, TT) PBS-BSA. Plates were incubated 1.5 h at room temperature (mycobacteria) or overnight at 4°C (ASCA, TT). For the GNL-binding ELISA, biotinylated GNL (Vector Laboratories, Burlingame, CA, USA) was added at 5 µg/mL to the blocked plates. Plates were washed with PBS-BSA + 0.05% Tween 20 (PBST-BSA). For ASCA and TT ELISA peroxidase-coupled anti-human IgG (Sigma) was added 1/5000 in PBST-BSA and plates incubated for 1 h at room temperature. The plates incubated with biotinylated GNL were further incubated with peroxidase-coupled streptavidin (BD Biosciences). After washing ELISA was developed with TMB substrate (Sigma) for 15-30 min. in the dark. Reaction was stopped with 0.5 mol/L sulphuric acid and plates read at 450 nm. A cut-off value discriminating between negativity and positivity for anti-mycobacterial IgG was defined using the average extinction values for the healthy population

(without the clearly positive individuals as defined by  $A_{450} \geq 0.3$ ) and addition of 3 standard deviations. To determine the MBL oligomer concentration sera were diluted 1/100 and assessed in an MBL-oligomer ELISA kit according to the manufacturer's instructions (The Antibodyshop, Gentofte, Denmark).

## Affinity purification of ASCA

*S. cerevisiae* mannan was separated on an 8% polyacrylamide gel. The separated mannan was transferred to Hybond-ECL nitrocellulose membrane (Amersham GE Healthcare Europe, Otelfingen, Switzerland) and the ASCA-reactive material was localized by Western blot on a small section of the membrane. The ASCA-reactive region of the remaining membrane was cut into 8 equal pieces and the pieces blocked in TBS + 2% BSA.

Eight highly ASCA-positive sera from Crohn's patients were diluted 1/5 in TBS + 2% BSA and incubated with one piece of the membrane overnight at 4°C, on a rocking platform. After incubation the membranes were thoroughly washed with TBS and 1× with H<sub>2</sub>O and bound antibodies eluted with 0.2 mol/L glycine, pH 2.8. The eluate was neutralized with Tris-base pH 8.0 and the eluted antibodies stabilized with 0.1% BSA.

## Affinity purification of anti-*M. smegmatis* antibodies

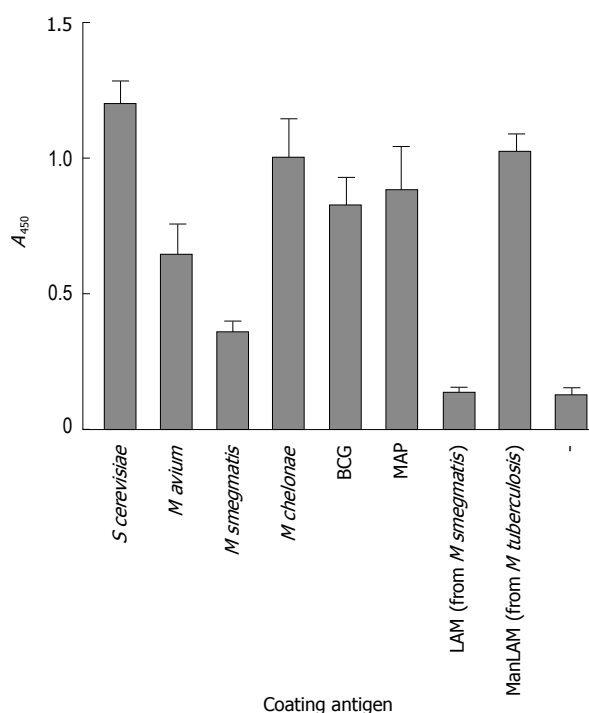
1 cm × 1 cm pieces of Hybond-ECL nitrocellulose membrane were decorated with *M. smegmatis* lysate and sequentially incubated overnight at 4°C and for 2 h at rt. Membranes were rinsed and blocked in TBS + 2% BSA. Incubation with sera and elution of bound antibodies was performed as described above.

## Immunization of mice

C57BL/6 mice were reared in individually ventilated cages (IVC) under specified pathogen-free conditions. Housing and experimental procedures were in accordance with the European regulations on animal experimentation (FELASA). BCG culture was adjusted to a density of  $A_{600} = 0.5$  MacFarland (10<sup>8</sup> cells per mL) with 0.9% sterile NaCl and 100 µL (10<sup>7</sup> viable bacteria) injected into the tail vein per mouse. Mice were 8 wk old and sex-matched. Viability of the mycobacteria was confirmed by reculturing the remaining bacterial suspension. Four weeks after infection serum was prepared from whole blood samples after clipping the tail tips. By week 5, the mice were boosted by iv with the same amount of mycobacteria and sera collected 4 and 13 wk later. Anti-BCG and ASCA IgM and IgG were determined by ELISA. To that end, samples were diluted 1/250 and anti mouse IgM and IgG HRP antibodies (Sigma) used 1/1000 and 1/500, respectively.

## Statistical analysis

Raw data were imported into a statistical package program (STATA Versiön 9.0, Texas). Results of numerical data are presented as mean ± SE. Categorical data are summarized as the percentage of the group total. Two-sided Fisher's exact test ( $n < 20$ ) or the Chi square test ( $n \geq 20$ ) was used to explore associations of



**Figure 1** Reactivity of *Galanthus nivalis* lectin (GNL) with mycobacterial lysates and purified LAM or ManLAM. ELISA plates were coated with yeast mannan, mycobacterial lysates, purified LAM or ManLAM or coating buffer alone without antigen (-), and binding of biotinylated GNL to these antigens assessed by further incubation with peroxidase-coupled streptavidin followed by TMB substrate reaction. Shown are mean values ± SE of 3 individual experiments.

categorical data in 2 independent groups. The Wilcoxon rank sum test was used to explore associations of numerical data in 2 independent groups. A  $P < 0.05$  was considered statistically significant. Associations between numerical data were evaluated using the Spearman rank correlation coefficient.

## RESULTS

### The dominant ASCA epitope terminal $\alpha$ -1,3 linked mannose is differentially present in lysates of different mycobacterial species

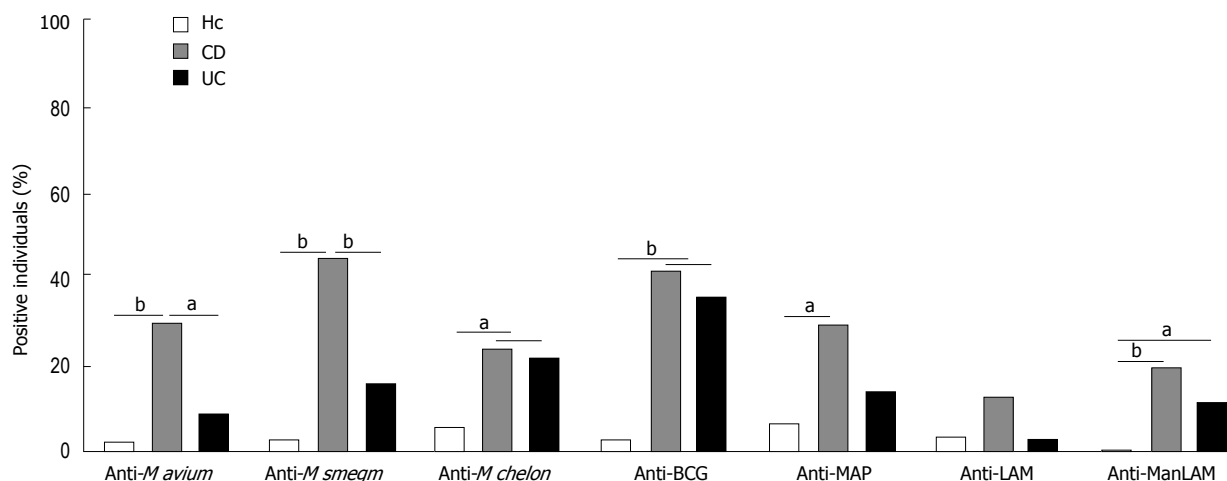
Phosphopeptidomannan with  $\alpha$ -1,3 mannose ( $\alpha$ -1,2 mannose  $\alpha$ -1,2 mannose)<sub>n</sub> ( $n = 1$  or  $2$ ) sugar residues represent a prominent epitope recognized by ASCA from CD patients<sup>[9,13]</sup>. The snowdrop lectin *Galanthus nivalis* agglutinin or lectin (GNL) has been shown to have high specificity for such terminal mannose sugar residues<sup>[16,17]</sup> and has been used to detect ASCA epitopes from microorganisms directly or in infected tissue<sup>[11,20]</sup>. After coating ELISA plates with mycobacterial lysates, purified LAM or ManLAM, or, as a positive control, yeast mannan, GNL differentially bound to these antigens. Besides yeast mannan, GNL strongly bound to lysate from *M chelonae* which is in perfect agreement with published observations<sup>[18]</sup>. Marked binding was further observed with lysates from BCG and MAP followed by *M avium* and *M smegmatis*. While no binding was observed to purified LAM, GNL strongly bound to ManLAM (Figure 1).

### Higher frequencies of anti-mycobacterial IgG-positive individuals among patients with IBD compared to healthy controls

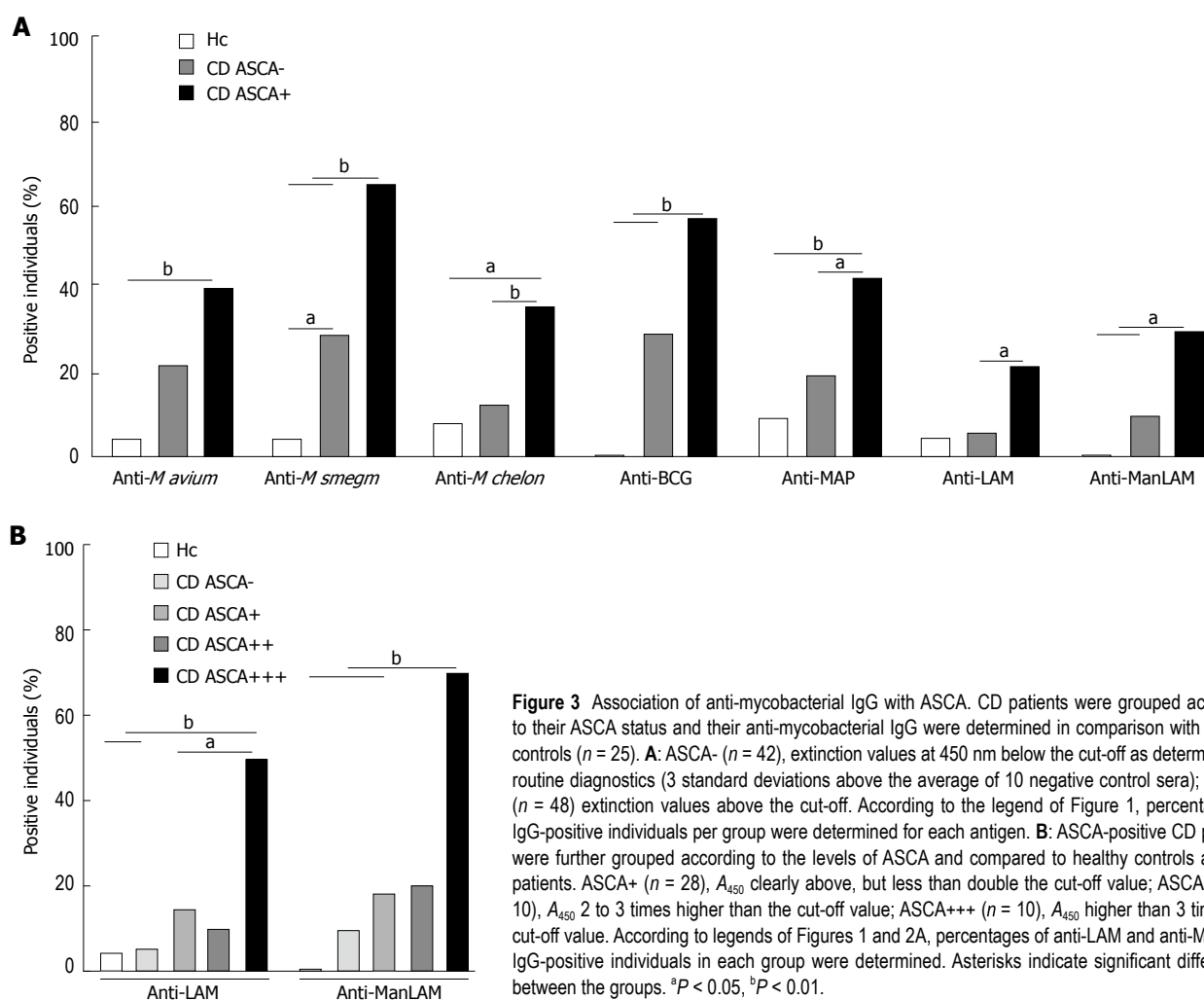
$A_{450}$  extinction values of the anti-mycobacterial ELISA obtained with sera from the healthy population were used to define cut-off values for anti-mycobacterial IgG as described in the materials and methods section. According to these cut-off levels, between 13% (anti-LAM) and 45% (anti-*M smegmatis*) of CD patients were designated anti-mycobacterial IgG positive while in the group of healthy controls only 0 (anti-ManLAM) to 6% (anti-*M chelonae*, anti-MAP) were positive (Figure 2). Between 2% (anti-LAM) and 36% (anti-BCG) of UC patients were anti-mycobacterial IgG positive. Despite similar age groups, only one of the 35 healthy volunteers was tested positive for anti-BCG IgG while 42% of CD patients and 36% of UC patients were above the cut-off. Furthermore, while none of the healthy controls reached positivity for anti-ManLAM IgG, 19% of CD and 11% of UC patients did. Using the Chi-square test, differences in frequencies of positive individuals between the groups of CD patients and healthy controls were significant for IgG against all mycobacterial lysates tested and purified ManLAM. Differences between UC patients and controls were significant for *M chelonae*, BCG and ManLAM. Finally, compared to UC patients, CD patients were significantly more frequently positive for anti-*M avium* and anti-*M smegmatis* IgG (Figure 2).

### Anti-mycobacterial IgG correlate with ASCA

To test whether seroreactivity against mycobacterial antigens in general and mycobacterial mannans in particular may correlate with that against yeast mannans, we grouped CD patients into ASCA-negative and ASCA-positive categories. ASCA-positive CD patients showed the highest frequencies of seropositivities against all mycobacterial antigens tested. Between 21% (anti-LAM) and 65% (anti-*M smegmatis*) of ASCA-positive CD patients were anti-mycobacterial IgG positive, while only 5% (anti-LAM) to 29% (anti-*M smegmatis*) of ASCA-negative CD patients had anti-mycobacterial IgG titers above the cut-off (Figure 3A). Compared to healthy controls, even the proportion of anti-LAM-positive individuals was significantly elevated in ASCA-positive CD patients (21% *vs* 4%). In addition, ASCA-positive CD patients were also significantly more frequently positive for IgG against *M smegmatis*, *M chelonae*, BCG, MAP and purified ManLAM when compared with ASCA-negative CD patients. On the other hand, ASCA-negative CD patients were significantly more frequently positive than healthy controls only for anti-*M smegmatis* IgG. When ASCA-positive CD patients were further grouped according to the levels of their ASCA titers, we observed a weak trend of increasing titers of IgG against mycobacterial lysates with increasing ASCA titers (data not shown). In contrast, seropositivity against purified LAM or ManLAM was almost exclusively and strongly elevated in the highest ASCA-positive subgroup. While less than 20% of ASCA-negative, or weakly positive CD patients were positive for anti-LAM



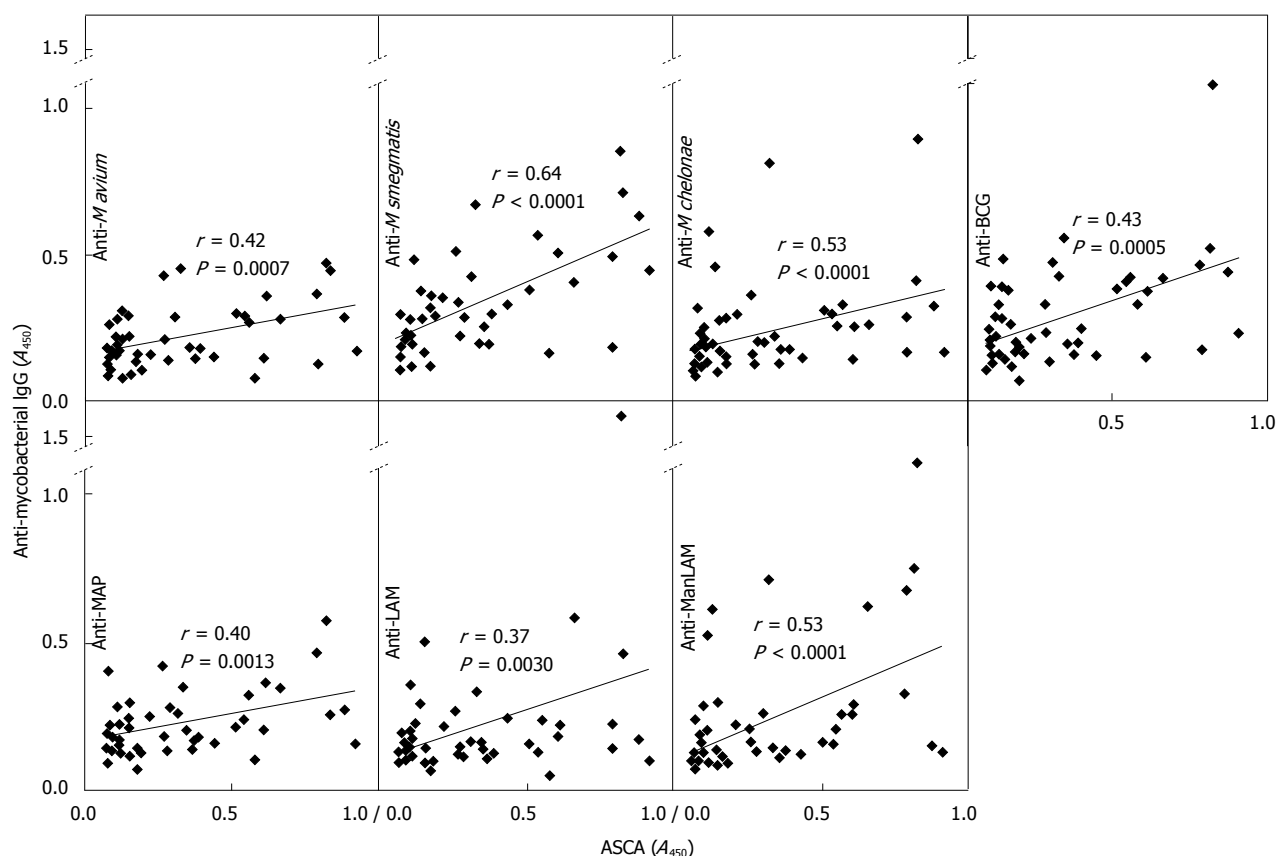
**Figure 2** Determination of anti-mycobacterial serum IgG in IBD and healthy controls. ELISA for different mycobacterial lysates and purified LAM or ManLAM were performed with sera from 105 CD patients, 45 UC patients and 35 controls. A negative to positive discrimination level was defined by a cut-off value representing the average of all  $A_{450}$  values from the healthy controls (excluding the clearly positive individuals with an  $A_{450} \geq 0.3$ ) plus 3 standard deviations. With these cut-off values, individuals positive for antibodies against the various mycobacterial antigens were identified and are shown as a percent of the total population. Asterisks indicate significant differences between the populations. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ .



**Figure 3** Association of anti-mycobacterial IgG with ASCA. CD patients were grouped according to their ASCA status and their anti-mycobacterial IgG were determined in comparison with healthy controls ( $n = 25$ ). **A:** ASCA- ( $n = 42$ ), extinction values at 450 nm below the cut-off as determined by routine diagnostics (3 standard deviations above the average of 10 negative control sera); ASCA+ ( $n = 48$ ) extinction values above the cut-off. According to the legend of Figure 1, percentages of IgG-positive individuals per group were determined for each antigen. **B:** ASCA-positive CD patients were further grouped according to the levels of ASCA and compared to healthy controls and UC patients. ASCA+ ( $n = 28$ ),  $A_{450}$  clearly above, but less than double the cut-off value; ASCA++ ( $n = 10$ ),  $A_{450}$  2 to 3 times higher than the cut-off value; ASCA+++ ( $n = 10$ ),  $A_{450}$  higher than 3 times the cut-off value. According to legends of Figures 1 and 2A, percentages of anti-LAM and anti-ManLAM IgG-positive individuals in each group were determined. Asterisks indicate significant differences between the groups. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ .

and anti-ManLAM IgG, 50% and 70% of the highest ASCA-positive CD patients were positive for anti-LAM ( $P \leq 0.0362$ , *vs* ASCA-low or ASCA-negative patients) and anti-ManLAM IgG ( $P \leq 0.0047$ , *vs* ASCA-low or ASCA-negative patients; Figure 3B), respectively. To

better visualize the extent of correlations between anti-mycobacterial IgG and ASCA, we plotted the extinction values obtained in the mycobacterial ELISA against those of the ASCA ELISA (Figure 4). All correlations were highly significant and ASCA correlated best with



**Figure 4** Correlation of ASCA with anti-mycobacterial IgG titers. Sera from 60 CD patients were randomly chosen and used for determination of ASCA titers and anti-mycobacterial IgG levels in the same experiment. Each dot represents one serum sample with the  $A_{450}$  obtained in the ASCA-specific ELISA (x-axis) and that obtained with ELISA specific for IgG against different mycobacterial lysates or purified LAM or ManLAM (y-axis).

IgG against *M. smegmatis* ( $r = 0.64$ ,  $P < 0.0001$ ), followed by anti-BCG and anti-ManLAM IgG ( $r = 0.53$ ,  $P < 0.0001$ ).

#### ASCA-positivity and MBL-deficiency synergistically associate with anti-mycobacterial IgG

Since deficiency for MBL associates with the ASCA-positive subgroup of CD patients [27, 28] we asked whether deficiency for MBL might also associate with elevated levels of anti-mycobacterial IgG. We found that similar to ASCA-positivity, MBL-deficiency was associated with higher proportions of anti-mycobacterial IgG-positive individuals for some but not all mycobacterial antigens tested (Figure 5), intriguingly, MBL-negativity synergistically contributed to increased frequencies of anti-mycobacterial IgG-positive individuals in the ASCA-positive/MBL-negative as compared to the ASCA-/MBL-double positive subgroup of CD patients. The synergistic effect that MBL-negativity and ASCA-positivity had regarding increased frequencies of anti-mycobacterial IgG positive individuals was most obvious when the ASCA-positive/MBL-negative group was compared with the ASCA-negative/MBL-positive group of CD patients: between 2.7-fold (from 13.0% to 35.3% for anti-*M. chelonae*) and 7.4-fold (from 8.7% to 64.7% for anti-*M. avium*) increased proportion of anti-mycobacterial IgG-positive individuals.

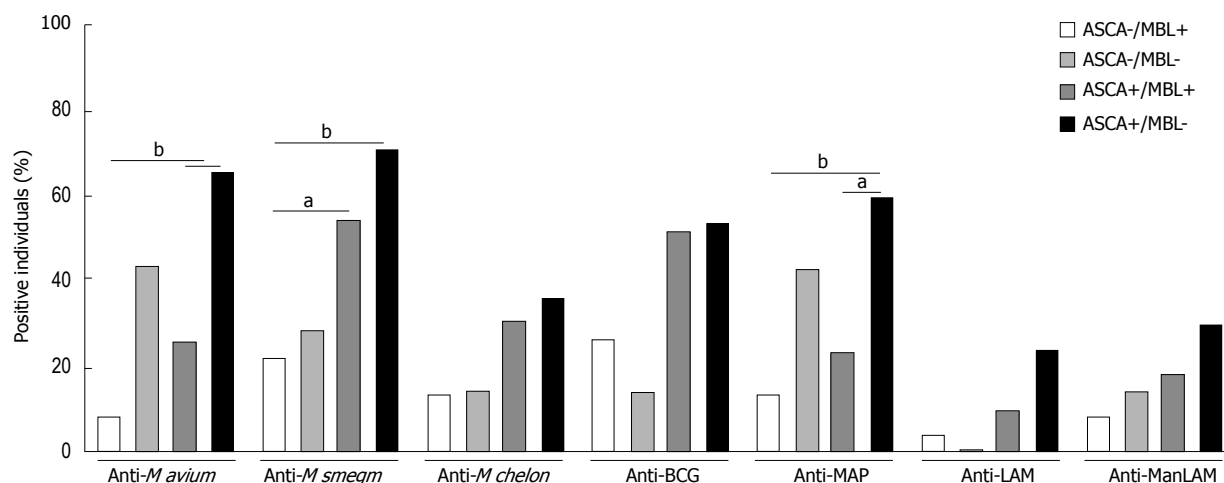
#### Anti-mycobacterial antibodies bind to yeast mannan

To determine potential cross-reactivities between anti-mycobacterial antibodies and ASCA, we affinity-purified eight highly ASCA-positive sera from CD patients on yeast mannan and compared original sera with the corresponding affinity purified antibodies for reactivity with yeast mannan (positive control), mycobacterial lysates and purified LAM and ManLAM. A tetanus toxoid-specific ELISA confirmed that affinity-purified serum antibodies were virtually free of contaminating, non-yeast mannan-specific IgG. Affinity-purified ASCA from individual patients showed individual reactivity patterns. While three of eight patients' ASCA reacted markedly with all mycobacterial preparations (patients No. 1, 4 and 7), 5 patients showed restricted reactivity (Figure 6A). On average, affinity-purified antibodies showed the highest degree of reactivity with LAM ( $73\% \pm 34\%$  of original sera) and lowest with MAP lysate ( $35\% \pm 16\%$  of original sera). The same 8 highly ASCA-positive sera were also affinity purified on *M. smegmatis* lysate and assessed for reactivity with yeast mannan and tetanus toxoid. All sera showed strongly reduced reactivity with yeast mannan after affinity-purification (Figure 6B).

#### Infection of mice with BCG leads to transient production of immunoglobulins that cross-react with *S. cerevisiae* mannan

To test, whether experimentally induced antibodies





**Figure 5** ASCA-positivity and MBL-negativity synergistically associate with anti-mycobacterial seroreactivity. CD patients' sera were grouped according to their ASCA and MBL status. ASCA-negative and ASCA-positive patients were separated into MBL-expressors ( $> 500$  ng/mL,  $n = 23$  and  $39$ , respectively) and MBL-low/deficient ( $\leq 500$  ng/mL,  $n = 7$  and  $17$ , respectively) individuals. From each group, average IgG levels against mycobacterial lysates or purified LAM or ManLAM  $\pm$  SEM were determined. Percentages of individuals per group with anti-mycobacterial IgG titers above the cut-off levels are shown. Significant differences are marked with asterisks. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ .

against mycobacteria may cross-react with *S cerevisiae* mannan, we infected mice with  $10^7$  live BCG, followed by an equal booster injection after 5 wk. Figure 7 shows that infection with BCG induced marked levels of BCG-lysate-specific IgM and moderate levels of specific IgG after 4 wk and up to 13 wk after the booster injection. Intriguingly, BCG infection led to transiently elevated titers of ASCA IgM and IgG which declined to background or near background levels within 2 mo post booster injection.

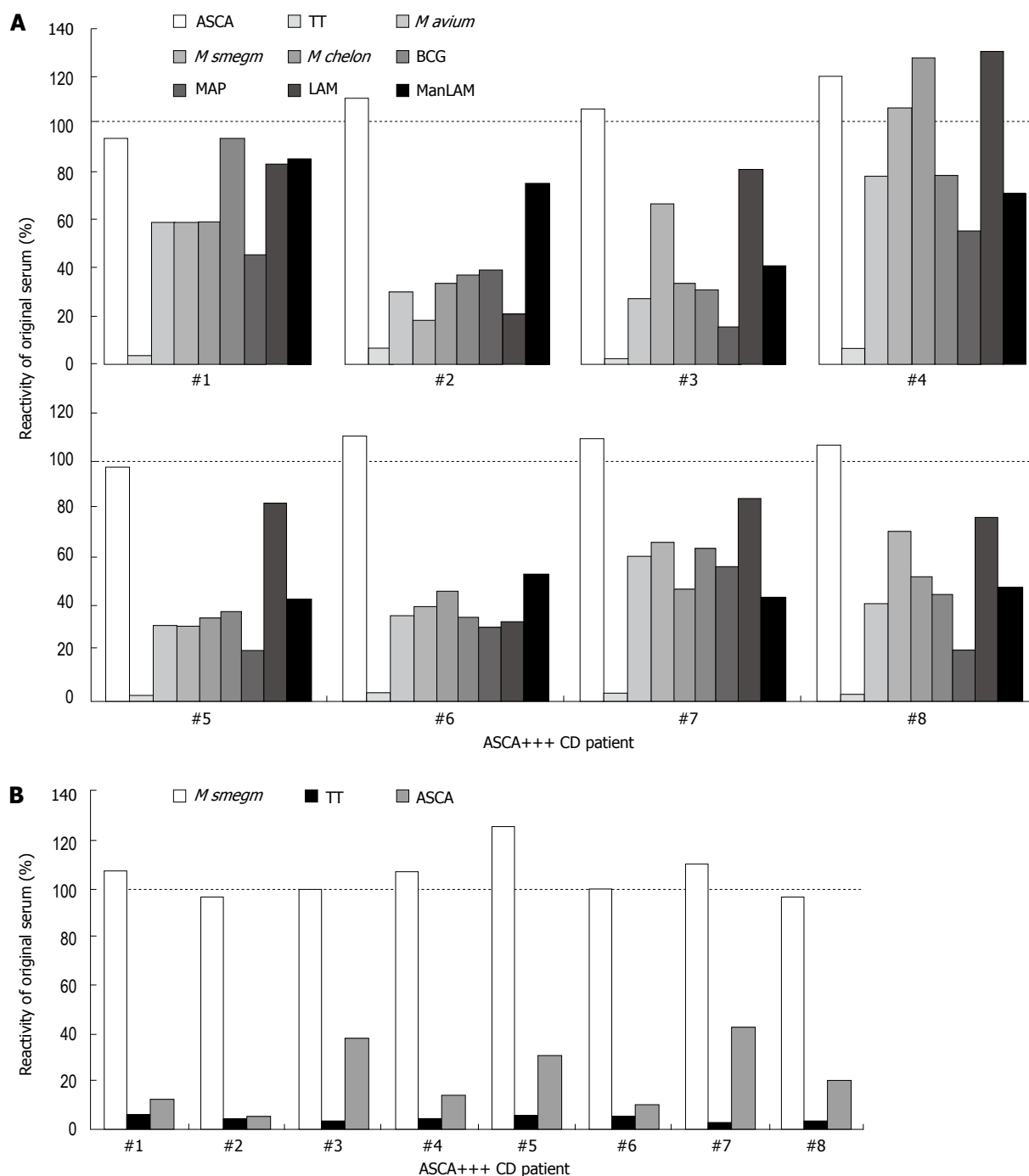
#### Higher frequency of anti-mycobacterial IgG-positive individuals in the subgroup of CD patients with fistulizing disease

In order to address the possible significance of anti-mycobacterial IgG in CD, patients were grouped according to the Montreal classification into UC-like (purely inflammatory), or stenosing or fistulizing disease phenotype (the latter two summarized as complicated disease). Sera from these groups of CD patients were compared for anti-mycobacterial IgG and ASCA by ELISA. ASCA were included because earlier reports indicate that ASCA associate with complicated disease<sup>[33,34]</sup> and increased risk for surgery months<sup>[35]</sup>. There was a weak tendency for patients with complicated disease to more often express anti-mycobacterial IgG compared to patients with UC-like disease (data not shown). On the other hand, a markedly higher frequency of patients expressed antibodies against mycobacterial lysates, but not purified LAM or ManLAM, when only the subgroup of patients with fistulizing disease was compared with UC-like disease (Figure 8). These differences were statistically significant for anti-*M avium* ( $P = 0.024$ ), anti-*M smegmatis* ( $P = 0.004$ ) and anti-MAP IgG ( $P = 0.045$ ). In contrast, the proportion of patients with stenoses expressing IgG against mycobacterial lysates was not markedly elevated compared to those with UC-like disease. Both subgroups with complicated

disease had a markedly higher proportion of individuals being positive for ASCA compared to UC-like disease. Here, the difference was statistically significant for the subgroup with stenoses compared to UC-like disease ( $P = 0.008$ , Figure 8). Finally, compared to patients with UC-like disease or stenoses, patients with fistulizing disease on average showed more individual seroreactivities per patient against the mycobacterial antigens tested ( $2.5$  vs  $1.5/1.6$ ,  $P = 0.050$ , Figure 8B).

## DISCUSSION

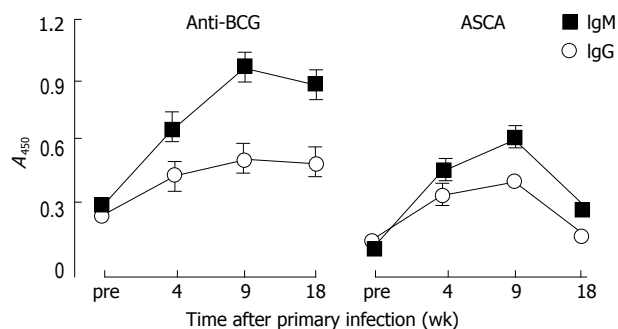
In the present study we have chosen a number of mycobacterial strains with more or less ubiquitous occurrence and - with the exception of BCG-originally isolated from patients suffering from mycobacterial infections to serve as antigens for ELISA with IBD patients' and control sera in order to assess a possible relationship between anti-mycobacterial antibodies and ASCA in CD. In addition, we used purified lipoarabinomannans with or without a richly mannosylated arabinose moiety (LAM from *M smegmatis* and ManLAM from *M tuberculosis*, respectively) because of similar oligomannose side chains as found in mannan from *S cerevisiae*, the specific antigen for ASCA. In agreement with published data<sup>[19,20]</sup>, terminal  $\alpha$ -1,3 linked mannose which is part of the dominant ASCA epitope was present at different extents in our mycobacterial preparations. In particular this epitope was strongly present in ManLAM from *M tuberculosis* while it was completely absent in LAM from *M smegmatis*. In accordance with that, *M smegmatis* lysate showed the weakest binding to GNL among all lysates tested. With the exception of non-mannose-capped LAM, we found significantly higher proportions of anti-mycobacterial IgG-positive individuals in CD compared to the healthy control group. We focused on IgG because initial screenings generally showed much lower levels



**Figure 6** ASCA strongly cross-react with mycobacterial antigens in a subgroup of ASCA-positive CD patients. **A:** Eight sera from highly ASCA-positive CD patients were incubated with membrane sections of yeast mannan Western blots containing the ASCA-reactive material, and bound antibodies were eluted. Eluted antibodies were titrated to yield comparable extinction values in the ASCA ELISA as with the original serum (approx. 100%, empty columns) and used at the corresponding dilution to assess reactivities against mycobacterial antigens. As a specificity control for affinity purification, eluted antibodies were also tested in a tetanus toxoid (TT) ELISA. **B:** The same 8 patients' sera were also affinity purified on nitrocellulose membranes decorated with *M. smegmatis* lysate. In analogy to A, eluted antibodies were adjusted for equal reactivity with *M. smegmatis* as the original sera (approx. 100%, empty columns) and tested at the corresponding dilution for reactivities against yeast mannan and TT.

or absence of specific IgA (data not shown). Previous studies have mainly focused on seroreactivities against selected antigens from MAP<sup>[22,36,37]</sup>. Naser *et al* have observed that a large proportion of CD, but neither UC patients nor controls had antibodies reactive with two recombinant antigens (75% and 89%, respectively) from their MAP genomic library<sup>[22]</sup>. In contrast, less than 30% of our CD patients showed broad MAP-specific

seroreactivity. Our results are comparable to those described by Polymeros *et al*, who found that sera of 42% of their small cohort of CD patients reacted with one or more MAP-derived peptides<sup>[37]</sup>. In our study UC patients showed-although less marked than CD patients - enhanced seroreactivity compared to healthy controls, in particular against some of the crude lysates. This is not surprising as enhanced seroreactivities against unusual,



**Figure 7** Antibody response of mice infected with *M bovis* BCG. C57BL/6 mice were intravenously infected with  $10^7$  viable *M bovis* BCG and the infection repeated after 5 wk. Serum was prepared from blood samples collected before infections, 4 wk after primary infection and 4 wk and 13 wk after secondary infection. Serum samples were tested for the presence of IgM (filled squares) and IgG (empty spheres) antibodies specific for *M bovis* BCG (left panel) and for *S cerevisiae* mannan (right panel). Results are shown as average  $A_{450}$  values of 4 animals  $\pm$  SE.

untypical or commensal antigens is not only a hallmark of CD but of IBD in general<sup>[38]</sup>.

While Polymeros *et al*<sup>[37]</sup> addressed a potential self-cross-reactive nature of anti-MAP antibodies in CD patients, in the present study, we were focusing on potential cross reactivities of CD patients' anti-mycobacterial antibodies with mannan from *S cerevisiae*. We could clearly demonstrate that the frequent seroreactivity against cell wall mannan from *S cerevisiae* (ASCA-positivity) in CD patients significantly correlated with seroreactivity not only against MAP, but also against antigens from all other mycobacteria that we tested. Importantly, correlations were best for *M smegmatis* lysate and purified ManLAM. The *M tuberculosis*-derived ManLAM used in the present study is characterized by highly mannosylated arabinose (similar to the ManLAM from BCG). While Stokes *et al* have also observed binding of GNL to *M tuberculosis*<sup>[39]</sup>, ManLAM from *M tuberculosis* should theoretically not contain the GNL-reactive  $\alpha$ -1,3 linked mannose residues according to biochemical analyses<sup>[40]</sup>. On the other hand, the phospho-myo-inositol-capped LAM of *M smegmatis* has no rich mannose-cap on its arabinose moiety<sup>[41,42]</sup> and shows no binding to GNL. Collectively, the good correlations between seroreactivities against *S cerevisiae* mannan and mycobacterial antigens shown in the present study do not solely depend on the presence of either the terminal  $\alpha$ -1,3 linked mannose residues or a rich mannose-cap. *M chelonae* is one of the rare mycobacterial strains with truly uncapped arabinose moieties on their LAM, also termed AraLAM or CheLAM<sup>[18]</sup>. On the other hand, this strain's lysate is very well recognized by GNL which may explain the increased reactivity with ASCA-positive CD patients' IgG.

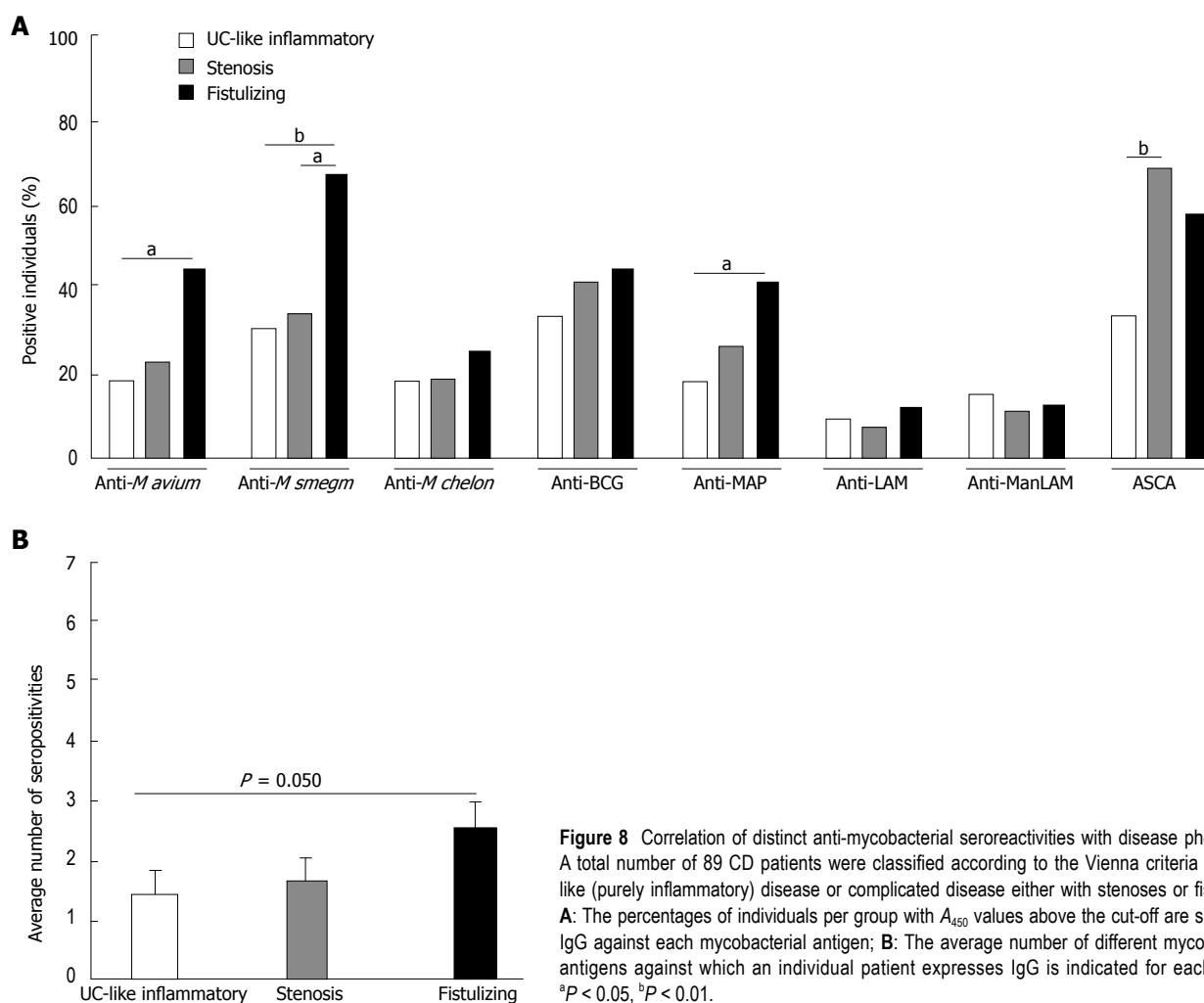
Even in the absence of the dominant ASCA epitope terminal  $\alpha$ -1,3 linked mannose there was a good and significant correlation of ASCA-positivity with seroreactivities against purified LAM, most strikingly if only those patients with high ASCA titers were considered.

Because in our cohort of CD patients MBL-deficiency associates with positivity for ASCA<sup>[27,28]</sup>, we wondered whether generation of anti-mycobacterial

antibodies may associate with this deficiency as well, and whether such an association may depend on certain strains with differential presence of the terminal  $\alpha$ -1,3 linked mannose motive and/or distinctly capped LAM. Indeed, we found an association of MBL deficiency with the prevalence of anti-mycobacterial antibodies. However, this association was not confined to strains with mannose-capped LAM and was not apparent for antibodies to BCG with rich mannose caps or *M chelonae* with the best binding to GNL apart from yeast mannan. It is known that MBL facilitates the entry of mycobacteria into host cells<sup>[43,44]</sup>. Therefore, in MBL-deficient persons, a stronger systemic immune response may be expected since the mycobacterial (cell wall) antigens are not rapidly eliminated by phagocytes.

The strong association of anti-mycobacterial antibodies with high ASCA titers with the observed synergistic effect of MBL-deficiency may be explained in different ways. First, it may be that these individuals have, due to a genetic predisposition, an increased reactivity to environmental (mannosylated) antigens. Alternatively there could be a true antibody cross-reactivity between the mannan antigens of *S cerevisiae* and mycobacteria. To address this question we affinity-purified ASCA from highly ASCA-positive CD patients and could show that these purified antibodies exert variable degrees of cross-reactivities between yeast mannan and mycobacterial lysates or purified (Man) LAM. On the other hand, affinity-purification of antibodies against *M smegmatis* lysate - the lysate that showed the highest degree of binding with affinity-purified ASCA-led to strongly reduced reactivity with yeast mannan compared to the original serum. Thus, while in some patients anti-mycobacterial IgG are mainly due to cross-reactive ASCA, in others, ASCA and anti-mycobacterial antibodies have separate specificities. In either case, the fact that affinity-purified anti-*M smegmatis* IgG only show weak or no reactivity with yeast mannan suggests that such cross-reactive antibodies only account for a minor fraction of total ASCA. This constellation makes it very unlikely that mycobacterial antigens play a role in the induction of ASCA. Since our purified LAM does not contain the terminal  $\alpha$ -1,3 mannose motif but shows a high degree of reactivity with affinity-purified ASCA, it has to be discussed whether the spectrum of antigens recognized by CD patients' ASCA goes beyond terminal  $\alpha$ -1,3 linked mannose side chains and may encompass other antigens such as peptide epitopes from the protein part of the mannan. In this context, it is of interest that according to a recent study CD patients contain antibodies that cross-react to an individual extent between  $\beta$ 2-glycoprotein I and yeast phosphopeptidomannans<sup>[45]</sup>.

Our experimental mouse model was to test whether mycobacterial infection is theoretically able to trigger induction of ASCA. For infection of mice we have chosen BCG because it is a commercial vaccination strain and is well established for infection and immunization studies in mice. Our finding that ASCA were only transiently expressed and declined by 2 mo post-booster



**Figure 8** Correlation of distinct anti-mycobacterial seroreactivities with disease phenotype. A total number of 89 CD patients were classified according to the Vienna criteria into UC-like (purely inflammatory) disease or complicated disease either with stenoses or fistulizing. **A:** The percentages of individuals per group with  $A_{450}$  values above the cut-off are shown for IgG against each mycobacterial antigen; **B:** The average number of different mycobacterial antigens against which an individual patient expresses IgG is indicated for each group. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$ .

infection while BCG-specific IgM and IgG remained high, supports our conclusion from the results of the affinity-purification study that an immune reactivity to mycobacterial antigens is unlikely to trigger the induction of a stable phenotype of ASCA-positivity.

We were interested whether seroreactivity to mycobacterial antigens may associate with a certain disease phenotype. We observed that more patients with complicated disease (stenosing or fistulizing) expressed antibodies against mycobacterial lysates compared to patients with purely inflammatory disease. Since we observed strong associations of anti-mycobacterial antibodies with ASCA-positivity and ASCA have been shown to associate with complicated disease<sup>[33-35]</sup>, our findings were not unexpected. However, the fact that this trend was clearly confined to the subgroup with fistulizing disease, while ASCA-positivity even better associated with stenoses, is surprising. Possibly, ingested mycobacteria more easily gain access to systemic immune compartments for priming if the bowel wall is transmurally damaged. Regarding ASCA, the origin of this unusual immune response is still unknown and whatever it is may actually be involved in the progression to more severe CD phenotypes. Finally, patients with fistulizing disease more frequently showed seroreactivities against multiple mycobacterial antigens compared to those with UC-like disease. This

observation fits to data published by others showing a more severe phenotype of disease in patients with increasing numbers of seroreactivities to various intestinal (commensal) antigens<sup>[46]</sup>.

In conclusion, we were able to demonstrate that ASCA-positive patients had significantly more immune reactivities to mycobacterial antigens. In a subgroup of ASCA-positive CD patients, anti-mycobacterial immunoglobulins at least partially represent cross-reactive ASCA, while in others there seem to be separate ASCA and anti-mycobacterial antibodies that do not cross-react. Furthermore, purified anti-*M. smegmatis* IgG showed low or no binding to yeast mannan. Therefore, we postulate that our results reflect more the predisposition of CD patients to develop increased immune reactivities to various ubiquitous antigens in general and mannosylated antigens in particular, rather than a role of mycobacteria in the induction of ASCA.

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## COMMENTS

## Background

A subgroup of patients with Crohn's disease (CD) develops antibodies against *S cerevisiae* cell wall mannan (ASCA). The mechanism of ASCA generation is still unclear. It is possible that some opportunistic or pathogenic infectious microorganism may be the initial inducer of this unusual antibody response because similar (cell wall) mannans also occur in other microorganisms such as mycobacteria with their lipoarabinomannan (LAM).

## Research frontiers

It has been shown that the opportunistic pathogen *Candida albicans* is able to experimentally induce ASCA (Standaert-Vitse *et al* 2006) and we have shown that Crohn's patients' ASCA cross-react with cell wall mannans from different yeast strains including *C albicans* (Schaffer *et al* 2007). In contrast to yeast, there exists a highly controversial debate on a possible role for mycobacteria in the etiopathogenesis of Crohn's disease.

## Innovations and breakthroughs

Our study clearly shows that (1) mycobacterial infection is very unlikely the origin of ASCA since anti-mycobacterial antibodies and ASCA in an individual patient are either non-overlapping or the former only represents a minor part of all antibodies recognizing yeast mannan; and (2) the correlation of antibodies against mycobacterial antigens with those against yeast mannan reflects - apart from pure cross-reactivity in some patients - increased predisposition for adaptive immune responses against ubiquitous antigens, especially observed in patients with a severe disease phenotype.

## Applications

The findings in the present study represent an important basis for further research on the role of antimicrobial immune responses in the pathogenesis of Crohn's disease. Furthermore, antimicrobial antibody patterns may define distinct subgroups of Crohn's patients requiring individual treatment approaches.

## Peer review

Similar to ASCA, seroreactivity against mycobacteria may define CD patients with complicated disease and a predisposition for immune responses against ubiquitous antigens. Mycobacterial infection does not likely play a role in ASCA induction.

## REFERENCES

- 1 **Duchmann R**, Kaiser I, Hermann E, Mayet W, Ewe K, Meyer zum Buschenfelde KH. Tolerance exists towards resident intestinal flora but is broken in active inflammatory bowel disease (IBD). *Clin Exp Immunol* 1995; **102**: 448-455
- 2 **Sartor RB**. Targeting enteric bacteria in treatment of inflammatory bowel diseases: why, how, and when. *Curr Opin Gastroenterol* 2003; **19**: 358-365
- 3 **Greenstein RJ**. Is Crohn's disease caused by a mycobacterium? Comparisons with leprosy, tuberculosis, and Johne's disease. *Lancet Infect Dis* 2003; **3**: 507-514
- 4 **Sandborn WJ**. Serologic markers in inflammatory bowel disease: state of the art. *Rev Gastroenterol Disord* 2004; **4**: 167-174
- 5 **Reumaux D**, Sendid B, Poulain D, Duthilleul P, Dewit O, Colombel JF. Serological markers in inflammatory bowel diseases. *Best Pract Res Clin Gastroenterol* 2003; **17**: 19-35
- 6 **Main J**, McKenzie H, Yeaman GR, Kerr MA, Robson D, Pennington CR, Parratt D. Antibody to *Saccharomyces cerevisiae* (bakers' yeast) in Crohn's disease. *BMJ* 1988; **297**: 1105-1106
- 7 **Seibold F**. ASCA: genetic marker, predictor of disease, or marker of a response to an environmental antigen? *Gut* 2005; **54**: 1212-1213
- 8 **Seibold F**, Stich O, Hufnagl R, Kamil S, Scheurlen M. Anti-*Saccharomyces cerevisiae* antibodies in inflammatory bowel disease: a family study. *Scand J Gastroenterol* 2001; **36**: 196-201
- 9 **Sendid B**, Colombel JF, Jacquinet PM, Faille C, Fruit J, Cortot A, Lucidarme D, Camus D, Poulain D. Specific antibody response to oligomannosidic epitopes in Crohn's disease. *Clin Diagn Lab Immunol* 1996; **3**: 219-226
- 10 **Wheeler RT**, Kupiec M, Magnelli P, Abeijon C, Fink GR. A *Saccharomyces cerevisiae* mutant with increased virulence. *Proc Natl Acad Sci USA* 2003; **100**: 2766-2770
- 11 **Standaert-Vitse A**, Jouault T, Vandewalle P, Mille C, Seddik M, Sendid B, Mallet JM, Colombel JF, Poulain D. *Candida albicans* is an immunogen for anti-*Saccharomyces cerevisiae* antibody markers of Crohn's disease. *Gastroenterology* 2006; **130**: 1764-1775
- 12 **Schaffer T**, Muller S, Flogerzi B, Seibold-Schmid B, Schoepfer AM, Seibold F. Anti-*Saccharomyces cerevisiae* mannan antibodies (ASCA) of Crohn's patients crossreact with mannan from other yeast strains, and murine ASCA IgM can be experimentally induced with *Candida albicans*. *Inflamm Bowel Dis* 2007; **13**: 1339-1346
- 13 **Young M**, Davies MJ, Bailey D, Gradwell MJ, Smestad-Paulsen B, Wold JK, Barnes RM, Hounsell EF. Characterization of oligosaccharides from an antigenic mannan of *Saccharomyces cerevisiae*. *Glycoconj J* 1998; **15**: 815-822
- 14 **Chatterjee D**. The mycobacterial cell wall: structure, biosynthesis and sites of drug action. *Curr Opin Chem Biol* 1997; **1**: 579-588
- 15 **Nigou J**, Gilleron M, Puzo G. Lipoarabinomannans: from structure to biosynthesis. *Biochimie* 2003; **85**: 153-166
- 16 **Shibuya N**, Goldstein IJ, Van Damme EJ, Peumans WJ. Binding properties of a mannose-specific lectin from the snowdrop (*Galanthus nivalis*) bulb. *J Biol Chem* 1988; **263**: 728-734
- 17 **Kaku H**, Goldstein IJ, Oscarson S. Interactions of five D-mannose-specific lectins with a series of synthetic branched trisaccharides. *Carbohydr Res* 1991; **213**: 109-116
- 18 **Guerardel Y**, Maes E, Ellass E, Leroy Y, Timmerman P, Besra GS, Loch C, Strecker G, Kremer L. Structural study of lipomannan and lipoarabinomannan from *Mycobacterium chelonae*. Presence of unusual components with alpha 1,3-mannopyranose side chains. *J Biol Chem* 2002; **277**: 30635-30648
- 19 **Michell SL**, Whelan AO, Wheeler PR, Panico M, Easton RL, Etienne AT, Haslam SM, Dell A, Morris HR, Reason AJ, Herrmann JL, Young DB, Hewinson RG. The MPB83 antigen from *Mycobacterium bovis* contains O-linked mannose and (1->3)-mannobiose moieties. *J Biol Chem* 2003; **278**: 16423-16432
- 20 **Mpofu CM**, Campbell BJ, Subramanian S, Marshall-Clarke S, Hart CA, Cross A, Roberts CL, McGoldrick A, Edwards SW, Rhodes JM. Microbial mannan inhibits bacterial killing by macrophages: a possible pathogenic mechanism for Crohn's disease. *Gastroenterology* 2007; **133**: 1487-1498
- 21 **Shafraan I**, Piromalli C, Decker JW, Sandoval J, Naser SA, El-Zaatari FA. Seroreactivities against *Saccharomyces cerevisiae* and *Mycobacterium avium* subsp. *paratuberculosis* p35 and p36 antigens in Crohn's disease patients. *Dig Dis Sci* 2002; **47**: 2079-2081
- 22 **Naser SA**, Hulten K, Shafraan I, Graham DY, El-Zaatari FA. Specific seroreactivity of Crohn's disease patients against p35 and p36 antigens of *M. avium* subsp. *paratuberculosis*. *Vet Microbiol* 2000; **77**: 497-504
- 23 **Turner MW**. Mannose-binding lectin (MBL) in health and disease. *Immunobiology* 1998; **199**: 327-339
- 24 **Vasta GR**, Quesenberry M, Ahmed H, O'Leary N. C-type lectins and galectins mediate innate and adaptive immune functions: their roles in the complement activation pathway. *Dev Comp Immunol* 1999; **23**: 401-420
- 25 **Jack DL**, Klein NJ, Turner MW. Mannose-binding lectin: targeting the microbial world for complement attack and opsonophagocytosis. *Immunol Rev* 2001; **180**: 86-99
- 26 **Dommett RM**, Klein N, Turner MW. Mannose-binding lectin in innate immunity: past, present and future. *Tissue Antigens* 2006; **68**: 193-209
- 27 **Seibold F**, Konrad A, Flogerzi B, Seibold-Schmid B, Arni S, Juliger S, Kun JF. Genetic variants of the mannan-binding lectin are associated with immune reactivity to mannans in

- Crohn's disease. *Gastroenterology* 2004; **127**: 1076-1084
- 28 **Seibold F**, Boldt AB, Seibold-Schmid B, Schoepfer AM, Flogerzi B, Muller S, Kun JF. Deficiency for mannan-binding lectin is associated with antibodies to *Saccharomyces cerevisiae* in patients with Crohn's disease and their relatives. *Gut* 2007; **56**: 152
- 29 **Best WR**, Beckett JM, Singleton JW, Kern F Jr. Development of a Crohn's disease activity index. National Cooperative Crohn's Disease Study. *Gastroenterology* 1976; **70**: 439-444
- 30 **Schroeder KW**, Tremaine WJ, Ilstrup DM. Coated oral 5-aminosalicylic acid therapy for mildly to moderately active ulcerative colitis. A randomized study. *N Engl J Med* 1987; **317**: 1625-1629
- 31 **Satsangi J**, Silverberg MS, Vermeire S, Colombel JF. The Montreal classification of inflammatory bowel disease: controversies, consensus, and implications. *Gut* 2006; **55**: 749-753
- 32 **Kocourek J**, Ballou CE. Method for fingerprinting yeast cell wall mannans. *J Bacteriol* 1969; **100**: 1175-1181
- 33 **Vasiliauskas EA**, Kam LY, Karp LC, Gaiennie J, Yang H, Targan SR. Marker antibody expression stratifies Crohn's disease into immunologically homogeneous subgroups with distinct clinical characteristics. *Gut* 2000; **47**: 487-496
- 34 **Dassopoulos T**, Frangakis C, Cruz-Correa M, Talor MV, Burek CL, Datta L, Nouvet F, Bayless TM, Brant SR. Antibodies to *saccharomyces cerevisiae* in Crohn's disease: higher titers are associated with a greater frequency of mutant NOD2/CARD15 alleles and with a higher probability of complicated disease. *Inflamm Bowel Dis* 2007; **13**: 143-151
- 35 **Forcione DG**, Rosen MJ, Kisiel JB, Sands BE. Anti-*Saccharomyces cerevisiae* antibody (ASCA) positivity is associated with increased risk for early surgery in Crohn's disease. *Gut* 2004; **53**: 1117-1122
- 36 **Olsen I**, Wiker HG, Johnson E, Langeeggen H, Reitan LJ. Elevated antibody responses in patients with Crohn's disease against a 14-kDa secreted protein purified from *Mycobacterium avium* subsp. *paratuberculosis*. *Scand J Immunol* 2001; **53**: 198-203
- 37 **Polymeros D**, Bogdanos DP, Day R, Arioli D, Vergani D, Forbes A. Does cross-reactivity between *mycobacterium avium paratuberculosis* and human intestinal antigens characterize Crohn's disease? *Gastroenterology* 2006; **131**: 85-96
- 38 **Bossuyt X**. Serologic markers in inflammatory bowel disease. *Clin Chem* 2006; **52**: 171-181
- 39 **Stokes RW**, Norris-Jones R, Brooks DE, Beveridge TJ, Doxsee D, Thorson LM. The glycan-rich outer layer of the cell wall of *Mycobacterium tuberculosis* acts as an antiphagocytic capsule limiting the association of the bacterium with macrophages. *Infect Immun* 2004; **72**: 5676-5686
- 40 **Chatterjee D**, Lowell K, Rivoire B, McNeil MR, Brennan PJ. Lipoarabinomannan of *Mycobacterium tuberculosis*. Capping with mannosyl residues in some strains. *J Biol Chem* 1992; **267**: 6234-6239
- 41 **Chatterjee D**, Khoo KH. *Mycobacterial* lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. *Glycobiology* 1998; **8**: 113-120
- 42 **Vercellone A**, Nigou J, Puzo G. Relationships between the structure and the roles of lipoarabinomannans and related glycoconjugates in tuberculosis pathogenesis. *Front Biosci* 1998; **3**: e149-e163
- 43 **Soborg C**, Madsen HO, Andersen AB, Lillebaek T, Kok-Jensen A, Garred P. Mannose-binding lectin polymorphisms in clinical tuberculosis. *J Infect Dis* 2003; **188**: 777-782
- 44 **Bonar A**, Chmiela M, Rudnicka W, Rozalska B. Mannose-binding lectin enhances the attachment and phagocytosis of mycobacteria in vitro. *Arch Immunol Ther Exp (Warsz)* 2005; **53**: 437-441
- 45 **Krause I**, Blank M, Cervera R, Font J, Matthias T, Pfeiffer S, Wies I, Fraser A, Shoenfeld Y. Cross-reactive epitopes on beta2-glycoprotein-I and *Saccharomyces cerevisiae* in patients with the antiphospholipid syndrome. *Ann N Y Acad Sci* 2007; **1108**: 481-488
- 46 **Arnott ID**, Landers CJ, Nimmo EJ, Drummond HE, Smith BK, Targan SR, Satsangi J. Sero-reactivity to microbial components in Crohn's disease is associated with disease severity and progression, but not NOD2/CARD15 genotype. *Am J Gastroenterol* 2004; **99**: 2376-2384

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CLINICAL RESEARCH

## Methylenetetrahydrofolate reductase C677T genotype affects promoter methylation of tumor-specific genes in sporadic colorectal cancer through an interaction with folate/vitamin B<sub>12</sub> status

Pooneh Mokarram, Fakhraddin Naghibalhossaini, Mehdi Saberi Firoozi, Seyed Vahid Hosseini, Ahmad Izadpanah, Heshmetalah Salahi, Seyed Ali Malek-Hosseini, Abdoulrasool Talei, Mehra Mojallal

Pooneh Mokarram, Fakhraddin Naghibalhossaini, Department of Biochemistry, Shiraz University of Medical Sciences, School of Medicine, Shiraz 71345, Iran

Mehdi Saberi Firoozi, Department of Internal Medicine and Gastroenterohepatology Research Centre, Shiraz University of Medical Sciences, Shiraz 71345, Iran

Seyed Vahid Hosseini, Ahmad Izadpanah, Department of Surgery (colorectal ward) and Gastroenterohepatology Research Centre, Shiraz University of Medical Sciences, Shiraz 71345, Iran

Heshmetalah Salahi, Seyed Ali Malek-Hosseini, Department of Surgery and Organ Transplantation Research Centre, Namazee Hospital, Shiraz University of Medical Sciences, Shiraz 71345, Iran

Abdoulrasool Talei, Department of Surgery and Institute of Cancer Research, Shiraz University of Medical Sciences, Shiraz 71345, Iran

Mehra Mojallal, Pathology Laboratory, Dena Hospital, Shiraz 71345, Iran

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**Author contributions:** Naghibalhossaini F designed research and wrote the paper; Hosseini SV, Saberi Firoozi M, Izadpanah A, Salahi H, Malek-Hosseini SA, Talei A, and Mojallal M provided specimens, reagents and analytical tools; Mokarram P performed research.

**Correspondence to:** Fakhraddin Naghibalhossaini, Department of Biochemistry, Shiraz University of Medical Sciences, School of Medicine, Zand Street, Shiraz 71345, Iran. fakhraddin.naghibalhossaini@elf.mcgill.ca

Telephone: +98-711-2303029 Fax: +98-711-2303029

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151 sporadic colorectal cancer patients. The promoter methylation of tumor-related genes was determined by methylation-specific PCR. Eighty six patients from whom fresh tumor samples were obtained and 81 controls were also examined for serum folate and vitamin B<sub>12</sub> concentrations by a commercial radioimmunoassay kit.

**RESULTS:** We found 29.1% of cases had tumors with at least one methylated gene promoter. In case-case comparison, we did not find a significant association between methylation in tumors and any single genotype. However, in comparison to controls with the CC genotype, an increased risk of tumor methylation was associated with the CT genotype (OR = 2.5; 95% CI, 1.1-5.6). In case-case comparisons, folate/vitamin B<sub>12</sub> levels were positively associated with tumor methylation. Adjusted odds ratios for tumor methylation in cases with high (above median) *versus* low (below median) serum folate/vitamin B<sub>12</sub> levels were 4.9 (95% CI, 1.4-17.7), and 3.9 (95% CI, 1.1-13.9), respectively. The frequency of methylated tumors was significantly higher in high methyl donor than low methyl donor group, especially in those with *MTHFR* CT (*P* = 0.01), and CT/TT (*P* = 0.002) genotypes, but not in those with the CC genotype (*P* = 1.0).

**CONCLUSION:** We conclude that high concentrations of serum folate/vitamin B<sub>12</sub> levels are associated with the risk of promoter methylation in tumor-specific genes, and this relationship is modified by *MTHFR* C677T genotypes.

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**Key words:** *Methylenetetrahydrofolate reductase*; Folate; Vitamin B<sub>12</sub>; Methylation; Colorectal cancer

**Peer reviewer:** Shu Zheng, Professor, Scientific Director of Cancer Institute, Zhejiang University, Secondary Affiliated Hospital, Zhejiang University, 88# Jiefang Road, Hangzhou 310009, Zhejiang Province, China

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### Abstract

**AIM:** To evaluate joint effects of *Methylenetetrahydrofolate reductase* (*MTHFR*) C677T genotypes, and serum folate/vitamin B<sub>12</sub> concentrations on promoter methylation of tumor-associated genes among Iranian colorectal cancer patients.

**METHODS:** We examined the associations between *MTHFR* C677T genotype, and promoter methylation of *P16*, *hMLH1*, and *hMSH2* tumor-related genes among

genotype affects promoter methylation of tumor-specific genes in sporadic colorectal cancer through an interaction with folate/vitamin B<sub>12</sub> status. *World J Gastroenterol* 2008; 14(23): 3662-3671 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3662.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3662>

## INTRODUCTION

Colon cancer (CLC) is one of the most common cancers in the world, with high rates in Western countries<sup>[1]</sup>. A significant increase in CLC incidence with the predominant localization in the left colon has also been reported in Iran over the last decade<sup>[2,3]</sup>. However, little is known about the molecular mechanism of CLC in this region.

One of the pathways by which that CLC can progress involves transcriptional silencing by hypermethylation of CpG islands referred as methylator phenotype (CIMP<sup>+</sup>)<sup>[4]</sup>. The CIMP<sup>+</sup> in CLC is characterized by frequent hypermethylation of specific CpG sites, including those present in the promoter regions of tumor suppressor genes such as the cell cycle regulator, *p16* and genes involved in DNA mismatch repair like *bMLH1*<sup>[4]</sup>. The *bMLH1* promoter region is methylated in about 90% of microsatellite unstable (MSI-positive) colon cancers that leads to the silencing of *bMLH1* expression<sup>[5]</sup>. The CIMP<sup>+</sup> phenotype may be the result of more widespread aberration in methyl-group metabolism in cancer cells.

Interaction of the epigenome with the environment, including nutrition, can alter patterns of gene expression. It has been proposed that polymorphisms in folate-metabolizing enzymes and genes involved in DNA methylation are associated with colon cancer. *MTHFR* is a key enzyme regulating folate metabolism, which affects DNA methylation and synthesis. *MTHFR* converts 5, 10-methylenetetrahydrofolate to 5-methyl tetrahydrofolate, which is required for homocysteine methylation to methionine. Methionine is then activated to S-adenosylmethionine, a universal methyl donor in numerous transmethylation reactions, including methylation of DNA, RNA, proteins, and other molecules<sup>[6]</sup>. The *MTHFR* gene is polymorphic with single nucleotide variants within codon 677 in exon 4 (C to T, Ala to Val). This variant encodes a thermolabile enzyme with reduced activity that leads to a reduced plasma folate level<sup>[7]</sup>.

Several case-control studies have shown a reduced risk of CLC for homozygous *MTHFR* TT individuals. The protective effect appears to depend on an adequate level of dietary folate intake, gender, age, and location of the tumor in the proximal or distal colon<sup>[8,9]</sup>. In some circumstances, the *MTHFR*-TT genotype seems to increase the risk of CLC<sup>[10,11]</sup>. It has been suggested that deficient activity of *MTHFR* affects DNA methylation status through an interaction with folate status<sup>[12]</sup>. Several data provide evidence that individuals with the common C677T mutation in the *MTHFR* gene and with low levels of folate had a diminished level of DNA methylation

compared with those with the C/C wild type. Folate deficiency may be involved in carcinogenesis through impaired synthesis and repair of DNA, or by causing global hypomethylation of DNA, a possible early event in carcinogenesis<sup>[13]</sup>. Although a protective role against cancer was suggested for the high dietary folate intake, epidemiological evidence has not consistently shown a protective effect of high folate intake against CLC<sup>[14,15]</sup>. There are few studies addressing joint effects of *MTHFR* C677T genotypes, and methyl donor coenzymes status on promoter methylation of tumor-associated genes in CLC<sup>[16,17]</sup>. In the current study we investigated the role of *MTHFR* C677T genotype, and serum folate/vitamin B<sub>12</sub> concentrations on methylation of CpG islands at *p16*, *bMLH1*, and *bMSH2* tumor-associated genes among Iranian sporadic CLC patients.

## MATERIALS AND METHODS

### Study population, and samples

A total of 151 sporadic primary CLC tumor samples (86 fresh and 65 formalin fixed and paraffin embedded) as well as corresponding normal mucosa were collected from surgical patients at 3 hospitals of the Shiraz University of Medical Sciences in Shiraz, Southern Iran from July, 2003 to September, 2005. Institutional review board approval was granted for this study. The fresh samples were snap frozen in liquid nitrogen immediately after resection and stored at -70°C until processing. All samples were evaluated and subjected to histological diagnosis by an expert pathologist, who also selected representative tissue sections for DNA extraction, and further molecular analyses. The splenic flexure was used as the anatomical boundary to define proximal and distal CLC. Sociodemographic characteristics such as age and gender were obtained by completion of a detailed questionnaire.

### Extraction of DNA and *MTHFR* genotyping

Genomic DNA was extracted from micro-dissected formalin-fixed, paraffin embedded tumor samples and adjacent normal tissues using the pinpoint slide DNA isolation kit (ZYMORESEARCH, CA, USA). We used the standard phenol/chloroform method for DNA extraction from fresh tumor samples. Genotyping of *MTHFR* at codon 677 of DNA from control and CLC cases was performed using a modification of the mutagenically separated PCR (MS-PCR) method described by Hill and FitzPatrick<sup>[18]</sup>. Genotyping for *MTHFR* involved analysis of PCR product size by electrophoresis on 3% agarose gels. PCR reactions were carried out in a volume of 50 µL containing 50 ng DNA, 1 × polymerization buffer (MBI Fermentas, Lithuania), 1.5 mmol/L MgCl<sub>2</sub>, 0.2 mmol/L dNTP, and 1.5 U Taq polymerase. The primers and concentrations used for PCR reactions were as follows: forward mutant (29 bases) 0.35 µmol/L 5'-CACTTGAAGGAGAAGGTGTCTGCGGGACT-3', forward normal (49 bases) 0.19 µmol/L 5'-GCTTTGAGGCTGACCTGAAGA-CCTTGAAGGAGAAG GTGTCTGCGGCAGC-3'



and the reverse primer (20 bases) 0.23  $\mu\text{mol/L}$  5'-TCACCTGGATGGGAAAGATC-3'. The two forward primers are complementary to the normal (677C) and mutant (677T) alleles and differed in length by 20 bases at their 5' ends. The cycling parameters were 5 min at 95°C followed by 35 cycles of 45 s at 95°C, 1 min at 55°C, and 45 s at 72°C followed by a single 10-min extension at 72°C. Twenty  $\mu\text{L}$  of each reaction mixture was separated on agarose gel and stained with ethidium bromide and visualized under UV illumination.

### Serum folate and vitamin B<sub>12</sub> measurement and methylation specific PCR (MSP)

Folate and vitamin B<sub>12</sub> measurements were limited to sera from 86 patients with freshly studied tumors and 81 age and sex matched normal controls, selected among healthy volunteers from the general population with no history of any cancer. Blood samples were drawn from patients before operation and serum was prepared within two hours of blood collection. Sera were frozen immediately at -70°C until used. The concentrations of folate and vitamin B<sub>12</sub> in each specimen were measured in duplicate by a commercial radioimmunoassay kit (SimulTRAC-SNB RIA, DRG International Inc. USA) using a gamma counter (Contron, Switzerland). We determined the *p16*, *bMLH1*, and *bMSH2* promoter methylation status by chemical treatment with sodium bisulfite and subsequent MSP as described<sup>[19]</sup>. In brief, this technique uses bisulfite modification to convert unmethylated, but not methylated, cytosine to uracil. MSP utilizes this difference to amplify specifically either methylated or unmethylated DNA. The sequences of primers used for amplification of the promoter region of each of the 3 genes were as follows: *p16* methylated, sense 5'-TTATTAGAGGGTGGGGC-GGATCGC-3' and antisense 5'-GACCCCGAACC GCGACCGTAA-3', which produce a 150 bp fragment; *p16* unmethylated: sense 5'-TTATTAGAGGGTGGGGTGGATTGT-3' and antisense 5'-CAACCCCAAACCACAACCATAA-3', which produce a 151 bp fragment; *bMLH1* methylated, sense 5'-ACGTAGACG-TTTTATTAGGGTCGC-3' and antisense 5'-CCTCATCGTAACTACCCGCG-3', which produce a 112 bp fragment; *bMLH1* unmethylated, sense 5'-TTTTGATGTAGATGTTTTATTAGGGTTGT-3' and antisense 5'-ACCACCTCATCATAACTACCCACA-3', which produce a 124 bp fragment; *bMSH2* methylated, sense 5'-TCGTGGTTCGGACGTCGTTTC-3' and antisense 5'-CAACGTCTCCTTCGACTACACCGG-3', which produce a 133 bp fragment; *bMSH2* unmethylated, sense 5'-GGTTGTTGTGGTTGGATGTTGTTT-3' and antisense 5'-CAACTACAACATCTCCTTCAAC TACACCA-3', which produce a 144 bp fragment. The hot-started PCR reactions were performed in a 50  $\mu\text{L}$  reaction volume containing 25 pmol of each of sense and antisense primer, 0.2 mmol/L dNTPs, and 80 ng bisulfite-modified DNA in 1× PCR buffer provided by Taq enzyme supplier. The reaction mixture was denatured at 95°C for 5 min, after which 1.5 U Taq polymerase was added; then amplified by 40 cycles, each consisting of 30 s denaturation at 95°C, 45 s annealing

Table 1 Frequency distributions of selected characteristics in CLC patients and control subjects

Variables	Cases			Control (n = 81)	P <sup>1</sup>
	Proximal	Distal	Total (n = 151)		
Gender					0.8
Male (%)	28 (31.1)	62 (68.9)	90 (59.6)	50 (61.7)	
Female (%)	32 (52.5)	29 (47.5)	61 (40.4)	31 (38.3)	
Smoking status					0.1
Smokers <sup>2</sup> (%)	22 (34.4)	42 (65.6)	64 (42.4)	25 (30.9)	
Non-smokers	38 (43.7)	49 (56.3)	87 (57.6)	56 (69.1)	
Age groups (yr)					0.4
< 60 (%)	24 (36.4)	42 (63.6)	66 (43.7)	40 (49.4)	
≥ 60 (%)	36 (42.4)	49 (57.6)	85 (56.3)	41 (50.6)	
Median (range)	61 (40-87)	60 (28-90)	60 (28-90)	60 (28-89)	
Mean (SD)	62.22 (10.97)	59.24 (12.7)	60.42 (12)	58.98 (15.66)	

<sup>1</sup>Fisher's exact test; <sup>2</sup>Current and former smokers.

at 58°C, and 30 s polymerization at 72°C, followed by a single 10-min extension at 72°C.

### Statistical analysis

Statistical analysis was performed using the SPSS version 11.5 software package (Chicago, IL). Associations between methylation of loci and clinical, biological and genotypic features were evaluated using Chi square and Fisher's exact test as appropriate. Logistic regression was used to calculate odds ratio (OR) and 95% confidence intervals (95% CI). We adjusted for covariates, specifically including age, gender, and smoking status. Comparing serum folate and vitamin B<sub>12</sub> levels in cases and controls was performed using two-sided *t*-test, Mann-Whitney test, and Kruskal-Wallis test appropriately.

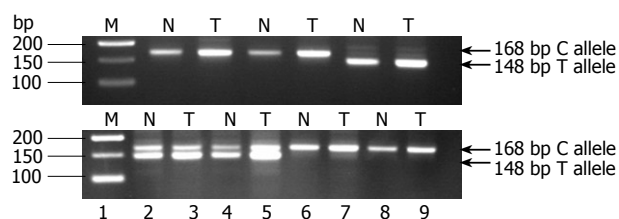
## RESULTS

### Distribution of selected characteristics of cases and controls

Selected characteristics of the study population are presented in Table 1. One hundred and fifty one patients and 81 controls entered the study. The distribution was similar in cases and controls by virtue of the study design. Sixty percent (91) of patients had distal CLC and 40% (60) had proximal CLC. Cases were more likely to be males and to be non-smokers. No statistically significant differences were found between cases and controls or between proximal and distal cancer cases with respect to distributions of age, and smoking status. The frequency of distal CLC in males and females was 68.9% and 47.5% (Table 1), respectively, indicative of a significantly higher left CLC incidence in males than females (OR = 2.65; 95% CI, 1.3-5.2).

### MTHFR genotypes and the methylation status of tumor-associated genes promoter

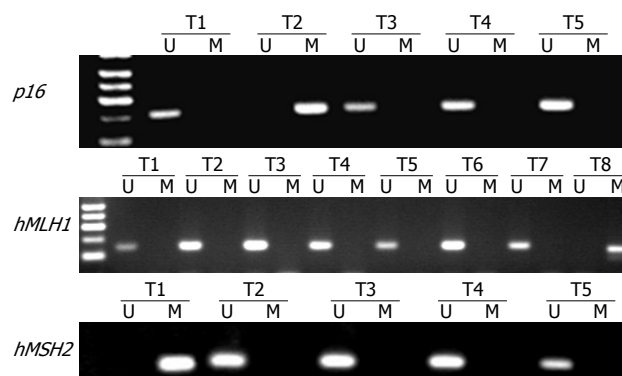
Illustrative examples of genotyping of *MTHFR* gene are shown in Figure 1. In 42 patients for whom we performed genotyping in both the cancer tissue and adjacent normal tissue, the typing results were identical in the two samples. CpG island promoter



**Figure 1** Representative example of MS-PCR assay for genotyping of codon 677 of *MTHFR* gene. For PCR-primers and reaction conditions see methods. In case of the *MTHFR* 677 C allele, a product with 168 base pairs (bp) in length was generated, whereas the *MTHFR* 677 T allele yielded a 148-bp product. The differently sized allele-specific PCR products were separated by agarose gel electrophoresis. In 42 patients genotyping was performed in both cancer tissue (T) and adjacent normal tissue (N). Lanes 2-5 in the lower panel show heterozygote (CT) samples. M: DNA size marker.

hypermethylation was analyzed in the primary tumors by methylation specific PCR as described in “MATERIALS AND METHODS”. Illustrative examples are shown in Figure 2. Table 2 summarizes the association of promoter methylation of genes and *MTHFR* genotype, and other clinical-biological characteristics of CLC patients. Several studies have reported age-dependent variation in the frequency of the *MTHFR* genotypes<sup>[20,21]</sup>. Therefore, we divided both CLC and control groups into  $\geq 60$  and  $< 60$ -year old groups. The median age of CLC patients (60 years) was chosen for this division. The most frequently methylated locus was *p16* (19.9 %; 30 of 151), followed by *hMLH1* (13.2 %; 20 of 151), and *hMSH2* (2.6 %; 4 of 151). Eight of 151 (5.3 %) of tumors had both P16 and *hMLH1* CpG island hypermethylation while 2 of 151 (1.3%) had both *hMLH1* and *hMSH2* promoter hypermethylation. None of the tumors had simultaneous CpG island hypermethylation of all three genes. There were no significant differences in association of methylation of any individual gene investigated by age or sex of patients. The frequency of tumor methylation (tumors with at least one gene methylated) was 44/151 (29.1%). The latter group of tumors is collectively referred to as “methylated tumors”. A significantly higher risk of tumor methylation was found in females (OR = 2.3; 95% CI, 1.1-5.04) (Table 2). Gene promoter methylation was also strongly associated with tumor site, the highest frequency (more than 97%) of methylation occurring in the proximal tumors.

We confronted *MTHFR* genotype with the methylation of tumors using the CC genotype as the reference group. Results from case-case comparison, showed no statistically significant genotype dependent differences in the frequency of any specific gene promoter methylation (Table 2). In comparison to cases with the CC genotype, we did not find any significant association between tumor methylation or “methylated tumors”, defined above and any single genotype in the entire group of patients, but cases with the CT genotype were slightly more likely to have methylated tumors (OR = 1.9; 95% CI, 0.9-4.2). Results from the case-control comparison, showed that the CT genotype was significantly associated with tumor methylation in the entire group of patients (OR = 2.5; 95% CI, 1.1-5.6;



**Figure 2** Representative examples of MSP reactions for promoter methylation analysis of *p16*, *hMLH1*, and *hMSH2* genes in primary CLC tumors. The presence of a visible PCR product in those lanes marked U indicates the presence of unmethylated genes; the presence of a product in those lanes marked M indicates the presence of methylated genes. Lane 1 indicates the 50 bp DNA size marker.

Table 3). More than five-fold increased risk of tumor methylation was also observed for the CT genotype, in male CLC cases compared with age-matched male controls. The CT genotype also presented significantly increased tumor methylation in the proximal and in the older age group (OR = 2.7, 95% CI, 1.2-6.2; OR = 3.8, 95% CI, 1.2-12, respectively). The same trend was also observed for CT + TT genotypes. These results suggest that the C677T genotype of *MTHFR* can predispose some of CLC patients to the methylation of genes promoter.

### Serum folate/vitamin B<sub>12</sub> status and genes promoter methylation

Due to the previously observed interaction between folate and the *MTHFR* genotype in CLC<sup>[22]</sup>, we investigated the influence of serum folate/vitamin B<sub>12</sub> levels on tumor methylation in 86 fresh tissue samples in which their corresponding blood samples were also available. We observed no significant differences in serum folate/vitamin B<sub>12</sub> levels between cases and controls (Table 4). There were also no differences in association of serum folate/vitamin B<sub>12</sub> levels by sex, and tumor location. Comparing two age groups of patients, a trend for higher serum folate/vitamin B<sub>12</sub> levels was observed in older age group, with the older cases presenting 15% higher serum folate ( $P = 0.04$ ).

In case-control comparisons, we found no significant difference in serum folate/vitamin B<sub>12</sub> levels by *MTHFR* genotypes (data not shown). However, in case-case comparisons, serum folate levels appear to be associated with *MTHFR* genotypes (Table 5). Patients with the homozygous TT genotype had significantly lower concentrations of folate in their blood than those with CT or CC genotypes (Mann-Whitney test,  $P = 0.007$  and  $P = 0.03$ , respectively). We found no significant difference in serum vitamin B<sub>12</sub> concentrations between subjects with the CC and TT genotypes.

To test the association between serum folate/vitamin B<sub>12</sub> levels and methylation of genes promoter and tumors methylation, we stratified serum folate/

Table 2 Stratification analysis of tumors and genes promoter methylation frequencies

Variables	Methylation positive (%)						Methylated tumors <sup>2</sup> n (%)	OR (95% CI, P) <sup>3</sup>
	p16	P <sup>1</sup>	hMLH1	P <sup>1</sup>	hMSH2	P <sup>1</sup>		
Sex								
Male (90)	13 (14.4)	0.06	10 (11.1)	NS	4 (4.4)	NS	21 (23.3)	1
Female (61)	17 (27.9)		10 (16.4)		0 (0)		23 (37.7)	2.3 (1.1-5.04, 0.03)
Total (151)	30 (19.9)		20 (13.2)		4 (2.6)		44 (29.1)	
Age (yr)								
< 60 (66)	15 (22.7)	NS	7 (10.6)	NS	0 (0)	NS	19 (28.8)	1
≥ 60 (85)	15 (17.6)		13 (15.3)		4 (4.7)		25 (29.4)	1.1 (0.6-2.4, 0.7)
Tumor site								
Proximal (60)	29 (48.3)	0.00	20 (33.3)	0.00	4 (6.7)	0.02	43 (71.7)	1
Distal (91)	1 (1.1)		0 (0)		0 (0)		1 (1.1)	0.002 (0.00-0.023, 0.00)
Serum folate (86)								
Low (38)	2 (5.3)	0.04	4 (10.5)	NS	0 (0)	NS	4 (10.5)	1
High (48)	10 (20.8)		10 (20.8)		3 (6.3)		16 (33.3)	4.9 (1.4-17.7, 0.01)
Serum vitamin B <sub>12</sub> (86)								
Low (42)	2 (4.8)	0.02	5 (11.9)	NS	0 (0)	NS	5 (11.9)	1
High (44)	10 (22.7)		9 (20.5)		3 (6.8)		15 (34.1)	3.9 (1.1-13.9, 0.03)
MTHFR 677 genotype <sup>4</sup>								
CC (64)	13 (20.3)	NS	7 (10.9)	NS	0 (0)	NS	15 (23.4)	1
CT (80)	17 (21.3)	NS	12 (15)	NS	3 (3.8)	NS	28 (35)	1.92 (0.9-4.2, 0.09)
TT (7)	0 (0)	NS	1 (14.3)	NS	1 (14.3)	NS	1 (14.3)	0.7 (0.07-6.1, 0.7)
CT + TT (87)	17 (19.5)	NS	13 (14.9)	NS	4 (4.6)	NS	29 (33.3)	1.8 (0.8-3.9, 0.125)

<sup>1</sup>Fisher's exact test; <sup>2</sup>tumors with at least one methylated gene promoter; <sup>3</sup>the first category was taken as reference. Odds ratio adjusted for age, sex, and smoking status; <sup>4</sup>For genotype comparison CC used as the reference category. NS: Not significant.

Table 3 Association between MTHFR genotypes and tumor methylation in relation to clinical-biological features of CLC patients (case-control comparison, n)

Genotype (%)	Controls	Cases	Methylated	OR (95% CI, P) <sup>1,2</sup>	Unmethylated	OR (95% CI, P) <sup>1,2</sup>
Total	81	151	44		107	
CC	40 (49.4)	64 (42.4)	15 (34.1)	1	49 (45.8)	1
CT	31 (38.3)	80 (53)	28 (63.6)	2.5 (1.1-5.6, 0.02)	52 (48.6)	1.4 (0.7-2.6, 0.3)
CT or TT	41 (50.6)	87 (57.6)	29 (65.9)	2.1 (0.9-4.5, 0.07)	58 (54.2)	1.1 (0.7-2.1, 0.6)
Sex						
Male	50	90	21		69	
CC	28 (56)	33 (36.7)	5 (23.8)	1	28 (40.6)	1
CT	17 (34)	51 (56.7)	15 (71.4)	5.3 (1.6-18.2, 0.008)	36 (52.2)	1.9 (0.9-4.3, 0.09)
CT or TT	22 (44)	57 (63.3)	16 (76.2)	4.3 (1.3-14.3, 0.01)	41 (59.4)	1.7 (0.8-3.6, 0.1)
Female	31	61	23		38	
CC	12 (38.7)	31 (50.8)	10 (43.5)	1	21 (55.3)	1
CT	14 (45.2)	29 (47.5)	13 (56.5)	1.1 (0.3-3.6, 0.9)	16 (42.1)	0.7 (0.2-1.9, 0.5)
CT or TT	19 (61.3)	30 (49.2)	13 (56.5)	0.9 (0.3-2.8, 0.8)	17 (44.7)	0.5 (0.2-1.4, 0.2)
Age						
< 60	40	66	19		47	
CC	18 (45)	31 (47)	6 (31.6)	1	25 (53.2)	1
CT	18 (45)	34 (51.5)	13 (68.4)	1.4 (0.4-5.1, 0.6)	21 (44.7)	0.8 (0.3-1.9, 0.6)
CT or TT	22 (55)	35 (53)	13 (68.4)	1.1 (0.3-3.8, 0.9)	22 (46.8)	0.7 (0.3-1.6, 0.3)
≥ 60	41	85	25		60	
CC	22 (53.7)	33 (38.8)	9 (36)	1	24 (40)	1
CT	13 (31.7)	46 (54.1)	15 (60)	3.8 (1.2-12.0, 0.02)	31 (51.7)	2.6 (0.9-6.6, 0.09)
CT or TT	19 (46.3)	52 (61.2)	16 (64)	2.7 (0.9-8.2, 0.08)	36 (60)	1.9 (0.8-4.2, 0.1)
Site						
Proximal		60	43		17	
CC		22 (36.7)	15 (34.9)		7 (41.2)	
CT		37 (61.7)	27 (62.8)	2.7 (1.2-6.2, 0.02)	10 (58.8)	1.4 (0.5-4.8, 0.4)
CT or TT		38 (63.3)	28 (65.1)	2.1 (1.03-4.99, 0.05)	10 (58.8)	1.2 (0.4-3.7, 0.7)
Distal		91	1		90	
CC		42 (46.2)	0 (0)		42 (46.7)	
CT		43 (47.3)	1 (100)	ND	42 (46.7)	1.3 (0.7-2.5, 0.4)
CT or TT		49 (53.8)	1 (100)	ND	48 (53.3)	0.9 (0.5-1.6, 0.7)

<sup>1,2</sup>For odds ratio and 95% CI calculations, controls with CC genotype was used as reference category, odds ratio adjusted for age, sex and smoking status. ND: Not determined.

vitamin B<sub>12</sub> levels in two groups of low (below median), and high (above median) levels. The prevalence of

**Table 4** Associations between serum folate/vitamin B<sub>12</sub> concentrations and tumor methylation in relation to the clinical-biological characteristics of patients

Variables	<i>n</i> (%)	Folate (ng/mL) mean (range)	<i>P</i> <sup>1</sup>	High folate/Low folate <sup>2</sup> <i>n</i> (%)	<i>P</i> <sup>3</sup>	Vit. B <sub>12</sub> (pg/mL) mean (range)	<i>P</i> <sup>1</sup>	High B <sub>12</sub> /Low B <sub>12</sub> <sup>2</sup> <i>n</i> (%)	<i>P</i> <sup>3</sup>
Controls (81)		6.3 (3.8-12)	0.1 <sup>4</sup>	49 (60.5)/32 (39.5)	0.6 <sup>4</sup>	312.8 (108-995)	0.1 <sup>4</sup>	40 (49.4)/41 (50.6)	0.9 <sup>4</sup>
Total cases (86)		5.9 (2.1-12)		48 (55.8)/38 (44.2)		269 (50-681)		44 (51.2)/42 (48.8)	
Unmethylated	66 (76.7)	5.7 (2.4-12)		32 (48.5)/34 (51.5)		255.2 (79-681)		29 (43.9)/37 (56.1)	
Methylated	20 (23.3)	6.4 (2.1-10)	0.06	16 (80)/4 (20)	0.02	314.7 (50-571)	0.1	15 (75)/5 (25)	0.02
Sex									
Male (55)		5.9 (2.4-12)		29 (52.7)/26 (47.3)		274.2 (67-681)		28 (50.9)/27 (49.1)	
Unmethylated	43 (78.2)	5.8 (2.4-12)		19 (44.2)/24 (55.8)		325.5 (67-571)		18 (41.9)/25 (58.1)	
Methylated	12 (21.8)	6.5 (3.9-9.5)	0.1	10 (83.3)/2 (16.7)	0.02	254.4 (83-681)	0.04	10 (83.3)/2 (16.7)	0.02
Female (31)		5.7 (2.1-10)	0.6 <sup>5</sup>	19 (61.3)/12 (38.7)	0.5 <sup>5</sup>	259.7 (50-523)	0.7 <sup>5</sup>	16 (51.6)/15 (48.4)	1.00 <sup>5</sup>
Unmethylated	23 (74.2)	5.6 (3-8)		13 (56.5)/10 (43.5)		260.3 (79-521)		11 (47.8)/12 (52.2)	
Methylated	8 (25.8)	6.2 (2.1-10)	0.4	6 (75)/2 (25)	0.4	257.8 (50-523)	0.9	5 (62.5)/3 (37.5)	0.7
Age									
< 60 yr (36)		5.4 (2.1-8)		18 (50)/18 (50)		238.1 (50-571)		14 (38.9)/22 (61.1)	
Unmethylated	30 (83.3)	5.4 (3-8)		14 (46.7)/16 (53.3)		229.8 (79-465)		11 (36.7)/19 (63.3)	
Methylated	6 (16.7)	5.5 (2.1-7.9)	0.5	4 (66.6)/2 (33.3)	0.7	279.5 (50-571)	0.8	3 (50)/3 (50)	0.7
≥ 60 yr (50)		6.3 (2.4-12)	0.04 <sup>5</sup>	30 (60)/20 (40)	0.4 <sup>5</sup>	291.3 (67-681)	0.1 <sup>5</sup>	30 (60)/20 (40)	0.1 <sup>5</sup>
Unmethylated	36 (72)	6.1 (2.4-12)		18 (50)/18 (50)		276.3 (83-681)		18 (50)/18 (50)	
Methylated	14 (28)	6.8 (3.9-10)	0.1	12 (85.7)/2 (14.3)	0.03	329.7 (67-543)	0.1	12 (85.7)/2 (14.3)	0.03
Site									
Distal (62)		5.8 (2.4-12)		32 (51.6)/30 (48.4)		260.6 (79-681)		28 (45.2)/34 (54.8)	
Unmethylated	61 (98.4)	5.8 (2.4-12)		31 (50.8)/30 (49.2)		255.5 (79-681)		27 (44.3)/34 (55.7)	
Methylated	1 (1.6)	7.9	0.3	1 (100)/0 (0)	ND	271 (271)	0.1	1 (100)/0 (0)	ND
Proximal (24)		6.1 (2.1-10)	0.4 <sup>5</sup>	16 (66.7)/8 (33.3)	0.2 <sup>5</sup>	290.7 (50-543)	0.5 <sup>5</sup>	16 (66.7)/8 (33.3)	0.1 <sup>5</sup>
Unmethylated	5 (20.8)	5.3 (4.5-6.4)		1 (20)/4 (80)		251 (109-483)		2 (40)/3 (60)	
Methylated	19 (79.2)	6.3 (2.1-10)	0.06	15 (78.9)/4 (21.1)	0.03	301.2 (50-543)	0.5	14 (73.7)/5 (26.3)	0.3

<sup>1</sup>*P*-value from Mann-Whitney and *t*-test where appropriate; <sup>2</sup>Median was taken as cut off point for low and high categorical value for serum folate/vitamin B<sub>12</sub> levels (5.5 ng/mL, and 240 pg/mL, respectively); <sup>3</sup>*P* value from Fisher's exact test; <sup>4</sup>Case/control comparison; <sup>5</sup>Subgroups comparison, cases only. ND: Not determined.

**Table 5** Combined effects of *MTHFR* 677 genotype and serum folate/vitamin B<sub>12</sub> concentrations on risk of tumor methylation (case-case comparison)

<i>MTHFR</i> 677 genotype	<i>n</i> (%)	Folate (ng/mL) mean (range)	<i>P</i> <sup>1</sup>	High folate/Low folate <sup>2</sup> <i>n</i> (%)	<i>P</i> <sup>3</sup>	Vit. B <sub>12</sub> (pg/mL) mean (range)	<i>P</i> <sup>1</sup>	High B <sub>12</sub> /Low B <sub>12</sub> <sup>2</sup> <i>n</i> (%)	<i>P</i> <sup>3</sup>
Cases (86)									
CC (42)		5.8 (2.1-8.8)		25 (59.5)/17 (40.5)		254.6 (50-673)		20 (47.6)/22 (52.4)	
Unmethylated	34 (81)	5.9 (3.4-8.8)		20 (58.8)/14 (41.2)		267.3 (107-673)		16 (47.5)/18 (53.9)	
Methylated	8 (19)	5.4 (2.1-7.8)	0.6	5 (62.5)/3 (37.5)	1.00	200.9 (50-429)	0.2	4 (50)/4 (50)	1.00
CT (38)		6.3 (3-12)	0.5 <sup>5</sup>	22 (57.9)/16 (42.1)	1.00	296 (79-681)	0.4 <sup>5</sup>	21 (55.3)/17 (44.7)	0.5
Unmethylated	27 (71.1)	5.9 (3-12)		12 (44.4)/15 (55.6)		254.1 (79-681)		11 (40.7)/16 (53.9)	
Methylated	11 (28.9)	7.2 (5.3-10)	0.06	10 (90.9)/1 (9.1)	0.01	398.7 (150-571)	0.007	10 (90.9)/1 (9.1)	0.01
TT (6) <sup>4</sup>		4.2 (2.4-5.9)	0.03 <sup>5</sup>	1 (16.7)/5 (83.3)	0.08	198.5 (83-300)	0.4 <sup>5</sup>	3 (50)/3 (50)	1.00
Unmethylated	5 (83.3)	3.8 (2.4-5.1)		0/5 (100)		178.2 (83-258)		2 (40)/3 (60)	
Methylated	1 (16.7)	5.9		1 (100)/0		300		1 (100)/0	
CT + TT (44)		5.9 (2.4-12)	0.9 <sup>5</sup>	23 (52.3)/21 (47.7)	0.5	282.7 (79-681)	0.5 <sup>5</sup>	24 (54.5)/20 (45.5)	0.7
Unmethylated	32 (72.7)	5.6 (2.4-12)		12 (37.5)/20 (62.5)		242.3 (79-681)		13 (40.6)/19 (59.4)	
Methylated	12 (27.3)	7.1 (5.3-10)	0.002	11 (91.7)/1 (8.3)	0.002	390.5 (150-571)	0.002	11 (91.7)/1 (8.3)	0.003

<sup>1</sup>*P*-values for methylated and unmethylated cases, and genotype comparison, Mann-Whitney, *t*-test and Kruskal Wallis where appropriate; <sup>2</sup>Median was taken as cut off point for low and high categorical value for serum folate/vitamin B<sub>12</sub> levels (5.5 ng/mL, and 240 pg/mL, respectively); <sup>3</sup>*P*-value from Fisher's exact test; <sup>4</sup>Since we did not have enough TT cases, the association of serum methyl donors and tumor methylation was not determined in these individuals; <sup>5</sup>Comparison with the CC genotype, cases only.

hypermethylation within the promoter of *p16* gene, but not in either *bMLH1* or *bMSH2* genes, was higher in CLCs derived from patients with high serum folate (*P* = 0.04) and vitamin B<sub>12</sub> (*P* = 0.02) when compared with CLCs from patients with low serum folate/vitamin B<sub>12</sub> levels status (Table 2).

The association between tumor methylation and serum folate/vitamin B<sub>12</sub> levels is shown in Table 4. A small trend for higher levels of serum folate was found

in the entire group of patients with methylated tumors compared to those with unmethylated tumors (*P* = 0.06). The percentage of methylated tumors in patients with high serum methyl donors was also higher than those with low serum methyl donors (80% in high folate group, and 75% in high B<sub>12</sub> group; *P* = 0.02), whereas no such difference was found for unmethylated tumor group (Table 4). The OR for tumor methylation was 4.9 (95% CI, 1.4-17.7) for patients with high serum folate



versus low serum folate (Table 2). The risk of tumor methylation was also positively associated with serum vitamin B<sub>12</sub> status (OR = 3.9, 95% CI, 1.1-13.9). The high serum folate/vitamin B<sub>12</sub> levels were particularly associated with tumor methylation in males ( $P = 0.02$ ), but not in females ( $P = 0.4$ , Table 4). The association was also age and site dependent, being significant for older cases and those with proximal tumors (Table 4). Since we did not have enough distal methylated tumors, we could not examine the association of tumor methylation with serum folate status in such tumors.

### Joint effects of serum methyl donors, and *MTHFR* C677T genotypes on promoter methylation of tumor-associated genes

To investigate further whether the relationship between serum folate/vitamin B<sub>12</sub> status and DNA methylation is modified by the *MTHFR* genotype, we evaluated the joint effects of *MTHFR* codon 677 genotypes and serum folate/vitamin B<sub>12</sub> levels on tumor methylation. The combined effects of serum folate/vitamin B<sub>12</sub> levels and *MTHFR* polymorphism on tumor methylation are presented in Table 5. While the *CC* genotype showed no association with serum folate/vitamin B<sub>12</sub> levels with respect to tumors methylation, the *CT* and *CT/TT* genotypes of *MTHFR* exhibited a significant association of tumor methylation with high serum methyl donors. Insufficient *TT* cases eliminated the ability to examine their association with serum methyl donors and tumor methylation in these individuals. The frequency of methylated tumors was significantly different between cases with high and low serum methyl donors only in those with the *CT* and *CT/TT* genotype, but not with the *CC* genotype. More than 90% of methylated tumors in cases with *CT* and *CT/TT* genotypes had high serum methyl donors (Table 5). Among *CC* individuals no significant differences in mean serum folate/vitamin B<sub>12</sub> levels between cases with methylated and unmethylated tumors or an association between tumor methylation and folate/vitamin B<sub>12</sub> levels was observed. Therefore, the 677T allele seems to increase the risk of methylation associated with high serum folate/vitamin B<sub>12</sub>. We conclude that for the heterozygous or homozygous *C677T* genotypes, increased concentrations of folate and vitamin B<sub>12</sub> are associated with increased risks of tumor methylation. Our data suggest that the *MTHFR* *C677T* genotype might be a genetic modifier of the effect of the folate/vitamin B<sub>12</sub> status on the risk of methylation of genes promoter.

## DISCUSSION

It is well established that loss of proper gene expression in human cancer can occur through epigenetic mechanisms. The effect of a common polymorphism in the *MTHFR* gene (*C677T*) on colorectal cancer risk in relation to folate status is controversial. Both global DNA hypomethylation and gene promoter hypermethylation associated with the *MTHFR* *TT* genotype under low folate intake have been reported<sup>[12]</sup>.

In the present study, we investigated the association between the *MTHFR* *C677T* genotype and methylation of three putative tumor-associated genes, *p16*, *hMLH1*, and *hMSH2*, in 151 unselected series of sporadic CLC.

In our study (Table 2) the number of CLCs with at least one gene methylated was higher in females than males, and in those with proximal tumor location than those with distal tumors. Proximal tumor location, higher frequency in female subjects, and older age are characteristics that were previously associated with CIMP+ CLC<sup>[4,10]</sup>.

In comparison to controls, *MTHFR* *C677T* allele was associated with the elevation of tumor methylation in the entire group of cases, as well as in males and older patients (Table 3). Therefore, our finding is consistent with those reports in which increased genes promoter methylation was associated with the *MTHFR* *C677T* genotype in CLC<sup>[10,23]</sup>. We found no apparent association between methylation of any of the individual gene examined and the *MTHFR* genotypes. Therefore, DNA methylation at specific loci appears to be random. Consistent with a previous report that there are more frequent CIMP+ proximal tumors in subjects with alleles conferring low *MTHFR* enzyme activity<sup>[23]</sup>, the majority of methylated tumors with 677T variants in our study were also located in the proximal colon (Table 3).

Vitamin B<sub>12</sub> and folate are two important cofactors of methyl-group metabolism. We noted a trend for association between serum folate/vitamin B<sub>12</sub> levels and gene promoter methylation (Table 2). Higher serum folate and vitamin B<sub>12</sub> levels were strongly associated with promoter methylation of the key tumor suppressor gene *p16* ( $P = 0.04$ , and  $P = 0.02$ , respectively). There was also a trend, although not statistically significant, in the association between the serum folate/vitamin B<sub>12</sub> levels with promoter methylation of *hMLH1* and *hMSH2* genes.

Given the interaction between folate and the *MTHFR* genotype for CLC risk, we stratified the analyses of tumor methylation based on *MTHFR* genotypes and serum folate/vitamin B<sub>12</sub> status (Tables 4 and 5). In case-case comparisons, we found no significant difference in methyl donor status by age, sex, or tumor location. However, the serum folate level was significantly lower in cases homozygous for the *C677T* variant compared to those with the *CT* and *CC* genotypes ( $P = 0.04$ , Table 5). We found the *TT* variant of *MTHFR* associated with lower levels of folate in patient's sera. Although the blood folate level is mainly determined by dietary intake, the *MTHFR* *C677T* polymorphism might modify its metabolism and serum concentration<sup>[24]</sup>.

In our study, we found significant differences in the serum folate and vitamin B<sub>12</sub> levels in patients with methylated and unmethylated tumors (Table 4). None of the patients in our study used vitamin supplementation. Therefore, the increased tumor methylation observed in our study was associated with the high serum methyl donor status in physiological range. We noted also the same trend in the association between serum vitamin B<sub>12</sub> levels and tumor methylation (Table 4). Therefore, our data are consistent with those reports where a

positive association of dietary folate intake with DNA methylation and CLC risk was observed<sup>[25,26]</sup>.

Both folate deficiency and the *MTHFR* C677T polymorphism have been previously linked to global DNA hypomethylation in lymphocytes and colon tissue<sup>[27,28]</sup>. However, few studies have addressed the joint effects of methyl donors in blood and *MTHFR* genotypes on promoter-specific DNA methylation in malignancies<sup>[12,29]</sup>. Here, we assessed the association between methylation of genes promoter, the circulating levels of folate/vitamin B<sub>12</sub> and the influence of the *MTHFR* 677 genotypes in CLC patients. While no significant difference in serum folate/vitamin B<sub>12</sub> status was observed between those with methylated and unmethylated tumors in CC individuals, the CT, and CT/TT genotypes of *MTHFR* exhibited a significant positive correlation with elevated folate/vitamin B<sub>12</sub> levels for promoter methylation silencing (Table 5). Previously, an interaction between dietary folic acid and vitamin B<sub>12</sub> supplementation with promoter methylation in colorectal adenomas has been suggested, especially for subjects with *MTHFR* TT genotype<sup>[16,30]</sup>. Because we did not have enough TT cases, we could not evaluate correlation of serum folate levels with tumor methylation in these individuals. Among our study group there was only one TT individual with a methylated tumor who also had higher serum folate/vitamin B<sub>12</sub> level than TT cases with unmethylated tumors (Table 5). Genomic DNA methylation in leukocytes and in transformed human lymphoblasts was shown to be positively correlated to folate status in those with the TT genotype, but not with wild-type *MTHFR* CC genotype<sup>[12,27,31]</sup>. An inverse trend of serum and erythrocyte folate with DNA hypomethylation was also reported in normal colonic mucosa<sup>[28]</sup>.

In some studies, no interaction was reported between either *MTHFR* genotype and folate intake in association with CIMP + colon tumors<sup>[17,32]</sup>. Further investigation is needed focusing on ethnic variations in the relationships between the *MTHFR* polymorphism, folate intake, and tumors methylation in CLC. The majority of previous case-control studies have assessed dietary folate or vitamin B intake from questionnaires rather than their blood measurements, a procedure which is prone to some degree of miscalculation. Moreover, other factors like alcohol intake and iron status may be related to folate availability and biological activity<sup>[33]</sup>. In the present work, the direct measurement of serum folate/vitamin B<sub>12</sub> was correlated with CLC and tumor methylation. It has been previously reported that the colonic mucosal folate concentration correlates directly with serum folate concentration in the physiological range at each time point<sup>[34]</sup>. Therefore, the serum folate measurement could be an accurate reflection of the folate status in the colonic mucosa. High intracellular concentrations of folate intermediates are associated with aberrant methylation within promoter regions of cancer – associated genes in colorectal tumors<sup>[35]</sup>.

There is evidence that the epigenetic mechanism of gene silencing by methylation may play a differential role in proximal versus distal colon carcinogenesis. A different role for the *MTHFR* 677 TT genotype in the

tumorigenesis of proximal and distal CLC has been also suggested<sup>[8]</sup>. Our finding of an increased risk of tumor methylation associated with high serum folate/vitamin B<sub>12</sub> levels in those with proximal tumors, and in older patients (Table 4) might be related to the previous observation of a high concentration of folate in tumors from older patients and proximal CLC<sup>[35]</sup>.

Our results indicate that a high serum folate/vitamin B<sub>12</sub> in combination with a heterozygous or homozygous C677T *MTHFR* genotype, predisposes tumor-specific genes to promoter hypermethylation. Conversely, folate could be protective or have no effect in developing CLC in subjects with the wild type *MTHFR* 677 CC genotype. The *MTHFR* C677T mutation reduces MTHFR activity, which leads to lower levels of 5-methylTHF in individuals with a marginal folate status. However, in the presence of high folate levels, the negative effect of *MTHFR* TT on the efficiency of the methylation process might be masked possibly by maximizing the catalytic activity of MTHFR<sup>[36]</sup>. Indeed, under conditions of high folate status no differences in the Km or Vmax values were detected between the wild type and mutant enzymes<sup>[37]</sup>. Therefore, hypermethylation of CpG islands could occur in individuals with the *MTHFR* 677T allele under high folate status.

A major draw-back of the present study is the fact that the serum data collection occurred after the onset of tumor formation. Although, more studies are needed to determine whether *MTHFR* C677T genotypes, together with high serum folate/vitamin B<sub>12</sub> levels, could serve as risk factors for the CIMP + CLC subgroup, the findings of this study are in agreement with other recent reports which together provide additional evidence for caution in the mandatory fortification of cereals with folic acid.

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## COMMENTS

### Backgrounds

Transcriptional silencing of tumor suppressor genes by hypermethylation of capillary blood gases (CpG) islands located in the promoter region is very common in human colorectal cancer. *P16*, *hMLH1*, *hMSH2* are key tumor suppressor genes frequently silenced by promoter methylation in sporadic colon cancer (CLC). Methylentetrahydrofolate reductase (*MTHFR*) C677T genotype has been associated with reduced enzyme activity and altered cellular folate composition. In this study, we investigated the association between serum folate/vitamin B<sub>12</sub>, *MTHFR* C677T genotype, and promoter methylation of three tumor-associated genes in solid tumors among sporadic CLC patients.

### Research frontiers

Dietary folate/vitamin B<sub>12</sub> intake and *MTHFR* C677T genotype was suggested to protect against colorectal cancer. However, only a few studies have addressed the joint effects of circulating levels of folate/vitamin B<sub>12</sub> and the *MTHFR* C677T genotype on the risk of epigenetic inactivation of specific tumor suppressor genes in CLC patients.

### Innovations and breakthroughs

Our data indicate that serum folate/vitamin B<sub>12</sub> levels are directly associated with the DNA hypermethylation of CpG island within promoter of the tumor specific genes and to the C677T genotype of *MTHFR*. We identified that

the T allele of *MTHFR* has strong influence on the risk of tumor methylation associated with high serum folate/vitamin B<sub>12</sub> levels.

### Applications

The results from the study support other recent reports that high folate and vitamin B<sub>12</sub> status might serve as risk factors for CLC. This study provides additional evidence for caution in terms of CLC risk because of the mandatory fortification of cereals with folic acid in certain countries.

### Peer review

This is a population-based, case-controlled, molecular epidemiological study on the interaction of *MTHFR* C677T genotype and circulating folate/vitamin B<sub>12</sub> with the CpG island hypermethylation of tumor-associated genes in sporadic colorectal cancer. This result indicated that for the *MTHFR* C677T genotypes, increased concentrations of folate and vitamin B<sub>12</sub> are associated with increased risks of tumor methylation. This demonstration might give a suggestion to protect against colorectal cancer, at least, in Iranian sporadic CLC population.

## REFERENCES

- Parkin DM, Bray F, Ferlay J, Pisani P. Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001; **94**: 153-156
- Yazdizadeh B, Jarrahi AM, Mortazavi H, Mohagheghi MA, Tahmasebi S, Nahvijo A. Time trends in the occurrence of major GI cancers in Iran. *Asian Pac J Cancer Prev* 2005; **6**: 130-134
- Hosseini SV, Izadpanah A, Yarmohammadi H. Epidemiological changes in colorectal cancer in Shiraz, Iran: 1980-2000. *ANZ J Surg* 2004; **74**: 547-549
- Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP. CpG island methylator phenotype in colorectal cancer. *Proc Natl Acad Sci USA* 1999; **96**: 8681-8686
- Herman JG. Hypermethylation pathways to colorectal cancer. Implications for prevention and detection. *Gastroenterol Clin North Am* 2002; **31**: 945-958
- Cellarier E, Durando X, Vasson MP, Farges MC, Demiden A, Maurizis JC, Madelmont JC, Chollet P. Methionine dependency and cancer treatment. *Cancer Treat Rev* 2003; **29**: 489-499
- Frosst P, Blom HJ, Milos R, Goyette P, Sheppard CA, Matthews RG, Boers GJ, den Heijer M, Kluijtmans LA, van den Heuvel LP. A candidate genetic risk factor for vascular disease: a common mutation in methylenetetrahydrofolate reductase. *Nat Genet* 1995; **10**: 111-113
- Toffoli G, Gafa R, Russo A, Lanza G, Dolcetti R, Sartor F, Libra M, Viel A, Boiocchi M. Methylenetetrahydrofolate reductase 677 C-->T polymorphism and risk of proximal colon cancer in north Italy. *Clin Cancer Res* 2003; **9**: 743-748
- Choi SW, Mason JB. Folate status: effects on pathways of colorectal carcinogenesis. *J Nutr* 2002; **132**: 2413S-2418S
- van Rijnsoever M, Grieu F, Elsaleh H, Joseph D, Iacopetta B. Characterisation of colorectal cancers showing hypermethylation at multiple CpG islands. *Gut* 2002; **51**: 797-802
- Heijmans BT, Boer JM, Suchiman HE, Cornelisse CJ, Westendorp RG, Kromhout D, Feskens EJ, Slagboom PE. A common variant of the methylenetetrahydrofolate reductase gene (1p36) is associated with an increased risk of cancer. *Cancer Res* 2003; **63**: 1249-1253
- Friso S, Choi SW, Girelli D, Mason JB, Dolnikowski GG, Bagley PJ, Olivieri O, Jacques PF, Rosenberg IH, Corrocher R, Selhub J. A common mutation in the 5,10-methylene tetrahydrofolate reductase gene affects genomic DNA methylation through an interaction with folate status. *Proc Natl Acad Sci USA* 2002; **99**: 5606-5611
- Kim YI. Folate and DNA methylation: a mechanistic link between folate deficiency and colorectal cancer? *Cancer Epidemiol Biomarkers Prev* 2004; **13**: 511-519
- Bollheimer LC, Buettner R, Kullmann A, Kullmann F. Folate and its preventive potential in colorectal carcinogenesis. How strong is the biological and epidemiological evidence? *Crit Rev Oncol Hematol* 2005; **55**: 13-36
- Sanjoaquin MA, Allen N, Couto E, Roddam AW, Key TJ. Folate intake and colorectal cancer risk: a meta-analytical approach. *Int J Cancer* 2005; **113**: 825-828
- van den Donk M, van Engeland M, Pellis L, Witteman BJ, Kok FJ, Keijer J, Kampman E. Dietary folate intake in combination with *MTHFR* C677T genotype and promoter methylation of tumor suppressor and DNA repair genes in sporadic colorectal adenomas. *Cancer Epidemiol Biomarkers Prev* 2007; **16**: 327-333
- Curtin K, Slattery ML, Ulrich CM, Bigler J, Levin TR, Wolff RK, Albertsen H, Potter JD, Samowitz WS. Genetic polymorphisms in one-carbon metabolism: associations with CpG island methylator phenotype (CIMP) in colon cancer and the modifying effects of diet. *Carcinogenesis* 2007; **28**: 1672-1679
- Hill AE, FitzPatrick DR. MS-PCR assay to detect 677C-->T mutation in the 5,10-methylenetetrahydrofolate reductase gene. *J Inherit Metab Dis* 1998; **21**: 694-695
- Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc Natl Acad Sci USA* 1996; **93**: 9821-9826
- Matsushita S, Muramatsu T, Arai H, Matsui T, Higuchi S. The frequency of the methylenetetrahydrofolate reductase-gene mutation varies with age in the normal population. *Am J Hum Genet* 1997; **61**: 1459-1460
- Heijmans BT, Gussekloo J, Kluit C, Droog S, Lagaay AM, Knook DL, Westendorp RG, Slagboom EP. Mortality risk in men is associated with a common mutation in the methylene-tetrahydrofolate reductase gene (*MTHFR*). *Eur J Hum Genet* 1999; **7**: 197-204
- Ueland PM, Hustad S, Schneede J, Refsum H, Vollset SE. Biological and clinical implications of the *MTHFR* C677T polymorphism. *Trends Pharmacol Sci* 2001; **22**: 195-201
- Oyama K, Kawakami K, Maeda K, Ishiguro K, Watanabe G. The association between methylenetetrahydrofolate reductase polymorphism and promoter methylation in proximal colon cancer. *Anticancer Res* 2004; **24**: 649-654
- Jacques PF, Bostom AG, Williams RR, Ellison RC, Eckfeldt JH, Rosenberg IH, Selhub J, Rozen R. Relation between folate status, a common mutation in methylenetetrahydrofolate reductase, and plasma homocysteine concentrations. *Circulation* 1996; **93**: 7-9
- Van Guelpen B, Hultdin J, Johansson I, Hallmans G, Stenling R, Riboli E, Winkvist A, Palmqvist R. Low folate levels may protect against colorectal cancer. *Gut* 2006; **55**: 1461-1466
- Keyes MK, Jang H, Mason JB, Liu Z, Crott JW, Smith DE, Friso S, Choi SW. Older age and dietary folate are determinants of genomic and p16-specific DNA methylation in mouse colon. *J Nutr* 2007; **137**: 1713-1717
- Stern LL, Mason JB, Selhub J, Choi SW. Genomic DNA hypomethylation, a characteristic of most cancers, is present in peripheral leukocytes of individuals who are homozygous for the C677T polymorphism in the methylene tetrahydrofolate reductase gene. *Cancer Epidemiol Biomarkers Prev* 2000; **9**: 849-853
- Pufulete M, Al-Ghnam R, Rennie JA, Appleby P, Harris N, Gout S, Emery PW, Sanders TA. Influence of folate status on genomic DNA methylation in colonic mucosa of subjects without colorectal adenoma or cancer. *Br J Cancer* 2005; **92**: 838-842
- Kraunz KS, Hsiung D, McClean MD, Liu M, Osanyingbemi J, Nelson HH, Kelsey KT. Dietary folate is associated with p16(*INK4A*) methylation in head and neck squamous cell carcinoma. *Int J Cancer* 2006; **119**: 1553-1557
- van den Donk M, Pellis L, Crott JW, van Engeland M, Friederich P, Nagengast FM, van Bergeijk JD, de Boer SY, Mason JB, Kok FJ, Keijer J, Kampman E. Folic acid and vitamin B-12 supplementation does not favorably influence uracil incorporation and promoter methylation in rectal mucosa DNA of subjects with previous colorectal adenomas. *J Nutr* 2007; **137**: 2114-2120
- Chiang EP, Wang YC, Tang FY. Folate restriction and

- methylenetetrahydrofolate reductase 677T polymorphism decreases adoMet synthesis via folate-dependent remethylation in human-transformed lymphoblasts. *Leukemia* 2007; **21**: 651-658
- 32 **Clarizia AD**, Bastos-Rodrigues L, Pena HB, Anacleto C, Rossi B, Soares FA, Lopes A, Rocha JC, Caballero O, Camargo A, Simpson AJ, Pena SD. Relationship of the methylenetetrahydrofolate reductase C677T polymorphism with microsatellite instability and promoter hypermethylation in sporadic colorectal cancer. *Genet Mol Res* 2006; **5**: 315-322
- 33 **Konings EJ**, Goldbohm RA, Brants HA, Saris WH, van den Brandt PA. Intake of dietary folate vitamers and risk of colorectal carcinoma: results from The Netherlands Cohort Study. *Cancer* 2002; **95**: 1421-1433
- 34 **Kim YI**, Fawaz K, Knox T, Lee YM, Norton R, Libby E, Mason JB. Colonic mucosal concentrations of folate are accurately predicted by blood measurements of folate status among individuals ingesting physiologic quantities of folate. *Cancer Epidemiol Biomarkers Prev* 2001; **10**: 715-719
- 35 **Kawakami K**, Ruszkiewicz A, Bennett G, Moore J, Watanabe G, Iacopetta B. The folate pool in colorectal cancers is associated with DNA hypermethylation and with a polymorphism in methylenetetrahydrofolate reductase. *Clin Cancer Res* 2003; **9**: 5860-5865
- 36 **Moat SJ**, Ashfield-Watt PA, Powers HJ, Newcombe RG, McDowell IF. Effect of riboflavin status on the homocysteine-lowering effect of folate in relation to the MTHFR (C677T) genotype. *Clin Chem* 2003; **49**: 295-302
- 37 **Yamada K**, Chen Z, Rozen R, Matthews RG. Effects of common polymorphisms on the properties of recombinant human methylenetetrahydrofolate reductase. *Proc Natl Acad Sci USA* 2001; **98**: 14853-14858

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BASIC RESEARCH

## Ellagic acid induces apoptosis through inhibition of nuclear factor $\kappa$ B in pancreatic cancer cells

Mouad Edдерkaoui, Irina Odínokova, Izumi Ohno, Ilya Gukovsky, Vay Liang W Go, Stephen J Pandol, Anna S Gukovskaya

Mouad Edдерkaoui, Irina Odínokova, Izumi Ohno, Ilya Gukovsky, Vay Liang W Go, Stephen J Pandol, Anna S Gukovskaya, Department of Medicine, Veterans Affairs Greater Los Angeles Healthcare System and University of California, Los Angeles CA 90073, California, United States

Irina Odínokova, Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences, Pushchino, Moscow Region 142290, Russia

**Author contributions:** Edдерkaoui M and Gukovskaya AS designed research; Edдерkaoui M, Odínokova I, Ohno I and Gukovsky I performed research; Edдерkaoui M, Go VLW, Pandol SJ and Gukovskaya AS analyzed data; Edдерkaoui M and Gukovskaya AS wrote the paper.

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**Correspondence to:** Anna S Gukovskaya, PhD, VA Greater Los Angeles Healthcare System, West Los Angeles VA Healthcare Center, 11301 Wilshire Blvd, Bldg 258, Rm 340, Los Angeles CA 90073, United States. [agukovsk@ucla.edu](mailto:agukovsk@ucla.edu)  
Telephone: +1-310-4783711-41525 Fax: +1-310-2684578

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activation. Ellagic acid does not directly affect mitochondria. Ellagic acid dose-dependently decreased NF- $\kappa$ B binding activity. Furthermore, inhibition of NF- $\kappa$ B activity using I $\kappa$ B wild type plasmid prevented the effect of ellagic acid on apoptosis.

**CONCLUSION:** Our data indicate that ellagic acid stimulates apoptosis through inhibition of the pro-survival transcription factor NF- $\kappa$ B.

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**Key words:** Ellagic acid; Nuclear factor- $\kappa$ B; Apoptosis; Pancreatic cancer

**Peer reviewer:** Minoti Vivek Apte, Pancreatic Research Group, South Western Sydney Clinical School, the University of New South Wales, Level 2, Thomas and Rachel Moore Education Centre, Liverpool Hospital, New South Wales 2170, Liverpool, Australia

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### Abstract

**AIM:** To determine the effect of ellagic acid on apoptosis and proliferation in pancreatic cancer cells and to determine the mechanism of the pro-survival effects of ellagic acid.

**METHODS:** The effect of ellagic acid on apoptosis was assessed by measuring Phosphatidylserine externalization, caspase activity, mitochondrial membrane potential and DNA fragmentation; and proliferation by measuring DNA thymidine incorporation. Mitochondrial membrane potential was measured in permeabilized cells, and in isolated mitochondria. Nuclear factor  $\kappa$ B (NF- $\kappa$ B) activity was measured by electromobility shift assay (EMSA).

**RESULTS:** We show that ellagic acid, a polyphenolic compound in fruits and berries, at concentrations 10 to 50 mmol/L stimulates apoptosis in human pancreatic adenocarcinoma cells. Further, ellagic acid decreases proliferation by up to 20-fold at 50 mmol/L. Ellagic acid stimulates the mitochondrial pathway of apoptosis associated with mitochondrial depolarization, cytochrome C release, and the downstream caspase

### INTRODUCTION

Pancreatic cancer is a very aggressive disease and is the fourth most common cause of death in Western countries with almost the same rate of incidence and mortality per year<sup>[1,2]</sup>. Pancreatic cancer is very resistant to radio- and chemo-therapies. One reason for that is the resistance of pancreatic cancer cells to apoptosis<sup>[3]</sup>.

During the past decade, significant progress has been achieved in understanding the molecular mechanisms of apoptosis<sup>[4]</sup>. The information obtained suggests that the commitment to apoptosis occurs through activation of caspases, a unique family of cysteine proteases<sup>[4]</sup>. Caspases are synthesized as inactive precursors and are, generally, activated by proteases including caspases themselves. Thus, caspases can function in an activation cascade.

Cancer cells protect themselves from apoptosis by

upregulation of prosurvival mechanisms. Activation of the transcription factor NF- $\kappa$ B is a key pro-survival mechanism in cancer cells<sup>[5,6]</sup>. NF- $\kappa$ B is constitutively active in pancreatic cancer cells, and its inhibition leads to pancreatic cancer cell death and inhibition of tumor development<sup>[7]</sup>.

Ellagic acid (C<sub>14</sub>H<sub>6</sub>O<sub>8</sub>) is a polyphenolic compound present in fruits and berries such as pomegranates, strawberries, raspberries and blackberries. It has anticarcinogenic, antioxidant and antifibrosis properties<sup>[8-11]</sup>. The anticarcinogenic effect of ellagic acid was shown in several types of cancers including skin, esophageal, and colon cancers<sup>[11,12]</sup>. However, the effects of ellagic acid on pancreatic cancer have not been studied. Furthermore, the mechanisms mediating anticancer effect of ellagic acid, in general, remain unknown.

There is a growing interest in natural compounds for enhancing cancer prevention and treatment. In this study, we show that ellagic acid induces apoptosis and decreases proliferation in pancreatic cancer cells. We demonstrate that ellagic acid stimulates apoptosis in pancreatic cancer cells through inhibiting transcription factor NF- $\kappa$ B activity.

## MATERIALS AND METHODS

### Reagents

Ac-Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (Ac-DEVD-AMC) was from Peptide Institute, Inc (Osaka, Japan). Antibody against cytochrome C was from BD Biosciences (San Diego, CA). CAPE was from biomol (Plymouth meeting, PA). Ellagic acid and all other reagents were from Sigma Chemical (St. Louis, MO).

### Cell culture

Human pancreatic adenocarcinoma cell lines, the poorly differentiated MIA PaCa-2 and the moderately differentiated PANC-1, were obtained from the American Type Culture Collection (Manassas, VA). MIA PaCa-2 and PANC-1 cells were grown in 1/1 D-MEM/F-12 medium (GIBCO Invitrogen Corporation, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS), 4 mmol/L L-glutamine, and 1% antibiotic/antimycotic solution (Omega Scientific, Tarzana, CA). Cells were maintained at 37°C in a humidified atmosphere containing 5% CO<sub>2</sub> and were used between passages 4 and 12.

MIA PaCa-2 and PANC-1 cells were plated at a density of  $2 \times 10^6$ /mL on 100-mm culture dishes, cultured for up to 48 h in D-MEM/F-12 medium supplemented with FBS, glutamine and the antibiotic/antimycotic solution, collected, and processed for the specified analyses. Inhibitors or vehicle were added to the culture medium just before plating out the cells.

### Preparation of cytosolic and membrane fractions

Cells were resuspended in a lysis buffer (250 mmol/L sucrose, 20 mmol/L HEPES, 10 mmol/L KCl,

1 mmol/L Na-EGTA, 1 mmol/L Na-EDTA, 2 mmol/L MgCl<sub>2</sub>, pH 7.0), allowed to swell for 30 min at 4°C, and then disrupted by 80 strokes in a Dounce homogenizer. Homogenates were centrifuged at 1000 *g* for 5 min to pellet nuclei and cell debris. Supernatants were centrifuged at 16000 *g* for 30 min, and the cytosolic fractions (supernatants) were collected. Pellets (heavy membranes enriched with mitochondria) were lysed in RIPA buffer (0.15 mol/L NaCl, 50 mmol/L Tris, 1% deoxycholic acid, 1% Triton X-100, 0.1% sodium dodecyl sulfate, pH 7.2) for 1 h. To determine the quality of cytosolic and mitochondrial separation, both fractions were assessed by immunoblotting for the mitochondrial marker cytochrome C oxidase subunit IV (COX IV).

### Western blot analysis

Cells were incubated in a lysis buffer (0.5 mmol/L EDTA, 150 mmol/L NaCl, 50 mmol/L Tris, 0.5% Nonidet P-40, pH 7.5) for 30 min at 4°C. The lysis buffer was supplemented with 1 mmol/L PMSF, 5 g/mL each of protease inhibitors pepstatin, leupeptin, chymostatin, antipain, and aprotinin. Cell lysates were centrifuged for 10 min at 13000 *g*. Supernatants were collected and proteins were separated by SDS-PAGE (Invitrogen) and electrophoretically transferred to nitrocellulose membranes. Non-specific binding was blocked with 5% milk in Tris-buffered saline (4 mmol/L Tris base, 100 mmol/L NaCl, pH 7.5). Membranes were washed in Tris-buffered saline containing 0.05% Tween 20 (TTBS) and incubated for 2 h with the indicated primary antibodies and then for 1 h with horseradish peroxidase-conjugated secondary antibody. Blots were developed with the Supersignal Chemiluminescent Substrate (ECL) (Pierce).

### Measurements of apoptosis

**Apoptosis parameters were measured as previously described<sup>[13-16]</sup>:** Internucleosomal DNA fragmentation was measured by using Cell Death Detection ELISA<sup>Plus</sup> kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's instructions.

**Phosphatidylserine (PS) externalization:** PS externalization was analyzed with the Annexin-V (AnV)-FLUOS Staining Kit from Roche Biochemicals (Indianapolis, IN) as we described before<sup>[13]</sup>. Cells were collected and resuspended at a density of  $1 \times 10^6$  cells in 500  $\mu$ L of binding buffer containing 2  $\mu$ L AnV and 1  $\mu$ L propidium iodide (PI), incubated in the dark for 30 min at room temperature, and analyzed by flow cytometry.

**Effector caspase (DEVDase) activity:** DEVDase activity was measured by a fluorogenic assay in whole cell lysates using DEVD-AMC as a substrate, as we described before<sup>[13]</sup>. The lysate (50-100  $\mu$ g of protein) was incubated with 10  $\mu$ mol/L substrate

in a reaction buffer (25 mmol/L HEPES (pH 7.5), 10% sucrose, 0.1% CHAPS, 10 mmol/L DTT) at 37°C. Caspase substrate cleavage releases AMC, which emits fluorescent signal with 380 nm excitation and 440 nm emission. Fluorescence was calibrated using a standard curve for AMC.

#### **Measurement of mitochondrial membrane potential ( $\Delta\psi_m$ )**

Changes in  $\Delta\psi_m$  were detected with the potential-sensitive probes 3, 3' dihexyloxa-carbocyanine DiOC6 (3) (Molecular Probes, Eugene, OR). Cells were incubated with 100 nmol/L DiOC6 (3) for 30 min at 37°C in the dark, washed twice with PBS, and analyzed on a FACScan using FL-1. To completely dissipate  $\Delta\psi_m$ , cells were treated with the uncoupling agent CCCP (50  $\mu$ mol/L) for 1 h before DiOC6 (3) staining.

#### **Registration of mitochondrial respiration and membrane potential in cells permeabilized with digitonin**

MIA PaCa-2 cells ( $5 \times 10^6$ ) were washed twice with PBS and resuspended in 50  $\mu$ L DMEM/F12 medium without serum. The medium for mitochondrial functional assays contained 250 mmol/L sucrose, 22 mmol/L KCl, 22 mmol/L triethanolamine (pH 7.4), 3 mmol/L  $MgCl_2$ , 5 mmol/L  $KH_2PO_4$ , 0.5% BSA. Glutamate (10 mmol/L) and malate (2 mmol/L) were used as mitochondrial respiratory substrates. Digitonin at concentration 0.001% was added to cell suspension to permeabilize plasma membrane and to allow substrates and chemicals to reach mitochondria immediately after addition into reaction medium. The measurements were performed at 25°C. Membrane potential and oxygen consumption were monitored simultaneously in a 1-mL custom-made chamber. Oxygen consumption was measured using a Clark-type electrode (Instech Lab., Plymouth Meeting, PA) connected to an oxygen meter (Yellow Springs Instruments, Yellow Springs, OH). Mitochondrial membrane potential was registered in the presence of 1  $\mu$ mol/L tetraphenyl phosphonium ( $TPP^+$ ) using a  $TPP^+$ -sensitive electrode connected to an amplifier (Vernier Software, Beaverton, OR). An increase in  $\Delta\psi_m$  causes  $TPP^+$  uptake by mitochondria and, correspondingly, a decrease in external  $TPP^+$  measured with the electrode.

Another approach applied to measure  $\Delta\psi_m$  is by using  $\Delta\psi_m$ -sensitive fluorescent probe tetramethylrhodamine methyl ester (TMRM; Molecular Probes, Eugene, OR). Changes in the fluorescence intensity were measured in the cell suspension containing 0.5  $\mu$ mol/L TMRM in the 2-mL cuvette in a RF-1501 spectrofluorophotometer (Simadzu, Japan) with 543 nm excitation, and 578 nm emission.  $\Delta\psi_m$ -driven mitochondrial uptake of TMRM causes TMRM quenching, which results in decreased fluorescence intensity.

#### **Isolation of mitochondria**

Mitochondria from Mia PaCa-2 cells were isolated using

differential centrifugation. Cells (approximately  $2 \times 10^8$ ) were homogenized using motor-driven tissue grinder in the isolation buffer containing 320 mmol/L sucrose, 10 mmol/L Tris-HCl (pH 7.4), 0.5 mmol/L EGTA, 0.5 mmol/L EDTA and 0.2% BSA. Unbroken cells were spun down by centrifugation at 500 g for 5 min, followed by nuclei centrifugation at 2000 g for 3 min. The resulting supernatant containing mitochondria was spun down at 12500 g for 10 min; then mitochondria were washed in a washing buffer containing 320 mmol/L sucrose and 10 mmol/L Tris-HCL (pH 7.4); and finally the mitochondrial pellet was re-suspended in the washing buffer. The obtained mitochondrial suspension contained 8 to 12 mg protein/mL as determined by the Bradford protein assay (BioRad Laboratories). All isolation steps were performed at 4°C, and the mitochondria were kept on ice all the time. Mitochondrial membrane potential and oxygen consumption were measured using  $TPP^+$ -sensitive and Clark-type electrodes correspondingly in the same incubation buffer as described above for digitonin-permeabilized cells.

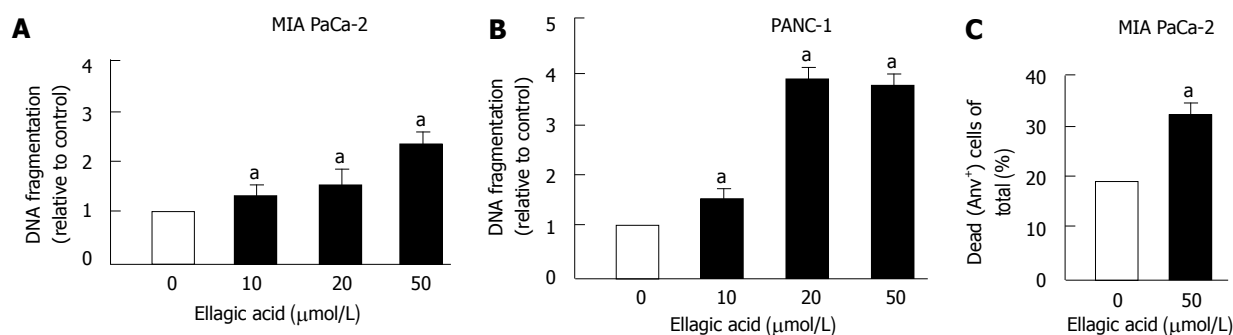
#### **Measurement of cytochrome C release from isolated mitochondria**

MIA PaCa-2 cell mitochondria were incubated in the absence or presence of ellagic acid for 10 min. Aliquots of mitochondrial suspension were collected, centrifuged at 13500 g for 10 min at 4°C, and cytochrome C levels in the mitochondria (pellet) and the medium (supernatant) were measured by Western blot as previously described<sup>[13]</sup>.

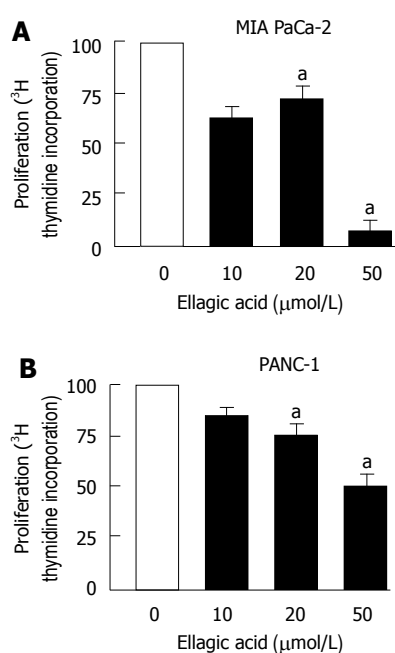
#### **Preparation of nuclear extracts and electromobility shift assay (EMSA)**

Preparation of nuclear extracts and EMSA have been described in detail<sup>[17-19]</sup>. Briefly, pancreatic cancer cells were lysed on ice in a hypotonic buffer A<sup>[17]</sup> supplemented with 1 mmol/L PMSF, 1 mmol/L DTT, and protease inhibitor cocktail containing 5  $\mu$ g/mL each of pepstatin, leupeptin, chymostatin, antipain, and aprotinin. Cells were left to swell on ice for a 20 min to 25 min period; 0.3% Igepal CA-630 was then added, and the nuclei were collected by microcentrifugation. The nuclear pellet was resuspended in a high-salt buffer C<sup>[17]</sup> supplemented with 1 mmol/L PMSF, 1 mmol/L DTT, and the protease inhibitor cocktail described above. After incubating at 4°C, membrane debris was pelleted by microcentrifugation for 10 min, and the clear supernatant (nuclear extract) was aliquoted and stored at -80°C. Protein concentration in the extracts was determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA).

For the EMSA, aliquots of nuclear extracts with equal amounts of protein (5-10  $\mu$ g) were mixed in 20- $\mu$ L reactions with a buffer containing 10 mmol/L HEPES (pH 7.8), 50 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L DTT, 10% glycerol, and 3  $\mu$ g poly (dI-dC). Binding reactions were started by the



**Figure 1** Ellagic acid stimulates apoptosis in pancreatic cancer cells. MIA PaCa-2 (A, C) and PANC-1 (B) cells were cultured for 48 h in the presence or absence of indicated doses of ellagic acid. Internucleosomal DNA fragmentation was measured using the Cell Death Detection ELISA kit (A, B); Dead cells were assessed by flow cytometry using AnV/PI staining (C). AnV<sup>+</sup>/PI<sup>+</sup> and AnV<sup>+</sup>/PI<sup>-</sup> cells were considered dying through apoptosis and/or secondary necrosis. Values are normalized to control (A, B). Values are mean  $\pm$  SE ( $n = 3$ ), <sup>a</sup> $P < 0.05$  vs control.



**Figure 2** Ellagic acid inhibits proliferation in pancreatic cancer cells. MIA PaCa-2 (A, C) and PANC-1 (B) cells were cultured for 48 h in the presence or absence of indicated doses of ellagic acid. Proliferation was assessed by measuring (<sup>3</sup>H) thymidine incorporation into DNA. The results are representative of at least 3 independent experiments, <sup>a</sup> $P < 0.05$  vs control.

addition of <sup>32</sup>P-labeled DNA probe and incubated at room temperature for 20 min. The oligo probe 5'-GCAGAGGGGACTTTCGAGA-3' containing  $\kappa$ B binding motif (underlined) was annealed to the complementary oligonucleotide and end-labeled by using T4 polynucleotide kinase. Samples were electrophoresed on a native 4.5% polyacrylamide gel at 200 V in 0.5 TBE buffer (1  $\times$  TBE: 89 mmol/L Tris base, 89 mmol/L boric acid, 2 mmol/L EDTA). Gels were dried and densitometrically quantified in the Phosphor-Imager (Molecular Dynamics, Sunnyvale, CA). In pancreatic cancer cells, the NF- $\kappa$ B band has two components: the upper component corresponds to the p50/p65 heterodimer and the lower component to the p50/p50 homodimer. In the present study, we quantified the total (combined) intensity of the NF- $\kappa$ B band.

### Cell transfection

For this a luciferase reporter gene system was used. Briefly, MIA PaCa-2 cells were simultaneously transfected with the 4KBwt-pRL-TK luciferase plasmid, which expresses the NF- $\kappa$ B inhibitor I- $\kappa$ B and pRL-TK luciferase (as a reference) using the Nucleofector™ II (Amaxa Inc, Gaithersburg, MD) according to the manufacturer protocol. The transfection efficiency and NF- $\kappa$ B transcriptional activity was assessed by using the Dual-Luciferase Reporter Assay System (Promega Corporation, Madison WI).

### Statistical analysis

Results are expressed as mean  $\pm$  SE from at least 3 independent experiments. Statistical analysis was done using the unpaired Student's *t*-test.  $P < 0.05$  was considered statistically significant.

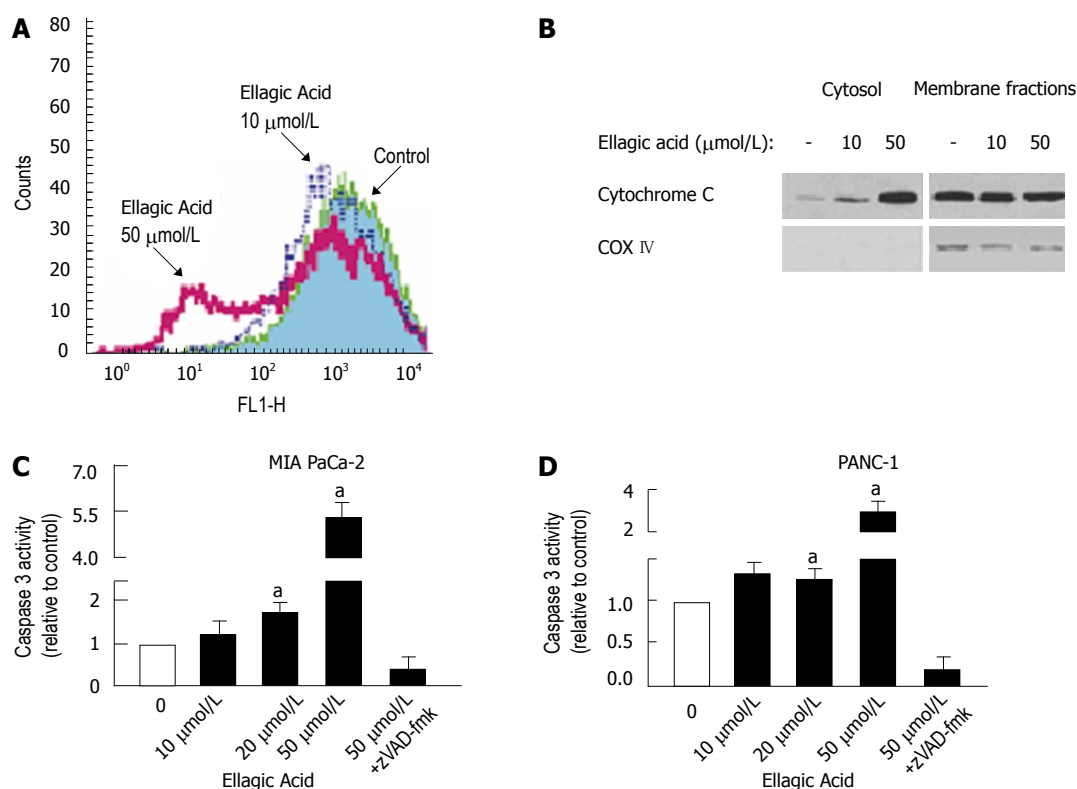
## RESULTS

### Ellagic acid stimulates apoptosis and inhibits proliferation of pancreatic cancer cells

Ellagic acid dose-dependently increased apoptosis in PaCa cells (Figure 1). To measure apoptosis we used 2 approaches. First, we showed that ellagic acid stimulates apoptotic internucleosomal DNA fragmentation in MIA PaCa-2 (Figure 1A) and PANC-1 cells (Figure 1B). Second, we used flow cytometry and AnV/PI staining to measure the percentage of dead cells as described in Experimental Procedures. We previously showed that the AnV<sup>+</sup>/PI<sup>-</sup> group includes cells at early stages of apoptosis, whereas the AnV<sup>+</sup>/PI<sup>+</sup> group includes both necrotic cells and apoptotic cells associated with secondary necrosis<sup>[13]</sup>. Ellagic acid increased the percentage of dead MIA PaCa-2 cells (i.e. stained positively for both AnV and PI or for AnV alone) (Figure 1C).

Next, we measured the effect of ellagic acid on proliferation of PaCa cells. The results in Figure 2 show that ellagic acid dose-dependently inhibited proliferation of both MIA PaCa-2 and PANC-1 cell lines as measured by <sup>3</sup>H thymidine incorporation (Figure 2A and B). The effect was most pronounced at 50  $\mu$ mol/L (almost 20 fold inhibition in MIA PaCa-2 cells).





**Figure 3** Ellagic acid induces loss of mitochondrial membrane potential, cytochrome C release, and caspase-3 activation in pancreatic cancer cells. MIA PaCa-2 (A-C) and PANC-1 (B) cells were cultured for 48 h in the presence or absence of indicated doses of ellagic acid or broad-spectrum caspase inhibitor zVAD-fmk (100  $\mu\text{mol/L}$ ). **A:** Changes in  $\Delta\psi_m$  were measured by flow cytometry using the potential-sensitive probe 3,3'-diethyloxa-carbocyanine DiOC6 (3); **B:** Cytochrome C release was assessed by measuring cytochrome C levels in both cytosolic and mitochondria-enriched membrane fractions using Western blot analysis. Blots were re-probed for cytochrome C oxidase (COX IV), a specific mitochondrial marker. Western blots of cytosolic fractions re-probed for actin to confirm equal protein loading; **C** and **D:** Caspase-3 activity was assessed by measuring the DEVDase (caspase-3 like) activities in cell lysates using a fluorometric assay with a specific substrate. The results are representative of at least 3 independent experiments. Values are normalized to control (**C** and **D**). Values are mean  $\pm$  SE ( $n = 3$ ), \* $P < 0.05$  vs control.

### Ellagic acid induces mitochondrial depolarization, cytochrome C release, and caspase activation in pancreatic cancer cells

To determine the signaling pathway mediating the proapoptotic effect of ellagic acid, we measured the effects of ellagic acid on mitochondrial membrane potential ( $\Delta\psi_m$ ), cytochrome C release, and caspase-3 activity. Ellagic acid decreased  $\Delta\psi_m$  as measured by flow cytometry using the potential-sensitive probe DiOC6 (3). Depolarization was already evident at 10  $\mu\text{mol/L}$ , and was very pronounced at 50  $\mu\text{mol/L}$  ellagic acid (Figure 3A). Ellagic acid also dose-dependently stimulated cytochrome C release, which manifests by its decrease in mitochondria-enriched membrane fractions and its increase in cytosolic fractions (Figure 3B). The increased cytochrome C release was associated with downstream activation of caspase-3 in MIA PaCa-2 (Figure 3C) and PANC-1 (Figure 3D) cells.

These data together indicate that ellagic acid induces the mitochondrial pathway of apoptosis associated with mitochondrial depolarization, cytochrome C release, and downstream caspase activation.

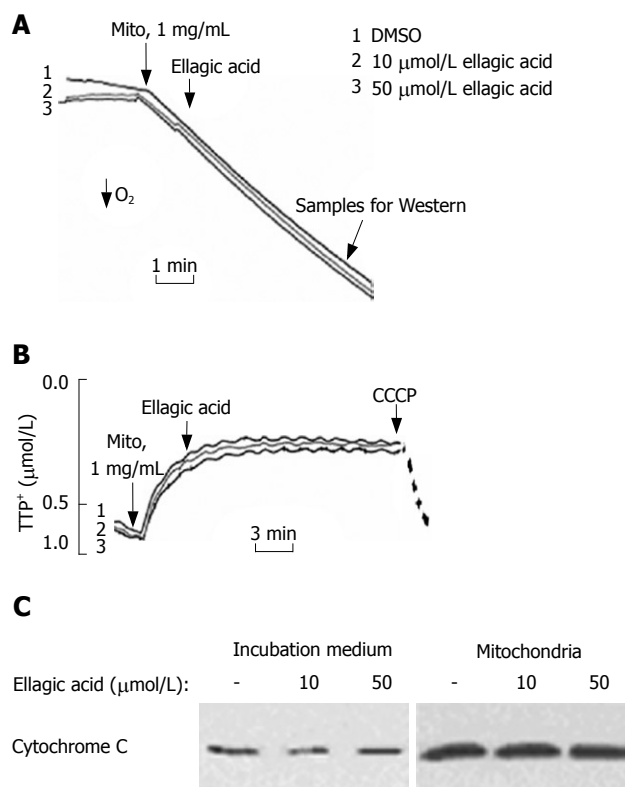
### Ellagic acid does not directly affect mitochondria function in pancreatic cancer cells

To test the effect of ellagic acid on mitochondria we isolated functional mitochondria from MIA PaCa-2 cells

and measured the effect of ellagic acid on mitochondria respiration, membrane potential, and cytochrome C release (Figure 4). The respiratory control ratio of isolated mitochondria with succinate as a respiratory substrate in all the experiments was greater than 4 in all the experiments. Ellagic acid affected neither oxygen consumption measured with Clark electrode (Figure 4A) nor  $\Delta\psi_m$  measured with TPP<sup>+</sup> electrode (Figure 4B). The protonophore CCCP, which we used as a positive control, depolarized mitochondria (Figure 4B). In agreement with these results, ellagic acid did not increase cytochrome C release from isolated mitochondria into the incubation medium (Figure 4C).

We next assessed the possibility of ellagic acid's effect in permeabilized cells. For this purpose, MIA PaCa-2 cells were permeabilized with digitonin as described by Ohno *et al*<sup>[20]</sup>. Ellagic acid did not induce any discernable effect on the mitochondrial membrane potential in digitonin-permeabilized cells as measured with TPP<sup>+</sup> electrode (Figure 5B) or by using TMRM fluorescent dye (Figure 5C). CCCP was applied to completely dissipate  $\Delta\psi_m$  (Figure 5B and C). Similarly, ellagic acid did not have any effect on mitochondrial respiration in permeabilized cells (Figure 5A).

The results in Figures 4 and 5 indicate that ellagic acid does not directly affect mitochondria functions in pancreatic cancer cells.

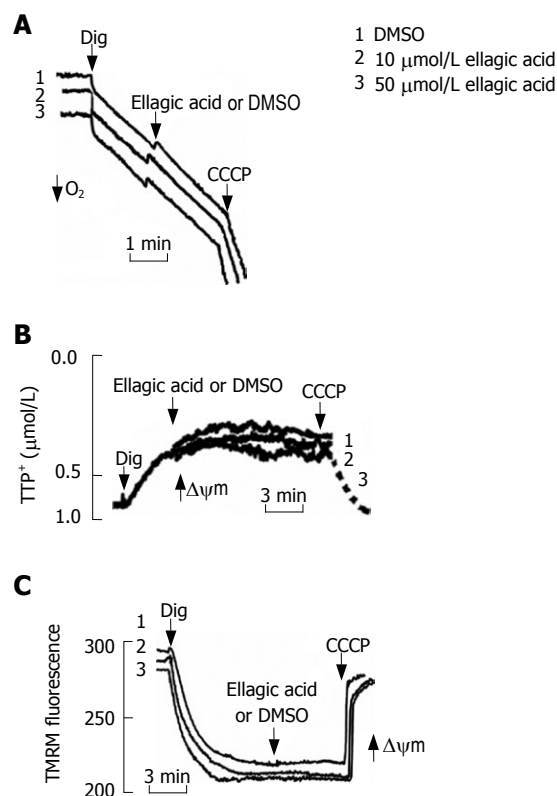


**Figure 4** Ellagic acid does not directly affect the function of isolated mitochondria. Mitochondria were isolated from MIA PaCa-2 cells cultured in the absence of ellagic acid. **A:** Oxygen consumption was measured using a Clark-type electrode connected to an oxygen meter; **B:** Mitochondrial membrane potential ( $\Delta\psi_m$ ) was monitored in the presence of 2  $\mu$ mol/L tetraphenyl phosphonium (TPP<sup>+</sup>) using a TPP<sup>+</sup>-sensitive electrode connected to an amplifier. Protonophore CCCP (10  $\mu$ mol/L) was added to dissipate  $\Delta\psi_m$ ; **C:** Cytochrome C levels were measured in the incubation medium and the mitochondrial pellet by Western blot analysis. The results are representative of 3 independent experiments.

### Ellagic acid and NF- $\kappa$ B inhibition act through the same mechanism to stimulate apoptosis

We hypothesized that ellagic acid induced the mitochondrial pathway of apoptosis through blocking a key upstream pro-survival mechanisms, namely NF- $\kappa$ B. NF- $\kappa$ B is a key transcription factor, which is usually activated and has anti-apoptotic role in cancer cells including pancreatic cancer<sup>[4]</sup>. We found that ellagic acid dose-dependently decreased NF- $\kappa$ B binding activity in both MIA PaCa-2 and PANC-1 cell lines (Figure 6A).

We further showed that a pharmacologic inhibitor of NF- $\kappa$ B caffeic acid phenethyl ester (CAPE) stimulated apoptosis in PaCa cells, and that in the presence of CAPE there was no additional stimulation of apoptosis by ellagic acid (50  $\mu$ mol/L) (Figure 6B). Further, transfection of MIA PaCa-2 cells with 4KBwt-pRL-TK plasmid completely blocked NF- $\kappa$ B transcriptional activity (Figure 6C), and at the same time stimulated apoptosis as measured by DNA fragmentation by > 5-fold (Figure 6D). The addition of ellagic acid to the transfected cells did not further increase DNA fragmentation (Figure 6D), confirming



**Figure 5** Ellagic acid does not directly affect mitochondria function in permeabilized MIA PaCa-2 cells. MIA PaCa-2 cells were permeabilized with 0.001% digitonin. **A:** Oxygen consumption was measured using a Clark-type electrode connected to an oxygen meter; **B:** Changes in  $\Delta\psi_m$  were monitored in the presence of 2  $\mu$ mol/L tetraphenyl phosphonium (TPP<sup>+</sup>) using a TPP<sup>+</sup>-sensitive electrode connected to an amplifier; **C:** Changes in  $\Delta\psi_m$  were monitored using the  $\Delta\psi_m$ -sensitive fluorescent probe tetramethylrhodamine methyl ester (TMRM); changes in the fluorescence intensities were measured using the excitation at 543 nm and the emission at 578 nm. Protonophore CCCP (10  $\mu$ mol/L) was added to dissipate  $\Delta\psi_m$ .

that ellagic acid causes apoptosis through inhibition of NF- $\kappa$ B.

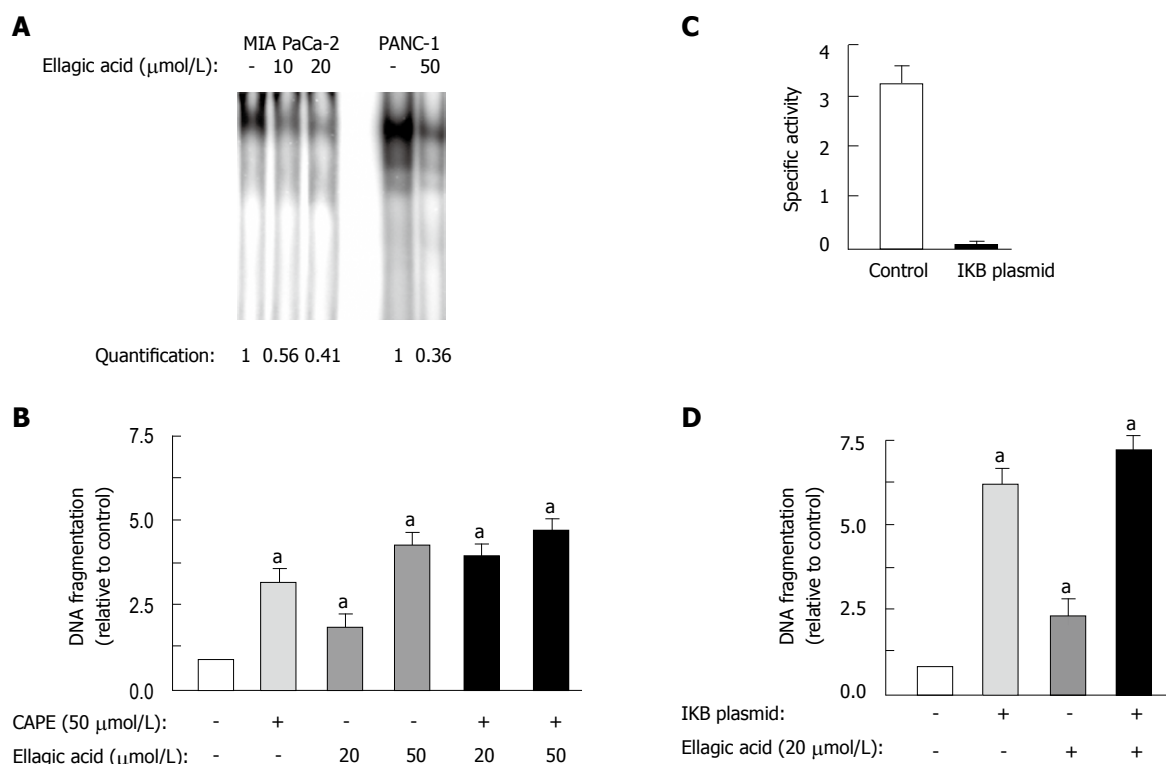
## DISCUSSION

Our study aimed to investigate the effect of ellagic acid on cell death and proliferation of pancreatic cancer cells and to determine the mechanism through which ellagic acid affects cell survival. We used the poorly differentiated MIA PaCa-2 and moderately differentiated PANC-1 human pancreatic carcinoma cell lines, which both display K-ras and p53 mutations characteristic of pancreatic cancer.

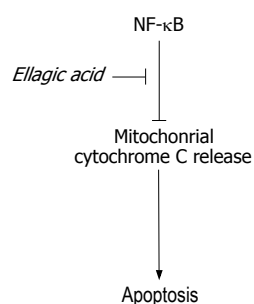
We found that ellagic acid: (1) stimulated apoptosis and inhibited proliferation of pancreatic cancer cells; (2) activated the mitochondrial death pathway associated with loss of  $\Delta\psi_m$ , cytochrome C release and caspase-3 activation without directly affecting the mitochondria; and (3) inhibited NF- $\kappa$ B activity in pancreatic cancer cells.

Mechanisms through which ellagic acid inhibits NF- $\kappa$ B remain to be investigated.

One mechanism through which NF- $\kappa$ B inhibits apoptosis is up-regulation of the anti-apoptotic Bcl-xL



**Figure 6** Ellagic acid decreases the activity of the transcription factor NF- $\kappa$ B. The effects of ellagic acid and pharmacological or molecular inhibition of NF- $\kappa$ B on apoptosis are not additive. MIA PaCa-2 (**A** and **B**) cells were cultured for 48 h in the presence or absence of indicated doses of ellagic acid. MIA PaCa-2 cells were transfected with 4KBwt-pRL-TK luciferase plasmid and pRL-TK luciferase as a control using the Nucleofector™ II electroporation system (**C** and **D**). **A**: NF- $\kappa$ B binding activity was measured as described in Experimental procedures; **B** and **D**: Internucleosomal DNA fragmentation was measured using the Cell Death Detection ELISA kit; **C**: NF- $\kappa$ B transcriptional activity was assessed using the dual-Luciferase Reporter Assay System assay. Results are representative of at least 3 independent experiments. Values are normalized to control (**B** and **D**). Values are mean  $\pm$  SE ( $n = 3$ ), <sup>a</sup> $P < 0.05$  vs control.



**Figure 7** Representative scheme of the proposed mechanism of induction of apoptosis by ellagic acid.

protein. Bcl-xL, in turn, blocks mitochondrial permeabilization resulting in inhibition of cytochrome C release as well as preventing mitochondrial depolarization<sup>[21-23]</sup>. Our data indicate that ellagic acid decreases NF- $\kappa$ B activity, leading to activation of the mitochondrial proapoptotic pathway and resulting in cytochrome C release and caspase activation. This scheme is depicted in Figure 7.

Our data indicate that ellagic acid inhibits proliferation of pancreatic cancer cells, similar to that in published data of other cells<sup>[21,24-28]</sup>. Ellagic acid completely abolished proliferation at high concentration, whereas it only increased apoptosis by 2.5-fold. The contribution of necrosis might account for the decreased proliferation.

Our results as well as the published data on the

potent inhibitory effect of ellagic acid on the vascular endothelial growth factor receptor and platelet-derived growth factor receptor leading to the inhibition of their signaling<sup>[29]</sup>, indicate that ellagic acid is a powerful phenolic compound with proapoptotic and anti-proliferation effects in cancer cells. Understanding the mechanism of action of ellagic acid will allow us to test the effect of the compound alone or in combination with other compounds on cancer growth and survival in an orthotopic model of pancreatic cancer<sup>[30]</sup>.

In summary, the present study shows that ellagic acid induces apoptosis and decreases proliferation in pancreatic cancer cells. This phenolic compound stimulates mitochondrial depolarization, cytochrome C release and caspase activation. Ellagic acid has no direct effect on mitochondria. One mechanism by which it stimulates the mitochondrial death pathway is through inhibiting the transcription factor NF- $\kappa$ B, a major prosurvival factor in pancreatic cancer cells. There is an increasing interest in the use of natural products for cancer treatments. Our results suggest a potential therapeutic role for ellagic acid in the treatment of pancreatic cancer.

## COMMENTS

### Background

Pancreatic cancer is very resistant to radio- and chemo-therapies. Recently,

there is a growing interest in natural compounds for enhancing cancer prevention and treatment. Ellagic acid is a phenolic compound present in fruits and berries such as pomegranates, strawberries, raspberries and blackberries. It has anticarcinogenic, antioxidant and antifibrosis properties. In the present study the authors investigate the effect of ellagic acid on pancreatic cancer cell proliferation and resistance to death.

### Research frontiers

The article focuses on the regulation of pancreatic cancer cell death by a phenolic compound, ellagic acid.

### Innovations and breakthroughs

The present study shows that ellagic acid induces apoptosis and decreases proliferation in pancreatic cancer cells. It was shown for the first time that ellagic acid stimulates mitochondrial depolarization, cytochrome C release and caspase activation. Ellagic acid has no direct effect on mitochondria. One mechanism by which it stimulates the mitochondrial death pathway is through inhibiting transcription factor NF- $\kappa$ B, a major prosurvival factor in pancreatic cancer cells.

### Applications

The data of this article demonstrate the anti-cancer properties of ellagic acid as well as its mechanism of action. By knowing the mechanism of action of ellagic acid, it can be used in combination with other drugs that target other pro-survival proteins to increase apoptosis in pancreatic cancer cells.

### Peer review

This is a carefully performed study with novel findings that have the potential for therapeutic application in pancreatic cancer. It examines the effect of a naturally occurring polyphenolic compound, ellagic acid, on pancreatic cancer cell function, in particular, apoptosis and proliferation. The authors report that ellagic acid stimulates apoptosis of two pancreatic cancer cell lines and the decreased proliferation of ellagic acid-treated cells.

## REFERENCES

- Giancotti FG, Ruoslahti E. Integrin signaling. *Science* 1999; **285**: 1028-1032
- Parker SL, Tong T, Bolden S, Wingo PA. Cancer statistics, 1997. *CA Cancer J Clin* 1997; **47**: 5-27
- Westphal S, Kalthoff H. Apoptosis: targets in pancreatic cancer. *Mol Cancer* 2003; **2**: 6
- Gukovskaya AS, Pandol SJ. Cell death pathways in pancreatitis and pancreatic cancer. *Pancreatology* 2004; **4**: 567-586
- Ayala GE, Dai H, Ittmann M, Li R, Powell M, Frolov A, Wheeler TM, Thompson TC, Rowley D. Growth and survival mechanisms associated with perineural invasion in prostate cancer. *Cancer Res* 2004; **64**: 6082-6090
- Dutta J, Fan Y, Gupta N, Fan G, Gelinas C. Current insights into the regulation of programmed cell death by NF- $\kappa$ B. *Oncogene* 2006; **25**: 6800-6816
- Li L, Aggarwal BB, Shishodia S, Abbruzzese J, Kurzrock R. Nuclear factor- $\kappa$ B and I $\kappa$ B kinase are constitutively active in human pancreatic cells, and their down-regulation by curcumin (diferuloylmethane) is associated with the suppression of proliferation and the induction of apoptosis. *Cancer* 2004; **101**: 2351-2362
- Mukhtar H, Das M, Khan WA, Wang ZY, Bik DP, Bickers DR. Exceptional activity of tannic acid among naturally occurring plant phenols in protecting against 7,12-dimethylbenzo(a)anthracene-, benzo(a)pyrene-, 3-methylcholanthrene-, and N-methyl-N-nitrosourea-induced skin tumorigenesis in mice. *Cancer Res* 1988; **48**: 2361-2365
- Thesiamma KC, Kuttan R. Inhibition of liver fibrosis by ellagic acid. *Indian J Physiol Pharmacol* 1996; **40**: 363-366
- Osawa T, Ide A, Su JD, Namiki M. Inhibition of lipid peroxidation by ellagic acid. *J. Agric. Food Chem* 1987; **35**: 808-812
- Stoner GD, Gupta A. Etiology and chemoprevention of esophageal squamous cell carcinoma. *Carcinogenesis* 2001; **22**: 1737-1746
- Larrosa M, Tomas-Barberan FA, Espin JC. The dietary hydrolysable tannin punicalagin releases ellagic acid that induces apoptosis in human colon adenocarcinoma Caco-2 cells by using the mitochondrial pathway. *J Nutr Biochem* 2006; **17**: 611-625
- Vaquero EC, Edderkaoui M, Nam KJ, Gukovsky I, Pandol SJ, Gukovskaya AS. Extracellular matrix proteins protect pancreatic cancer cells from death via mitochondrial and nonmitochondrial pathways. *Gastroenterology* 2003; **125**: 1188-1202
- Vaquero EC, Edderkaoui M, Pandol SJ, Gukovsky I, Gukovskaya AS. Reactive oxygen species produced by NAD(P)H oxidase inhibit apoptosis in pancreatic cancer cells. *J Biol Chem* 2004; **279**: 34643-34654
- Edderkaoui M, Hong P, Vaquero EC, Lee JK, Fischer L, Friess H, Buchler MW, Lerch MM, Pandol SJ, Gukovskaya AS. Extracellular matrix stimulates reactive oxygen species production and increases pancreatic cancer cell survival through 5-lipoxygenase and NADPH oxidase. *Am J Physiol Gastrointest Liver Physiol* 2005; **289**: G1137-G1147
- Edderkaoui M, Hong P, Lee JK, Pandol SJ, Gukovskaya AS. Insulin-like growth factor-I receptor mediates the prosurvival effect of fibronectin. *J Biol Chem* 2007; **282**: 26646-26655
- Mareninova OA, Sung KF, Hong P, Lugea A, Pandol SJ, Gukovsky I, Gukovskaya AS. Cell death in pancreatitis: caspases protect from necrotizing pancreatitis. *J Biol Chem* 2006; **281**: 3370-3381
- Gukovsky I, Gukovskaya AS, Blinman TA, Zaninovic V, Pandol SJ. Early NF- $\kappa$ B activation is associated with hormone-induced pancreatitis. *Am J Physiol* 1998; **275**: G1402-G1414
- Labrecque L, Lamy S, Chapus A, Mihoubi S, Durocher Y, Cass B, Bojanowski MW, Gingras D, Beliveau R. Combined inhibition of PDGF and VEGF receptors by ellagic acid, a dietary-derived phenolic compound. *Carcinogenesis* 2005; **26**: 821-826
- Chauvin C, De Oliveira F, Ronot X, Mousseau M, Leverve X, Fontaine E. Rotenone inhibits the mitochondrial permeability transition-induced cell death in U937 and KB cells. *J Biol Chem* 2001; **276**: 41394-41398
- Shimizu S, Narita M, Tsujimoto Y. Bcl-2 family proteins regulate the release of apoptogenic cytochrome c by the mitochondrial channel VDAC. *Nature* 1999; **399**: 483-487
- Dobbeling U. Transcription factor profiling shows new ways towards new treatment options of cutaneous T cell lymphomas. *Curr Drug Discov Technol* 2007; **4**: 24-30
- Saile B, Matthes N, El Armouche H, Neubauer K, Ramadori G. The bcl, NF $\kappa$ B and p53/p21WAF1 systems are involved in spontaneous apoptosis and in the anti-apoptotic effect of TGF- $\beta$  or TNF- $\alpha$  on activated hepatic stellate cells. *Eur J Cell Biol* 2001; **80**: 554-561
- Mertens-Talcott SU, Talcott ST, Percival SS. Low concentrations of quercetin and ellagic acid synergistically influence proliferation, cytotoxicity and apoptosis in MOLT-4 human leukemia cells. *J Nutr* 2003; **133**: 2669-2674
- Mertens-Talcott SU, Bomser JA, Romero C, Talcott ST, Percival SS. Ellagic acid potentiates the effect of quercetin on p21waf1/cip1, p53, and MAP-kinases without affecting intracellular generation of reactive oxygen species in vitro. *J Nutr* 2005; **135**: 609-614
- Chang WC, Yu YM, Chiang SY, Tseng CY. Ellagic acid suppresses oxidised low-density lipoprotein-induced aortic smooth muscle cell proliferation: studies on the activation of extracellular signal-regulated kinase 1/2 and proliferating cell nuclear antigen expression. *Br J Nutr* 2008; **99**: 709-714
- Ross HA, McDougall GJ, Stewart D. Antiproliferative



- activity is predominantly associated with ellagitannins in raspberry extracts. *Phytochemistry* 2007; **68**: 218-228
- 28 **Losso JN**, Bansode RR, Trappey A 2nd, Bawadi HA, Truax R. In vitro anti-proliferative activities of ellagic acid. *J Nutr Biochem* 2004; **15**: 672-678
- 29 **Labrecque L**, Lamy S, Chapus A, Mihoubi S, Durocher Y, Cass B, Bojanowski MW, Gingras D, Beliveau R. Combined inhibition of PDGF and VEGF receptors by ellagic acid, a dietary-derived phenolic compound. *Carcinogenesis* 2005; **26**: 821-826
- 30 **Eibl G**, Reber HA, Wente MN, Hines OJ. The selective cyclooxygenase-2 inhibitor nimesulide induces apoptosis in pancreatic cancer cells independent of COX-2. *Pancreas* 2003; **26**: 33-41

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## Experimental treatment of pancreatic cancer with two novel histone deacetylase inhibitors

Martin Haefner, Thilo Bluethner, Manuel Niederhagen, Christian Moebius, Christian Wittekind, Joachim Mossner, Karel Caca, Marcus Wiedmann

Martin Haefner, Thilo Bluethner, Joachim Mossner, Marcus Wiedmann, Department of Internal Medicine II, University of Leipzig, Philipp-Rosenthal-Str. 27, Leipzig 04103, Germany  
Manuel Niederhagen, Christian Wittekind, Institute of Pathology, University of Leipzig, Liebigstr. 26, Leipzig 04103, Germany

Christian Moebius, Department of Surgery II, University of Leipzig, Liebigstrasse 20a, Leipzig 04103, Germany

Karel Caca, Department of Internal Medicine I, Klinikum Ludwigsburg, Posilipstr. 4, Ludwigsburg 71640, Germany

Author contributions: Wiedmann M and Caca K designed research; Haefner M, Bluethner T and Niederhagen M performed research; Wittekind C contributed analytic tools; Moebius C and Mossner J analyzed data and corrected the manuscript; and Wiedmann M wrote the paper.

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Correspondence to: Dr. Marcus Wiedmann, Department of Internal Medicine II, University of Leipzig, Philipp-Rosenthal-Str. 27, Leipzig 04103,

Germany. [wiedm@medizin.uni-leipzig.de](mailto:wiedm@medizin.uni-leipzig.de)

Telephone: +49-341-9712230 Fax: +49-341-9712239

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**CONCLUSION:** Our findings suggest that NVP-LBH589 and NVP-LAQ824 are active against human pancreatic cancer, although the precise mechanism of *in vivo* drug action is not yet completely understood. Therefore, further preclinical and clinical studies for the treatment of pancreatic cancer are recommended.

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**Key words:** Histone deacetylase inhibitor; Pancreatic cancer; NVP-LAQ824; NVP-LBH589

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### Abstract

**AIM:** To investigate *in vitro* and *in vivo* treatment with histone deacetylase inhibitors NVP-LAQ824 and NVP-LBH589 in pancreatic cancer.

**METHODS:** Cell-growth inhibition by NVP-LAQ824 and NVP-LBH589 was studied *in vitro* in 8 human pancreatic cancer cell lines using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In addition, the anti-tumoral effect of NVP-LBH589 was studied in a chimeric mouse model. Anti-tumoral activity of the drugs was assessed by immunoblotting for p21<sup>WAF-1</sup>, acH4, cell cycle analysis, TUNEL assay, and immunohistochemistry for MIB-1.

**RESULTS:** *In vitro* treatment with both compounds significantly suppressed the growth of all cancer cell lines and was associated with hyperacetylation of nucleosomal histone H4, increased expression of p21<sup>WAF-1</sup>, cell cycle arrest at G2/M-checkpoint, and increased apoptosis. *In vivo*, NVP-LBH589 alone significantly reduced tumor mass and potentiated the efficacy of gemcitabine. Further analysis of the tumor specimens revealed slightly increased apoptosis and no significant reduction of cell proliferation.

### INTRODUCTION

Pancreatic cancer is the fifth to sixth leading cause of cancer death in Europe and the fourth leading cause of cancer death in the USA<sup>[1]</sup>. The lethality of this malignancy is demonstrated by the fact that the annual incidence is approximately equal to the annual deaths. Unfortunately, carcinoma of the pancreas is increasing in incidence, and its risk factors are poorly understood. Although surgical resection remains the only chance for cure, less than 10% of patients diagnosed with pancreatic cancer are eligible for curative (R0) resection, since up to 90% of patients will present with locally advanced or metastatic disease. In addition, there is a high rate of relapse, even in patients who receive adjuvant therapy<sup>[2]</sup>. A recent evaluation of the Finnish Cancer Registry, which recorded 4922 pancreatic cancer patients between 1990 and 1996, detected only 89 five year survivors (1.8%)<sup>[3]</sup>. Metastatic cancer tends to be a rapidly progressing disease, often accompanied by significant weight loss, abdominal pain, nausea, and/or depression. For decades, 5-fluorouracil (5-FU) was the most widely used chemotherapeutic agent in

metastatic pancreatic cancer. Today gemcitabine, a nucleoside analogue that is incorporated into replicating DNA resulting in premature chain termination and apoptosis, is the current standard of care<sup>[4]</sup>. In a phase III approval study 126 patients with metastatic disease who had not received prior chemotherapy were randomized to weekly gemcitabine ( $n = 63$ ) or weekly bolus 5-FU ( $n = 63$ )<sup>[5]</sup>. Overall survival in patients treated with gemcitabine was significantly improved compared with patients treated with 5-FU; However, there was no convincing gain in median survival time (median survival 5.7 mo *vs* 4.4 mo,  $P = 0.0025$ ). The primary efficacy measure in this study was clinical benefit response, a composite of patient-oriented parameters including pain, Karnofsky performance status, daily analgesic usage, and body weight. Clinical benefit was experienced in 23.8% of patients treated with gemcitabine compared with only 4.5% of the patients treated with 5-FU ( $P = 0.022$ ). Fixed-dose-rate (FDR) gemcitabine (1500 mg/m<sup>2</sup> at 10 mg/m<sup>2</sup> per minute) has also been investigated by Tempero *et al* in comparison to 2200 mg/m<sup>2</sup> gemcitabine over 30 min<sup>[6]</sup>. Although median survival time improved from 5.0 mo in the standard arm to 8.0 mo in the FDR arm ( $P = 0.013$ ), grade 3 and 4 toxicity increased significantly. Many combination regimens with gemcitabine have been tested in open-label phase II or III studies with higher response and progression-free survival rates, but no definitive benefit in overall survival, with the only exception being a combination with capecitabine<sup>[4,7]</sup>. As little progress has been made in the past decade, new strategies should focus on targeting cancer cells at the molecular level. Recently, in a randomized phase III placebo-controlled trial, Moore *et al* demonstrated that combining gemcitabine with EGFR inhibitor erlotinib was associated with a modest, but statistically significant survival benefit of 15 d<sup>[8]</sup>. In contrast, a recent phase III trial (SWOG S0205 study) failed to demonstrate a clinically significant advantage of the addition of cetuximab, an anti-EGFR monoclonal antibody, to gemcitabine for overall survival, progression free survival and response<sup>[9]</sup>. Another approach is targeting VEGF as a key player in tumor growth and resistance to therapy. In a phase II trial with 52 patients, a combination of VEGF inhibitor bevacizumab and gemcitabine yielded a 21% response rate and a median survival of 8.8 mo<sup>[10]</sup>. These data led CALGB to conduct a randomized, double-blind, placebo-controlled, phase III trial (CALGB 80303). However, the addition of bevacizumab to gemcitabine did not improve survival<sup>[11]</sup>. Inhibiting histone deacetylases (HDACs), which regulate interactions between histones and DNA together with histone acetylases (HATs) as counter-players, may be another promising molecular target. Clinical studies published so far have shown that HDAC inhibitors (HDACIs) can be administered safely in humans and that treatment of some cancers with such agents seems to be beneficial<sup>[12,13]</sup>. NVP-LAQ824 and NVP-LBH589 are new chemical entities belonging to a structurally novel class of cinnamic hydroxamic

acid compounds<sup>[14-17]</sup>, which are currently in phase I clinical evaluation in advanced refractory solid tumors and hematologic malignancies<sup>[18-22]</sup>. However, little is known about their potential efficacy in pancreatic cancer. Therefore, the objectives of the current study were to investigate the efficacy of *in vitro* and *in vivo* treatment with the novel pan-HDAC inhibitors NVP-LAQ824 and NVP-LBH589 and to evaluate effects of combination with gemcitabine.

## MATERIALS AND METHODS

### Materials

Eight human pancreatic cancer cell lines (Hs766T, As-PC-1, CFPAC-1, Capan-2, Panc-1, MiaPaca-2, HPAF-2 and L3.6pl) were examined<sup>[23-27]</sup>. All cell lines were cultured in a 37°C incubator with 50-100 mL/L CO<sub>2</sub> in appropriate media. The HDACIs NVP-LAQ824 and NVP-LBH589 were provided by Novartis (Basel, Switzerland) and dissolved in dimethyl sulfoxide (DMSO) (10 mmol/L stock). Hoechst dye, sodium butyrate and monoclonal (mc)  $\beta$ -actin antibody were purchased from Sigma (Sigma-Aldrich Chemie GmbH Munich, Germany), mc p21<sup>WAF-1/Cip-1</sup> from Cell Signaling (Cell Signaling Technology, Beverly, USA), mc acH4 antibody from Upstate (Upstate Biotechnology, Lake Placid, USA), mc MIB-1 antibody from Dako (Glostrup, Denmark), and gemcitabine (diluted in D5W and 50 mL/L DMSO) from our hospital pharmacy. Six to eight-wk-old female athymic NMRI nude mice were supplied by Taconic (Taconic Europe, Ry, Denmark) and held under pathogen-free conditions. Humane care was administered, and study protocols complied with the institutional guidelines.

### Inhibition of cell growth

Cytotoxic effects of both drugs were determined by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Chemie GmbH Munich, Germany) assay.  $1-5 \times 10^3$  cells were seeded in triplicate in 96-well plates (100  $\mu$ L/well) and allowed to attach overnight. The medium was then replaced with media (100  $\mu$ L) containing the designated drug or vehicle control (50 mL/L DMSO in D5W) followed by an incubation for 3 or 6 d. For the 6 d experiment, medium was changed after 3 d. Three hours before the end of the incubation period, 10  $\mu$ L of PBS containing MTT (5 g/L) was added to each well. Following this, the medium was removed. The precipitate was then resuspended in 100  $\mu$ L of lysis buffer (DMSO, 100 g/L SDS). Absorbance was measured on a plate reader at 590 nm using a reference wavelength of 630 nm. Each experiment was performed in triplicate.

### Immunoblotting

Cell culture monolayers were washed twice with ice-cold PBS and lysed with RIPA-buffer containing Tris-HCl (50 mmol/L, pH 7.4), NP-40 (10 g/L), sodium-desoxycholate (2.5 g/L), NaCl (150 mmol/L), EDTA (1 mmol/L), sodium-orthovanadate (1 mmol/L), and

one tablet of complete mini-EDTA-free protease inhibitor cocktail (Boehringer, Mannheim, Germany, in 10 mL buffer). Histones for anti-acH4 immunoblotting were isolated by acid extraction [cells were lysed in ice-cold lysis buffer (HEPES 10 mmol/L; pH 7.9), MgCl<sub>2</sub> (1.5 mmol/L), KCl (10 mmol/L), DTT (0.5 mmol/L), PMSF (1.5 mmol/L), and additional protease inhibitor]. One molar HCl was added to a final concentration of 0.2 mol/L, followed by an incubation on ice for 30 min and centrifugation at 13000 r/min for 10 min. The supernatant was retained and dialysed against 200 mL of 0.2 mol/L acetic acid twice for 1 h and against 200 mL H<sub>2</sub>O overnight). Proteins were quantified by Bradford protein assay (Bio-Rad, Munich, Germany) and stored at -80°C. 50 µg of cell or tissue lysates were separated on SDS-polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Freiburg, Germany). Membranes were then incubated in blocking solution [50 g/L dry milk in 10 mmol/L Tris-HCl, 140 mmol/L NaCl, 1 g/L Tween-20 (TBS-T)], followed by incubation with the primary antibody at 4°C overnight (50 g/L BSA in TBS-T). The membranes were then washed in TBS-T and incubated with horseradish peroxidase (HRPO)-conjugated secondary antibodies for 1 h at room temperature. Antibody detection was performed with an enhanced chemoluminescence reaction (SuperSignal West Dura, Pierce, Rockford, USA).

### Cell cycle analysis

Cells were seeded in T-25 flasks ( $2 \times 10^5$ ), treated with various concentrations of NVP-LAQ824 or NVP-LBH589 or vehicle control (50 mL/L DMSO in D5W) for 72 h, washed with PBS, trypsinized, centrifuged, and fixed in 750 mL/L ice-cold ethanol-phosphate-buffered saline containing 10 g/L EDTA. DNA was labeled with 100 mL/L propidium iodide. Cells were sorted by FACSscan analysis, and cell cycle profiles were determined using ModFitLT V2.0 software (Becton Dickinson, San Diego, USA). Each experiment was performed in triplicate.

### Animal studies

Tumors were induced by injecting  $5 \times 10^6$  HPAF-2 or L3.6pl cells in 200 µL PBS sc into the flank region of NMRI nude mice. Treatment was started when an average tumor volume of 150 mm<sup>3</sup> was reached (usually after 2 wk). The verum groups received either NVP-LBH589 (25 mg/kg, 5 × weekly) or gemcitabine (5 mg/kg, 1 × weekly) or a combination of both (NVP-LBH589 at 25 mg/kg, 5 × weekly plus gemcitabine at 5 mg/kg, 1 × weekly) ip, whereas the control group received placebo (carrier solution 50 mL/L DMSO in D5W) only. Treatment was continued for 28 consecutive days, tumors were measured daily with a Vernier caliper and tumor volumes were calculated using the formula tumor volume =  $0.5 \times L \times W^2$ , where *L* represents the length and *W* the width of the tumor. When treatment was finished, animals were sacrificed and tumors excised and weighed.

### TUNEL POD test

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (*in situ* cell death detection kit, POD) was used to detect apoptosis in paraffin sections from mouse tumor tissue. TUNEL was carried out following the manufacturer's instructions (Roche, Penzberg, Germany) as previously described<sup>[28]</sup>. Apoptotic cells (red) were counted under a light microscope after fluorescence signal conversion using peroxidase-conjugated antibody and peroxidase substrate (DAB, Roche, Penzberg, Germany). The number of positive cells was counted by an experienced pathologist (M.N.) in a total of 8 high power fields (HPFs) and expressed as mean percentage of total cells in these fields of the tumor. Necrotic tumor cells were excluded from the cell count.

### Immunohistochemical staining

For MIB-1 staining, we used paraffin sections following a protocol that has been described elsewhere<sup>[29]</sup>. The number of positive cells was counted by an experienced pathologist (M.N.) in a total of 4 HPFs and expressed as mean percentage of total cells in these fields of the tumor.

### Statistical analysis

Statistical calculations were performed using SPSS, version 10.0 (SPSS Inc., Chicago, USA). Numeric data were presented as mean value with SD or SEM. Inter-group comparisons were performed with the Student *t*-test and ANOVA. *P* < 0.05 was considered significant.

## RESULTS

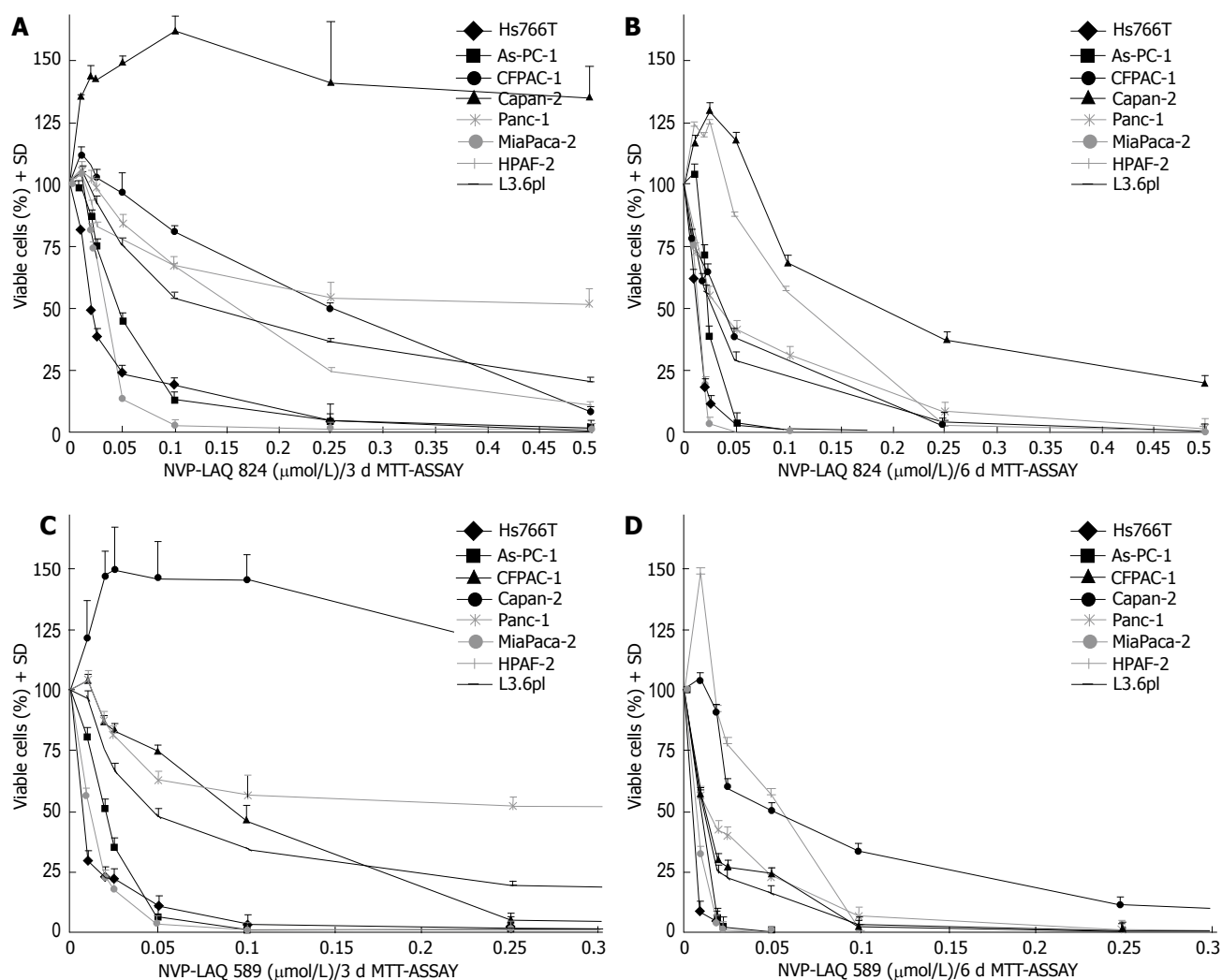
### Inhibition of cell growth

After 3 d of incubation, 7 of 8 tested cell lines were sensitive to NVP-LAQ824 (mean IC<sub>50</sub> (3 d) =  $0.18 \pm 0.24$  µmol/L) and even more to NVP-LBH589 (mean IC<sub>50</sub> (3 d) =  $0.09 \pm 0.14$  µmol/L). Only cell line Capan-2 demonstrated an IC<sub>50</sub> (3 d) value > 1 µmol/L for both compounds. Inhibition of cell growth was more pronounced if incubation time was extended to 6 d with a mean IC<sub>50</sub> value of  $0.06 \pm 0.07$  µmol/L for NVP-LAQ824 and  $0.03 \pm 0.02$  µmol/L for NVP-LBH589. After 6 d of incubation, cell line Capan-2 also became responsive (Figure 1 and Table 1). In addition, DMSO alone (the solvent for NVP-LAQ824 and NVP-LBH589) had no influence on cell growth (data not shown).

### Immunoblotting

Treatment of cell lines HPAF-2 and L3.6pl with 0.1 µmol/L NVP-LAQ824 or 0.1 µmol/L NVP-LBH589 for 24 h resulted in acetylation of histone H4 (Figure 2A and B). The same treatment caused an induction of p21<sup>WAF-1/CIP-1</sup> expression (Figure 2C and D). A dose increase to 0.2 µmol/L NVP-LAQ824 or NVP-LBH589 corresponded with an increase in histone H4 acetylation and p21<sup>WAF-1/CIP-1</sup> levels. Histone H4 acetylation was higher in treated HPAF-2 than L3.6pl cells, whereas p21<sup>WAF-1/CIP-1</sup> expression was slightly higher in treated L3.6pl cells.





**Figure 1** *In vitro* treatment of pancreatic cancer with NVP-LAQ824 and NVP-LBH589 (MTT assay). **A:** 3-d incubation with NVP-LAQ824 ( $n = 3$ ); **B:** 6-d incubation with NVP-LAQ824 ( $n = 3$ ); **C:** 3-d incubation with NVP-LBH589 ( $n = 3$ ); **D:** 6-d incubation with NVP-LBH589 ( $n = 3$ ).

**Table 1** Inhibition of cell growth by NVP-LAQ824 and NVP-LBH589

Cell line	IC <sub>50</sub> (μmol/L)			
	NVP-LAQ824		NVP-LBH589	
	3 d	6 d	3 d	6 d
MiaPaca-2	0.03	0.01	0.01	0.01
As-PC-1	0.05	0.02	0.02	0.01
Panc-1	0.70	0.04	0.40	0.02
Hs766T	0.02	0.01	0.01	0.01
CFPAC-1	0.25	0.04	0.09	0.02
HPAF-2	0.16	0.12	0.07	0.06
L3.6pl	0.05	0.02	0.03	0.04
Capan-2	> 1	0.19	> 1	0.05

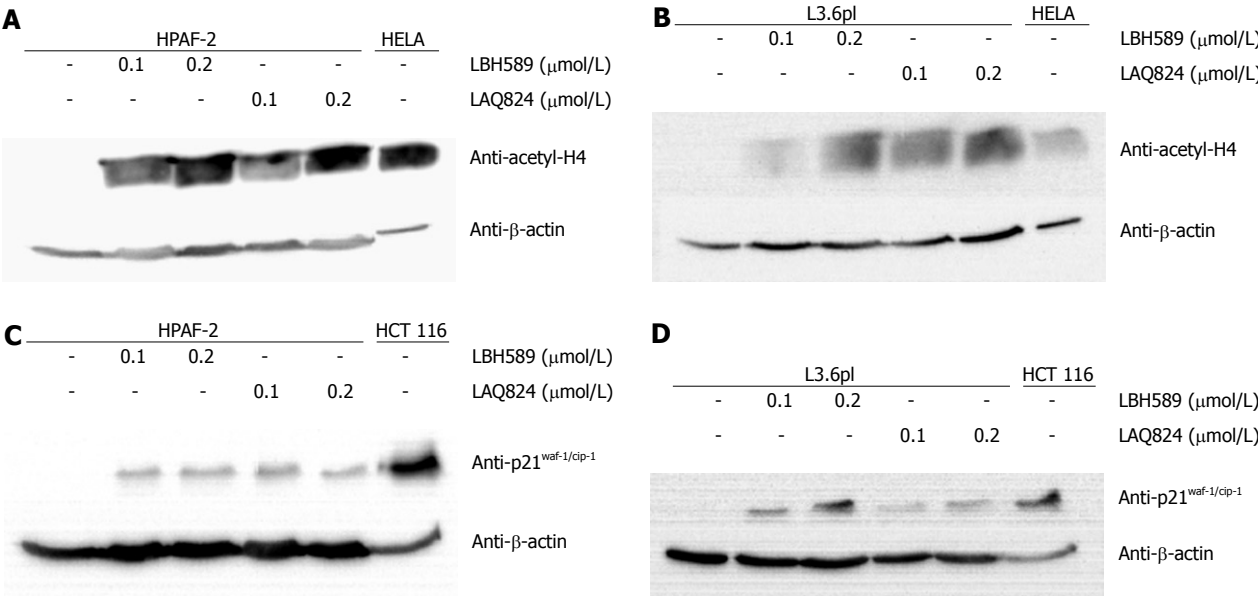
### Cell cycle analysis

Treatment of cell lines HPAF-2 and L3.6pl with 0.1 μmol/L NVP-LAQ824 or NVP-LBH589 for 72 h resulted in G2/M arrest. This arrest was, in general, more pronounced if the dose of NVP-LAQ824 or NVP-LBH589 was increased to 0.2 μmol/L. Percentual G2/M arrest was lower for 0.2 μmol/L than 0.1 μmol/L only for the treatment of HPAF-2 cells with NVP-LBH589. This phenomenon may derive from the fact, that at

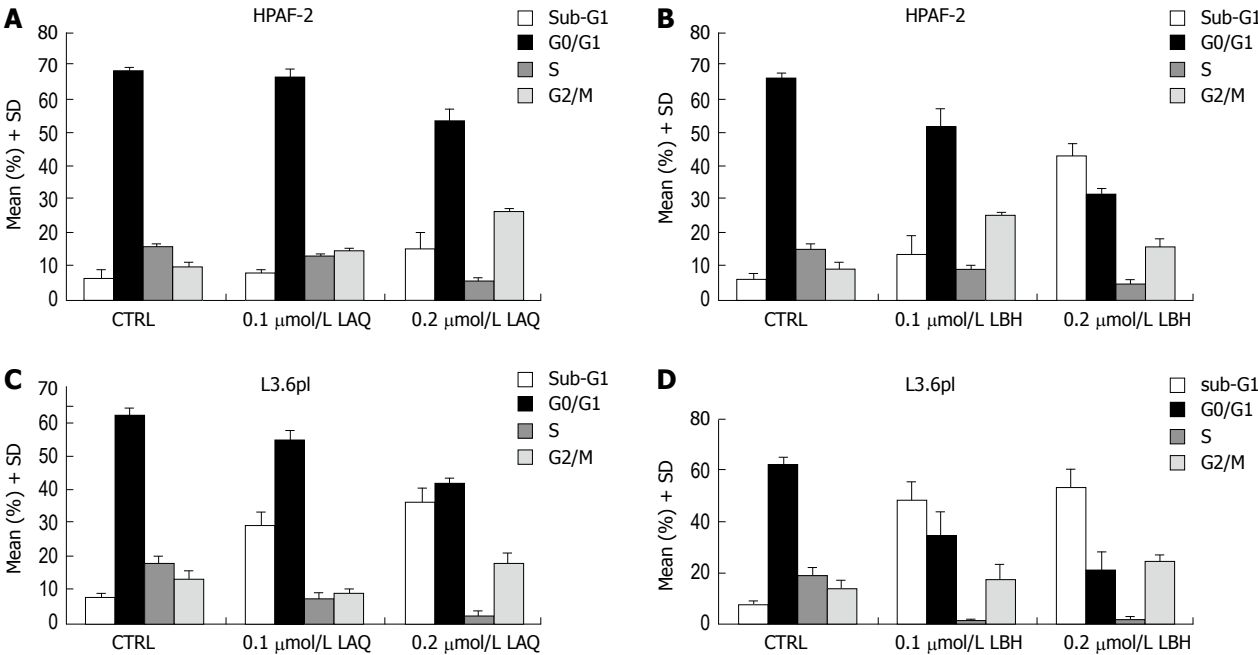
the same time the sub-G1-peak was much higher for 0.2 μmol/L. For both concentrations, the effect of NVP-LBH589 was stronger than the effect of NVP-LAQ824 with the aforementioned exception of 0.2 μmol/L NVP-LBH589 in HPAF-2 cells (Figure 3). In addition, incubation with NVP-LAQ824 or NVP-LBH589 for 72 h resulted in a dose-dependent significant increase in the sub-G1-peak, which was higher for NVP-LBH589 than NVP-LAQ824 and higher in L3.6pl than in HPAF-2 cells. This result correlated well with the fact that IC<sub>50</sub> values in the cell growth inhibition experiment (Figure 1) were lower for L3.6pl in comparison to HPAF-2 cells.

### Chimeric mouse model

Tumors were induced in nude mice by subcutaneous injection of HPAF-2 and L3.6pl cells. These cell lines were selected because they had the best growth capability in our nude mice in a pilot study. Treatment of mice consisted of ip injections with NVP-LBH589, gemcitabine, NVP-LBH589 plus gemcitabine (COMBO) or placebo (50 mL/L DMSO in D5W). Three days after commencement of NVP-LBH589 or COMBO treatment, HPAF-2 cell tumors showed a signifi-



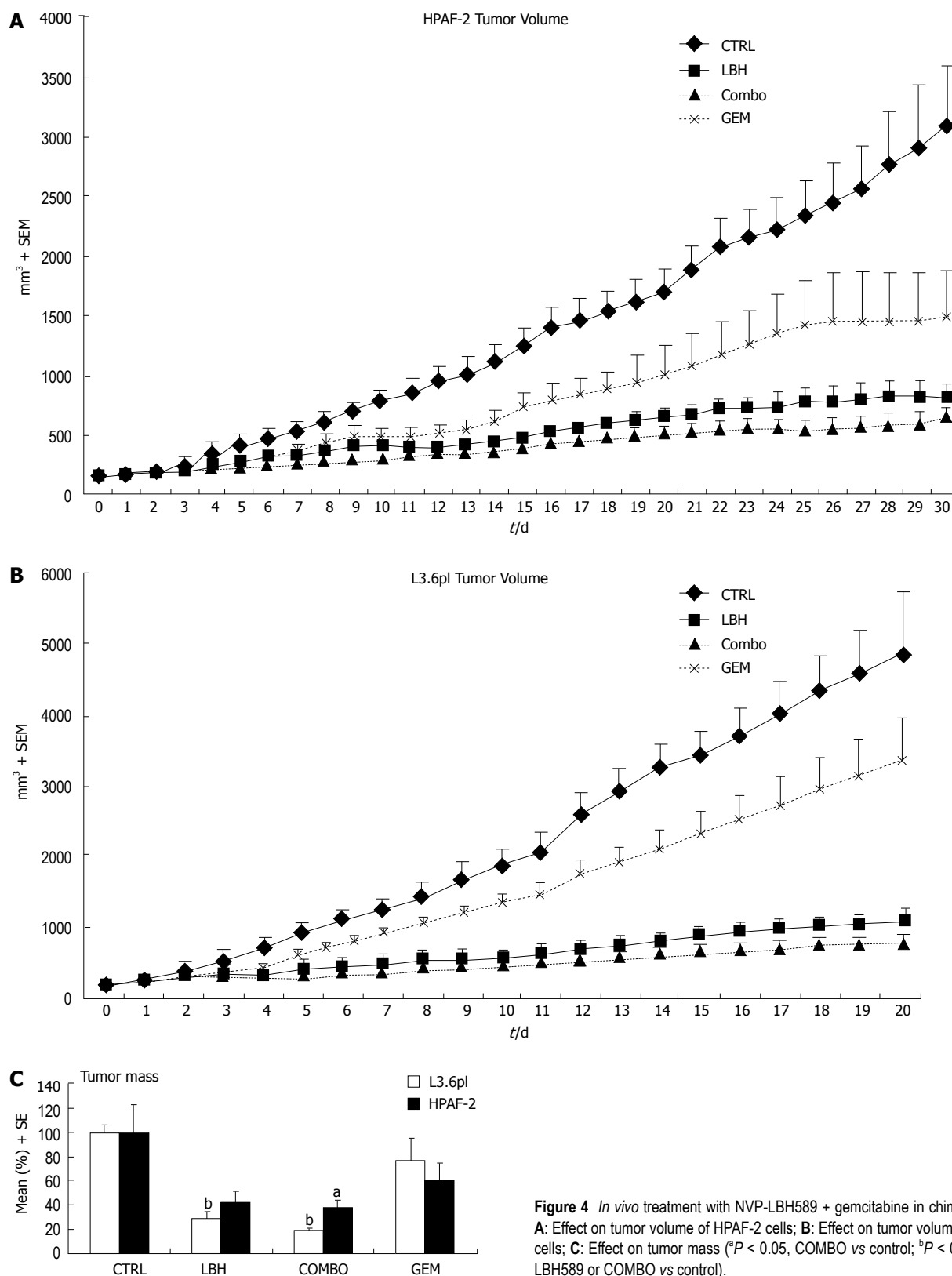
**Figure 2** Mechanism of drug action after *in vitro* treatment with NVP-LAQ824 and NVP-LBH589 for 24 h. **A** and **B**: Acetylation of histone H4. Protein extracts from HELA cells that were treated with 5 mmol/L sodium butyrate served as positive controls; **C** and **D**: p21<sup>waf-1/cip-1</sup> expression. Cell lysate from HCT 116 colon cancer cells served as positive control; **A-D**: Staining with  $\beta$ -actin antibody confirmed equal protein loading.



**Figure 3** Cell cycle analysis. **A**: Treatment of cell line HPAF-2 with 0.1 or 0.2  $\mu\text{mol/L}$  NVP-LAQ824 for 72 h ( $n = 3$ ); **B**: Treatment of cell line HPAF-2 with 0.1 or 0.2  $\mu\text{mol/L}$  NVP-LBH589 for 72 h ( $n = 3$ ); **C**: Treatment of cell line L3.6pl with 0.1 or 0.2  $\mu\text{mol/L}$  NVP-LAQ824 for 72 h ( $n = 3$ ); **D**: Treatment of cell line L3.6pl with 0.1 or 0.2  $\mu\text{mol/L}$  NVP-LBH589 for 72 h ( $n = 3$ ).

cantly reduced volume in comparison to control ( $n = 7$  for each group,  $P < 0.05$ ). Treatment of mice with gemcitabine alone resulted in a significant reduction of tumor volume compared to control after 4 d from commencement of treatment. These differences were maintained until the end of the experiment. COMBO therapy was significantly more efficient than gemcitabine treatment alone on treatment day 7, 8, 13, 14, 15, and 16 and was significantly more efficient than NVP-LBH589 therapy alone on treatment day 7 and 14 ( $P < 0.05$ ,

Figure 4A). Treatment of L3.6pl tumors with NVP-LBH589 or COMBO resulted in a significantly reduced volume in comparison to control after 4 d ( $P < 0.05$ ) and 3 d ( $P < 0.05$ ) from commencement of therapy, respectively ( $n = 7$  for each group). These differences were also maintained until the end of the experiment. Treatment of mice with gemcitabine alone resulted in a significant reduction of tumor volume compared to control at treatment day 12, 13, 16, 17, and 18 ( $P < 0.05$ ). COMBO therapy was significantly more

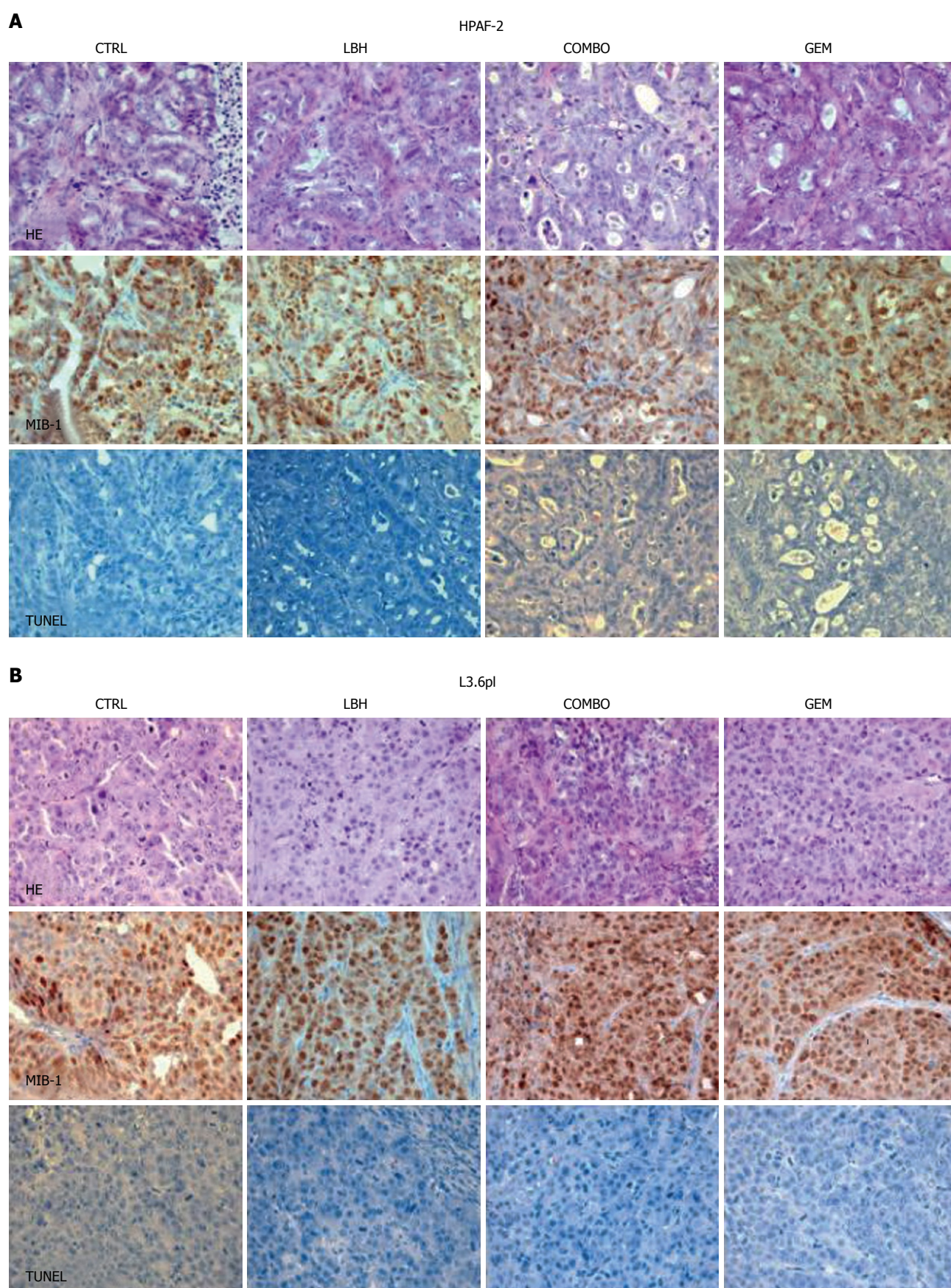


**Figure 4** *In vivo* treatment with NVP-LBH589 + gemcitabine in chimeric mice. **A:** Effect on tumor volume of HPAF-2 cells; **B:** Effect on tumor volume of L3.6pl cells; **C:** Effect on tumor mass (<sup>a</sup> $P < 0.05$ , COMBO vs control; <sup>b</sup> $P < 0.01$ , NVP-LBH589 or COMBO vs control).

efficient than gemcitabine treatment alone on treatment day 3-20 and was significantly more efficient than NVP-LBH589 therapy alone on treatment day 3 ( $P < 0.05$ ). NVP-LBH589 therapy was significantly more efficient than gemcitabine treatment alone on treatment day 5-20 ( $P < 0.05$ , Figure 4B). At the end of the experiment after 30 d, tumor mass in HPAF-2 cells bearing mice

was significantly diminished as compared to placebo after treatment with COMBO (-63%,  $P < 0.05$ ). In contrast, treatment of mice with gemcitabine (-24%,  $P = 0.45$ ) or NVP-LBH589 alone (-58%,  $P = 0.056$ ) did not result in any significant reduction of tumor mass as compared to control (Figure 4C). L3.6pl cell tumor mass in mice was significantly diminished after treatment





**Figure 5** Hematoxylin-eosin (HE), MIB-1 (proliferation marker) and TUNEL (apoptosis marker) staining of mouse tumors (SABC, x 40). **A:** Cell line HPAF-2; **B:** Cell line L3.6pl.

with either NVP-LBH589 (-70%,  $P < 0.01$ ) or COMBO (-81%,  $P < 0.01$ ), but not with gemcitabine (-24%,  $P = 0.28$ ),



**Table 2** MIB-1- and TUNEL-staining of mouse tumor specimens

Mean in %	HPAF-2		L3.6pl	
	MIB-1	Apoptosis	MIB-1	Apoptosis
CTRL	67.5	1.3	61.3	0
GEM	66.3	2.5	70	0
LBH	51.3	3.8	76.3	6.3
COMBO	55.0	3.8	78.8	3.8

respectively. In addition, the combination of NVP-LBH589 with gemcitabine was more effective at tumor mass reduction in comparison to gemcitabine alone ( $P < 0.05$ ). The L3.6pl animal experiment was stopped at day 21 for ethical reasons, since animals suffered from tumor burden. Regarding side effects of the different drugs used in HPAF-2 cell tumor bearing mice, weight loss was 2%, 0%, 13%, and 6%, in the control, gemcitabine, NVP-LBH589, and COMBO groups. There was a statistically significant difference between the control and NVP-LBH589 group ( $P < 0.05$ ) and between the gemcitabine and NVP-LBH589 group ( $P < 0.01$ ). Concerning side effects of the different drugs used in L3.6pl cell tumor bearing mice, weight loss was 23%, 17%, 12%, and 25%, in the control, gemcitabine, NVP-LBH589, and COMBO groups. There was a statistically significant difference between the control and NVP-LBH589 group ( $P < 0.05$ ).

In order to assess the anti-tumoral drug mechanism, paraffin sections of mouse tumors were stained with hematoxylin-eosin (H&E), MIB-1 (proliferation marker) and TUNEL (apoptosis marker) (Figure 5). Treatment with NVP-LBH589 and COMBO slightly reduced proliferation (reduced MIB-1 staining) and slightly induced apoptosis (increased TUNEL-staining) in HPAF-2 cell bearing mice, whereas proliferation was not decreased and apoptosis only slightly increased in L3.6pl cell bearing mice (Table 2).

## DISCUSSION

Analyzing palliative treatment data, a novel approach for patients with metastatic pancreatic cancer is urgently required. Targeting HDACs may be a new option for this tumor entity. Preliminary studies have demonstrated *in vitro* activity of HDACIs in pancreatic cancer cell lines. Natoni *et al*<sup>[30]</sup> showed that treatment with sodium butyrate, a carboxyl acid class inhibitor of HDACs, resulted in marked down-regulation of anti-apoptotic Bcl-xL protein expression, mitochondrial membrane depolarization, cytochrome c release from mitochondria, activation of caspase-9 and -3, and apoptosis induction. Garcia-Morales *et al*<sup>[31]</sup> reported HDACIs induced apoptosis in the pancreatic cancer cell lines IMIM-PC-1, IMIM-PC-2, and RWP-1 that are normally resistant to other antineoplastic drugs. This finding was previously observed by Sato *et al*<sup>[32]</sup> for five normally chemotherapy-resistant cell lines when treated with FR901228, a cyclic peptide HDACI belonging to the depsipeptides class. Recently, another class of HDACIs, the hydroxamic

acids, with representatives such as trichostatin A (TSA), suberoylanilide hydroxamic acid (vorinostat, SAHA), azelaic bis-hydroxamic acid (ABHA), scriptaid, oxamflatin, pyroxamide, m-carboxycinnamic acid bis-hydroxamide (CBHA), and the recently developed NVP-LAQ824, NVP-LBH589, and PXD101 have become the focus for further research, including pancreatic cancer. Gahr *et al*<sup>[33]</sup> used HDACI trichostatin A for *in vitro* treatment of pancreatic carcinoma cell lines YAP C and DAN G. They described an apoptosis rate of 71% and 66% after 72 h using a drug concentration of 1  $\mu\text{mol/L}$ . Moore *et al*<sup>[34]</sup> tested trichostatin A in PaCa44 cells using microarrays containing 22283 probe sets. One prominent feature was the increased ratio between the levels of expression of pro-apoptotic (BIM) and anti-apoptotic (Bcl-xL and Bcl-W) genes. In addition, Cecconi *et al*<sup>[35]</sup> reported for the same cell line PaCa44 that trichostatin A caused cell cycle arrest at the G2 phase and induced apoptotic cell death. Another hydroxamic acid, SAHA, induced growth inhibition in three pancreatic cell lines BxPC3, COLO-357, and PANC-1 by upregulating p21 and sequestering it in the cytoplasm<sup>[36]</sup>. In our current study, we investigated the two novel cinnamic hydroxamic acid compounds NVP-LAQ824 and NVP-LBH589 for *in vitro* treatment of 8 different human pancreatic cancer cell lines. Cell-growth inhibition by NVP-LAQ824 and NVP-LBH589 was studied by MTT assay. Treatment with both compounds significantly suppressed the growth of 7 cancer cell lines after 3 d of incubation and all cancer cell lines after 6 d of incubation. We hypothesize that the lack of response of Capan-2 cells after 3 d of treatment may be based on the status of the tumor suppressor p53. A genetic profile of 10 different human pancreatic cancer cell lines (6 of the 8 cell lines used in our experiment being amongst them) created by a group from John Hopkins University (<http://pathology2.jhu.edu/pancreas/geneticsweb/profiles.htm>) discovered p53 mutations in almost all cell lines, but not in Capan-2 cells. On the other hand, it has been shown that acetylation and deacetylation of p53 is likely to be part of the mechanism that controls its physiological activity. Whereas HDACs are capable of downregulating p53 function, HDAC inhibition can cause the opposite effect<sup>[37]</sup>. Interestingly, it has also been shown that HDAC inhibitors, such as FR901228 and trichostatin A, completely deplete mutant p53 in cancer cell lines and restore p53-like functions, which is highly toxic to cell lines with mutant p53<sup>[38]</sup>. Donadelli *et al* confirmed this finding in p53 gene mutated pancreatic cancer cell lines which were treated with trichostatin A. The compound induced G2 phase arrest and apoptotic cell death by activation of p21<sup>waf1</sup>, which is normally induced by p53<sup>[39]</sup>.

In previous *in vitro* studies, NVP-LAQ824 exhibited potent anti-proliferative activity against colon carcinoma ( $\text{IC}_{50} = 0.01 \mu\text{mol/L}$ ), and biliary tract cancer ( $\text{IC}_{50} = 0.11 \mu\text{mol/L}$ ) as well as against non-small cell lung carcinoma ( $\text{IC}_{50} = 0.15 \mu\text{mol/L}$ ), prostate cancer ( $\text{IC}_{50} = 0.018\text{--}0.023 \mu\text{mol/L}$ ), head and neck squamous

carcinoma ( $IC_{50} = 0.04\text{--}0.34\ \mu\text{mol/L}$ ), and human breast adenocarcinoma cells ( $IC_{50} = 0.03\text{--}0.039\ \mu\text{mol/L}$ ) after 72 h of exposure<sup>[16,40-42]</sup>. The *in vitro* effects of NVP-LAQ824 on hematologic malignancies have been examined in several human cell lines with a death rate of more than 90% following 48 h of drug incubation, with exposures as low as  $0.1\ \mu\text{mol/L}$ <sup>[43-45]</sup>. Our second compound NVP-LBH589, was even more effective *in vitro* for the treatment of human chronic myeloid leukemia blast crisis K562 and LAMA-84, multiple myeloma, and acute leukemia MV4-11 cells<sup>[15,46-48]</sup>.

The *in vitro* anti-tumoral drug mechanism in our study was assessed by immunoblotting for acH4 (surrogate marker for histone acetylation) p21<sup>WAF-1/CIP-1</sup>, and cell cycle analysis. Treatment with both compounds was associated with hyperacetylation of nucleosomal histone H4, increased expression of p21<sup>WAF-1/CIP-1</sup>, cell cycle arrest at G2/M-checkpoint, and significant induction of apoptosis (increased sub-G1-peak). Therefore, our results are very consistent with the *in vitro* results of the aforementioned studies by Natoni *et al.*<sup>[30]</sup>, Garcia-Morales *et al.*<sup>[31]</sup>, Sato *et al.*<sup>[32]</sup>, Gahr *et al.*<sup>[33]</sup>, Donadelli *et al.*<sup>[39]</sup>, Cecconi *et al.*<sup>[35]</sup>, and Arnold *et al.*<sup>[36]</sup>.

Encouraged by our *in vitro* results, we decided to test the most effective drug NVP-LBH589 *in vivo* in comparison to placebo using the chimeric mouse model. The NVP-LBH589 dose of 25 mg/kg (5 d/wk) was selected according to a study testing different iv doses of NVP-LAQ824 between 5 and 100 mg/kg (5 d/wk) in a similar chimeric mouse model using the human colon cancer cell line HCT 116<sup>[16]</sup>. *In vivo* data for NVP-LBH589 using human prostate carcinoma cell PC-3 xenografts became available only after completion of our study, and showed tumor reduction at a dose of 10 mg/kg per day<sup>[49]</sup>. In our experiments, NVP-LBH589 significantly reduced tumor mass in comparison to placebo and potentiated the efficacy of gemcitabine. In accordance with our observations, Gahr *et al.*<sup>[33]</sup> and Piacentini *et al.*<sup>[50]</sup> showed that a combination with gemcitabine potentiated the *in vitro* effects of trichostatin A in pancreatic cancer cells, demonstrating a synergistic effect between both agents. This phenomenon has been shown for *in vitro* cotreatment with SAHA, too, where the compound rendered pancreatic cancer cells sensitive to the inhibitory and proapoptotic effects of gemcitabine<sup>[36]</sup>. In human breast cancer cell lines SKBR-3 and BT-474, NVP-LAQ824 also enhanced gemcitabine-induced apoptosis *in vitro*<sup>[41]</sup>. For head and neck squamous carcinoma cells, the combination of NVP-LAQ824 with gemcitabine was more effective *in vitro* than a combination with docetaxel, paclitaxel, or cisplatin, especially when the cytotoxic agent was used first for 24 h followed by 48 h of NVP-LAQ824<sup>[40]</sup>. Unfortunately, in the first recently published randomized, double-blind, placebo-controlled multicenter-phase II trial, gemcitabine plus benzamide HDACI CI-994 (N-acetyldinaline) showed no advantage over gemcitabine alone in patients with advanced pancreatic cancer<sup>[51]</sup>. In this study, a total of 174 patients received combination therapy (CI-994, 6 mg/m<sup>2</sup> per day, day 1-21

plus gemcitabine, 1000 mg/m<sup>2</sup>, day 1, 8 and 15 each 28-d cycle) or placebo plus gemcitabine (1000 mg/m<sup>2</sup>, day 1, 8 and 15 each 28-d cycle). Median survival was 194 d (combination therapy) *vs* 214 d (gemcitabine) ( $P = 0.908$ ). The objective response rate was 12% *vs* 14% when investigator-assessed and 1% *vs* 6%, respectively, when assessed centrally. Time to treatment failure did not differ between the two arms ( $P = 0.304$ ). Quality of life scores at 2 mo were worse with the combination than with gemcitabine alone. Pain response rates were similar between the two groups. There was an increased incidence of neutropenia and thrombocytopenia with combination therapy. However, it is currently unknown whether these clinical observations are also true for the hydroxamic acids class of HDACIs. In addition, recent *in vitro* and *in vivo* data have shown synergistic effects of trichostatin A in combination with DNA methyltransferase inhibitors azacytidine<sup>[52,53]</sup> and zebularine<sup>[54]</sup> and proteasome inhibitor PS-341<sup>[55]</sup>, suggesting alternative combination partners for HDACIs. Whereas upregulation of tumor suppressors DUSP6<sup>[52]</sup> and MUC 2<sup>[53]</sup> is the proposed mechanism for the additional effect of DNA methyltransferase inhibitors, it is inactivation of NFkappaB signalling, downregulation of anti-apoptotic Bcl-xL and disruption of MAP kinase pathway for combination with the proteasome inhibitor PS-341<sup>[55]</sup>.

Regarding side effects of the different drugs used in our studies, there was no significant additional weight loss in the COMBO group as compared to placebo. Moreover, NVP-LBH589 alone only induced additional weight loss in the HPAF-2 cell experiment. Weight loss in general was apparently more pronounced in the L3.6pl than in the HPAF-2 cell experiment. This may be due to the fact that L3.6pl cells are a selected variant of COLO-357 cells with increased metastatic potential<sup>[24,56,57]</sup>. Regarding other studies, weight loss of animals was not previously reported for NVP-LAQ824<sup>[16]</sup>, but for NVP-LBH589<sup>[42]</sup>.

In order to assess *in vivo* anti-tumoral drug mechanisms, paraffin sections of mouse tumors were stained with hematoxylin-eosin (H&E), MIB-1 (proliferation marker) and TUNEL (apoptosis marker). Treatment with NVP-LBH589 and COMBO slightly reduced proliferation (reduced MIB-1 staining) and slightly induced apoptosis (increased TUNEL-staining) in HPAF-2 cell bearing mice, whereas proliferation was not decreased and apoptosis only slightly increased in L3.6pl cell bearing mice. Surprisingly, the calculated numbers were much smaller than expected from the *in vitro* experiments. This might be derived from the fact that other pathways, like inhibition of angiogenesis, which we were unable to study in our model due to insufficient tissue quality, may be more important for NVP-LBH589 action in the *in vivo* setting.

Our findings suggest that NVP-LBH589 and NVP-LAQ824 are active against human pancreatic cancer cells *in vitro*, mainly by inhibition of proliferation and induction of apoptosis. NVP-LBH589 is also active in the *in vivo* setting, although the precise mechanism of

drug action is not yet completely understood. Therefore, a clinical study testing NVP-LBH589 for the treatment of pancreaticobiliary cancer has just been initiated at our department.

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## COMMENTS

### Background

Pancreatic adenocarcinoma is essentially an incurable disease, with mortality closely approaching incidence. Single agent gemcitabine is currently considered the standard of care for the treatment of inoperable pancreatic cancer, providing a small but sizable benefit in survival and palliation of symptoms.

### Research frontiers

In the past ten years, several molecular-targeting agents have been introduced in the clinical setting. Despite promising results in phase II studies, randomized clinical trials exploring the new compounds, such as matrix-metalloprotease-inhibitors (MMPi), farnesyl transferase inhibitors (FTI), signal transduction inhibitors, and angiogenesis inhibitors, either alone or in combination with gemcitabine have been largely disappointing. Polo-like kinase 1 (PLK-1), death receptor 5 (DR5), and histone deacetylase (HDAC) inhibitors are currently under clinical evaluation as new treatment options.

### Innovations and breakthroughs

In 2003, fixed-dose-rate (FDR) gemcitabine (1500 mg/m<sup>2</sup> at 10 mg/m<sup>2</sup> per minute) improved median survival time from 5.0 mo in the standard arm to 8.0 mo in a randomized study; However, grade 3 and 4 toxicity increased significantly. In 2005, investigators of a phase III study found that the gemcitabine-capecitabine combination significantly improved overall survival over gemcitabine alone (hazard ratio 0.80; 95% CI 0.65-0.98; *P* = 0.026). Recently, a randomized phase III placebo-controlled trial demonstrated that combining gemcitabine with EGFR inhibitor erlotinib was associated with a modest, but statistically significant survival benefit of 15 d.

### Applications

The aim of our study was to investigate *in vitro* and *in vivo* treatment with the histone deacetylase inhibitors NVP-LAQ824 and NVP-LBH589 in pancreatic cancer. Our findings suggested that NVP-LBH589 and NVP-LAQ824 are active against human pancreatic cancer *in vitro*. In addition, NVP-LBH589 demonstrated significant *in vivo* activity and potentiated the efficacy of gemcitabine.

### Terminology

Histones (positively charged proteins) are the major components of chromatin. Histone acetylation and deacetylation modulate chromosome structure and regulate gene transcription. Two families of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), activate and repress gene expression, respectively. Aberrant HAT or HDAC activity is associated with various epithelial and hematologic cancers. HDACs may play an important role in human oncogenesis through HDAC-mediated gene silencing and interaction of HDACs with proteins involved in tumorigenesis. HDAC inhibition could potentially restore normal processes in transformed cells without affecting normal cells.

### Peer review

This paper addresses the use of histone deacetylase inhibitors in the treatment of pancreatic cancer *in vitro* and *in vivo*. It represents an important experimental assessment of novel agents in the treatment of a cancer for which effective therapy is currently lacking. It's a very interesting paper.

## REFERENCES

- 1 Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor

- A, Feuer EJ, Thun MJ. Cancer statistics, 2005. *CA Cancer J Clin* 2005; **55**: 10-30
- 2 Lockhart AC, Rothenberg ML, Berlin JD. Treatment for pancreatic cancer: current therapy and continued progress. *Gastroenterology* 2005; **128**: 1642-1654
- 3 Carpelan-Holmstrom M, Nordling S, Pukkala E, Sankila R, Luttges J, Kloppel G, Haglund C. Does anyone survive pancreatic ductal adenocarcinoma? A nationwide study re-evaluating the data of the Finnish Cancer Registry. *Gut* 2005; **54**: 385-387
- 4 Cardenes HR, Chiorean EG, Dewitt J, Schmidt M, Loehrer P. Locally advanced pancreatic cancer: current therapeutic approach. *Oncologist* 2006; **11**: 612-623
- 5 Burris HA 3rd, Moore MJ, Andersen J, Green MR, Rothenberg ML, Modiano MR, Cripps MC, Portenoy RK, Storniolo AM, Tarassoff P, Nelson R, Dorr FA, Stephens CD, Von Hoff DD. Improvements in survival and clinical benefit with gemcitabine as first-line therapy for patients with advanced pancreas cancer: a randomized trial. *J Clin Oncol* 1997; **15**: 2403-2413
- 6 Tempero M, Plunkett W, Ruiz Van Haperen V, Hainsworth J, Hochster H, Lenzi R, Abbruzzese J. Randomized phase II comparison of dose-intense gemcitabine: thirty-minute infusion and fixed dose rate infusion in patients with pancreatic adenocarcinoma. *J Clin Oncol* 2003; **21**: 3402-3408
- 7 Cunningham D, Chau I, Stocken D, Davies C, Dunn J, Valle J, Smith D, Steward W, Harper P, Neoptolemos J. Phase III randomised comparison of gemcitabine (GEM) versus gemcitabine plus capecitabine (GEM-CAP) in patients with advanced pancreatic cancer. *EJC Supplements* 2005; **3**: 4
- 8 Moore MJ, Goldstein D, Hamm J, Figer A, Hecht JR, Gallinger S, Au HJ, Murawa P, Walde D, Wolff RA, Campos D, Lim R, Ding K, Clark K, Voskoglou-Nomikos T, Ptasynski M, Parulekar W. Erlotinib plus gemcitabine compared with gemcitabine alone in patients with advanced pancreatic cancer: a phase III trial of the National Cancer Institute of Canada Clinical Trials Group. *J Clin Oncol* 2007; **25**: 1960-1966
- 9 Philip PA, Benedetti J, Fenoglio-Preiser C, Zalupski M, Lenz H, O'Reilly E, Wong R, Atkins J, Abbruzzese J, Blanke C. Phase III study of gemcitabine plus cetuximab versus gemcitabine in patients with locally advanced or metastatic pancreatic adenocarcinoma: SWOG S0205 study. *Journal of Clinical Oncology*, 2007 ASCO Annual Meeting Proceedings Part I 2007; **25**: LBA4509
- 10 Kindler HL, Friberg G, Singh DA, Locker G, Nattam S, Kozloff M, Taber DA, Karrison T, Dachman A, Stadler WM, Vokes EE. Phase II trial of bevacizumab plus gemcitabine in patients with advanced pancreatic cancer. *J Clin Oncol* 2005; **23**: 8033-8040
- 11 Kindler HL, Niedzwiecki D, Hollis D, Oraefo E, Schrag D, Hurwitz H, McLeod HL, Mulcahy MF, Schilsky RL, Goldberg RM. A double-blind, placebo-controlled, randomized phase III trial of gemcitabine (G) plus bevacizumab (B) versus gemcitabine plus placebo (P) in patients (pts) with advanced pancreatic cancer (PC): A preliminary analysis of Cancer and Leukemia Group B (CALGB). *Journal of Clinical Oncology*, 2007 ASCO Annual Meeting Proceedings Part I 2007; **25**: 4508
- 12 Hess-Stump H. Histone deacetylase inhibitors and cancer: from cell biology to the clinic. *Eur J Cell Biol* 2005; **84**: 109-121
- 13 Budillon A, Bruzzese F, Di Gennaro E, Caraglia M. Multiple-target drugs: inhibitors of heat shock protein 90 and of histone deacetylase. *Curr Drug Targets* 2005; **6**: 337-351
- 14 Remiszewski SW, Sambucetti LC, Bair KW, Bontempo J, Cesarz D, Chandramouli N, Chen R, Cheung M, Cornell-Kennon S, Dean K, Diamantidis G, France D, Green MA, Howell KL, Kashi R, Kwon P, Lassota P, Martin MS, Mou Y, Perez LB, Sharma S, Smith T, Sorensen E, Taplin F, Trogani N, Versace R, Walker H, Weltchek-Engler S, Wood A, Wu A, Atadja P. N-hydroxy-3-phenyl-

- 2-propenamides as novel inhibitors of human histone deacetylase with *in vivo* antitumor activity: discovery of (2E)-N-hydroxy-3-[4-[[[(2-hydroxyethyl)[2-(1H-indol-3-yl)ethyl]amino]methyl]phenyl]-2-propenamide (NVP-LAQ824). *J Med Chem* 2003; **46**: 4609-4624
- 15 **George P**, Bali P, Annavarapu S, Scuto A, Fiskus W, Guo F, Sigua C, Sondarva G, Moscinski L, Atadja P, Bhalla K. Combination of the histone deacetylase inhibitor LBH589 and the hsp90 inhibitor 17-AAG is highly active against human CML-BC cells and AML cells with activating mutation of FLT-3. *Blood* 2005; **105**: 1768-1776
  - 16 **Atadja P**, Gao L, Kwon P, Trogani N, Walker H, Hsu M, Yeleswarapu L, Chandramouli N, Perez L, Versace R, Wu A, Sambucetti L, Lassota P, Cohen D, Bair K, Wood A, Remiszewski S. Selective growth inhibition of tumor cells by a novel histone deacetylase inhibitor, NVP-LAQ824. *Cancer Res* 2004; **64**: 689-695
  - 17 **Edwards A**, Li J, Atadja P, Bhalla K, Haura EB. Effect of the histone deacetylase inhibitor LBH589 against epidermal growth factor receptor-dependent human lung cancer cells. *Mol Cancer Ther* 2007; **6**: 2515-2524
  - 18 **Giles F**, Fischer T, Cortes J, Garcia-Manero G, Beck J, Ravandi F, Masson E, Rae P, Laird G, Sharma S, Kantarjian H, Dugan M, Albitar M, Bhalla K. A phase I study of intravenous LBH589, a novel cinnamic hydroxamic acid analogue histone deacetylase inhibitor, in patients with refractory hematologic malignancies. *Clin Cancer Res* 2006; **12**: 4628-4635
  - 19 **Rowinsky EK**, Pacey S, Patnaik A, O'Donnell A, Mita MM, Atadja P, Peng B, Dugan M, Scott JW, De Bono JS. A phase I, pharmacokinetic (PK) and pharmacodynamic (PD) study of a novel histone deacetylase (HDAC) inhibitor LAQ824 in patients with advanced solid tumors. *J Clin Oncol* 2004; **22**: abstract 3022 (ASCO 2004)
  - 20 **Ottmann OG**, Deangelo DJ, Stone DJ, Pfeifer H, Lowenberg B, Atadja P, Peng B, Scott JW, Dugan M, Sonneveld P. A Phase I, pharmacokinetic (PK) and pharmacodynamic (PD) study of a novel histone deacetylase inhibitor LAQ824 in patients with hematologic malignancies. *J Clin Oncol* 2004; **22**: 3024
  - 21 **Sharma S**, Vogelzang NJ, Beck J, Patnaik A, Mita M, Dugan M, Hwang A, Masson E, Culver KW, Prince H. Phase I pharmacokinetic (PK) and pharmacodynamic (PD) study of LBH589, a novel deacetylase (DAC) inhibitor given intravenously on a new once weekly schedule. *J Clin Oncol* 2007; **25**: 14019
  - 22 **Prince HM**, George D, Patnaik A, Mita M, Dugan M, Butterfoss D, Masson E, Culver KW, Burris HA, Beck J. Phase I study of oral LBH589, a novel deacetylase (DAC) inhibitor in advanced solid tumors and non-hodgkin's lymphoma. *J Clin Oncol* 2007; **25**: 3500
  - 23 **Ryu B**, Jones J, Blades NJ, Parmigiani G, Hollingsworth MA, Hruban RH, Kern SE. Relationships and differentially expressed genes among pancreatic cancers examined by large-scale serial analysis of gene expression. *Cancer Res* 2002; **62**: 819-826
  - 24 **Bruns CJ**, Harbison MT, Kuniyasu H, Eue I, Fidler IJ. *In vivo* selection and characterization of metastatic variants from human pancreatic adenocarcinoma by using orthotopic implantation in nude mice. *Neoplasia* 1999; **1**: 50-62
  - 25 **Schoumacher RA**, Ram J, Iannuzzi MC, Bradbury NA, Wallace RW, Hon CT, Kelly DR, Schmid SM, Gelder FB, Rado TA. A cystic fibrosis pancreatic adenocarcinoma cell line. *Proc Natl Acad Sci USA* 1990; **87**: 4012-4016
  - 26 **Meck RA**, Clubb KJ, Allen LM, Yunis AA. Inhibition of cell cycle progression of human pancreatic carcinoma cells *in vitro* by L-(alpha S, 5S)-alpha-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid, Acivicin (NSC 163501). *Cancer Res* 1981; **41**: 4547-4553
  - 27 **Sipos B**, Moser S, Kalthoff H, Torok V, Lohr M, Kloppel G. A comprehensive characterization of pancreatic ductal carcinoma cell lines: towards the establishment of an *in vitro* research platform. *Virchows Arch* 2003; **442**: 444-452
  - 28 **Tannapfel A**, Geissler F, Kockerling F, Katalinic A, Hauss J, Wittekind C. Apoptosis and proliferation in relation to histopathological variables and prognosis in hepatocellular carcinoma. *J Pathol* 1999; **187**: 439-445
  - 29 **Tannapfel A**, Hahn HA, Katalinic A, Fietkau RJ, Kuhn R, Wittekind CW. Prognostic value of ploidy and proliferation markers in renal cell carcinoma. *Cancer* 1996; **77**: 164-171
  - 30 **Natoni F**, Diolordi L, Santoni C, Gilardini Montani MS. Sodium butyrate sensitises human pancreatic cancer cells to both the intrinsic and the extrinsic apoptotic pathways. *Biochim Biophys Acta* 2005; **1745**: 318-329
  - 31 **Garcia-Morales P**, Gomez-Martinez A, Carrato A, Martinez-Lacaci I, Barbera VM, Soto JL, Carrasco-Garcia E, Menendez-Gutierrez MP, Castro-Galache MD, Ferragut JA, Saceda M. Histone deacetylase inhibitors induced caspase-independent apoptosis in human pancreatic adenocarcinoma cell lines. *Mol Cancer Ther* 2005; **4**: 1222-1230
  - 32 **Sato N**, Ohta T, Kitagawa H, Kayahara M, Ninomiya I, Fushida S, Fujimura T, Nishimura G, Shimizu K, Miwa K. FR901228, a novel histone deacetylase inhibitor, induces cell cycle arrest and subsequent apoptosis in refractory human pancreatic cancer cells. *Int J Oncol* 2004; **24**: 679-685
  - 33 **Gahr S**, Ocker M, Ganslmayer M, Zopf S, Okamoto K, Hartl A, Leitner S, Hahn EG, Herold C. The combination of the histone-deacetylase inhibitor trichostatin A and gemcitabine induces inhibition of proliferation and increased apoptosis in pancreatic carcinoma cells. *Int J Oncol* 2007; **31**: 567-576
  - 34 **Moore PS**, Barbi S, Donadelli M, Costanzo C, Bassi C, Palmieri M, Scarpa A. Gene expression profiling after treatment with the histone deacetylase inhibitor trichostatin A reveals altered expression of both pro- and anti-apoptotic genes in pancreatic adenocarcinoma cells. *Biochim Biophys Acta* 2004; **1693**: 167-176
  - 35 **Cecconi D**, Scarpa A, Donadelli M, Palmieri M, Hamdan M, Astner H, Righetti PG. Proteomic profiling of pancreatic ductal carcinoma cell lines treated with trichostatin-A. *Electrophoresis* 2003; **24**: 1871-1878
  - 36 **Arnold NB**, Arkus N, Gunn J, Korc M. The histone deacetylase inhibitor suberoylanilide hydroxamic acid induces growth inhibition and enhances gemcitabine-induced cell death in pancreatic cancer. *Clin Cancer Res* 2007; **13**: 18-26
  - 37 **Juan LJ**, Shia WJ, Chen MH, Yang WM, Seto E, Lin YS, Wu CW. Histone deacetylases specifically down-regulate p53-dependent gene activation. *J Biol Chem* 2000; **275**: 20436-20443
  - 38 **Blagosklonny MV**, Trostel S, Kayastha G, Demidenko ZN, Vassilev LT, Romanova LY, Bates S, Fojo T. Depletion of mutant p53 and cytotoxicity of histone deacetylase inhibitors. *Cancer Res* 2005; **65**: 7386-7392
  - 39 **Donadelli M**, Costanzo C, Faggioli L, Scupoli MT, Moore PS, Bassi C, Scarpa A, Palmieri M. Trichostatin A, an inhibitor of histone deacetylases, strongly suppresses growth of pancreatic adenocarcinoma cells. *Mol Carcinog* 2003; **38**: 59-69
  - 40 **Tran H**, TShoaf SL. Improved efficacy with sequential use of histone deacetylase inhibitor, LAQ824, with common chemotherapeutic agents in head and neck squamous carcinoma cell lines. *Proc Amer Assoc Cancer Res* 2005; **46**: 5095
  - 41 **Fuino L**, Bali P, Wittmann S, Donapaty S, Guo F, Yamaguchi H, Wang HG, Atadja P, Bhalla K. Histone deacetylase inhibitor LAQ824 down-regulates Her-2 and sensitizes human breast cancer cells to trastuzumab, taxotere, gemcitabine, and epothilone B. *Mol Cancer Ther* 2003; **2**: 971-984
  - 42 **Bluethner T**, Niederhagen M, Caca K, Serr F, Witzigmann H, Moebius C, Mossner J, Wiedmann M. Inhibition of histone deacetylase for the treatment of biliary tract cancer: a new effective pharmacological approach. *World J*



- Gastroenterol* 2007; **13**: 4761-4770
- 43 **Catley L**, Weisberg E, Tai YT, Atadja P, Remiszewski S, Hideshima T, Mitsiades N, Shringarpure R, LeBlanc R, Chauhan D, Munshi NC, Schlossman R, Richardson P, Griffin J, Anderson KC. NVP-LAQ824 is a potent novel histone deacetylase inhibitor with significant activity against multiple myeloma. *Blood* 2003; **102**: 2615-2622
  - 44 **Bhalla KN**, Nimmanapalli R, Fuino L, Tao J, Lee H. Histone deacetylase inhibitor LAQ824 down regulates BCR-ABL levels and induces apoptosis of imatinib mesylate -sensitive or -refractory BCR-ABL positive human leukemia cells. *Proc Am Soc Clin Oncol* 2003; **22**: 2322
  - 45 **Rosato RR**, Almenara JA, Maggio SC, Atadja P, Dent P, Grant S. Potentiation of LAQ824-mediated lethality by the cyclin-dependent kinase inhibitor roscovitine in human leukemia cells proceeds through an XIAP- and reactive oxygen species (ROS)-dependent mechanism. *Proc Amer Assoc Cancer Res* 2005; **46**: 5327
  - 46 **Maiso P**, Carvajal-Vergara X, Ocio EM, Lopez-Perez R, Mateo G, Gutierrez N, Atadja P, Pandiella A, San Miguel JF. The histone deacetylase inhibitor LBH589 is a potent antimyeloma agent that overcomes drug resistance. *Cancer Res* 2006; **66**: 5781-5789
  - 47 **Catley L**, Weisberg E, Kiziltepe T, Tai YT, Hideshima T, Neri P, Tassone P, Atadja P, Chauhan D, Munshi NC, Anderson KC. Aggresome induction by proteasome inhibitor bortezomib and alpha-tubulin hyperacetylation by tubulin deacetylase (TDAC) inhibitor LBH589 are synergistic in myeloma cells. *Blood* 2006; **108**: 3441-3449
  - 48 **Fiskus W**, Prnpat M, Bali P, Balasis M, Kumaraswamy S, Boyapalle S, Rocha K, Wu J, Giles F, Manley PW, Atadja P, Bhalla K. Combined effects of novel tyrosine kinase inhibitor AMN107 and histone deacetylase inhibitor LBH589 against Bcr-Abl-expressing human leukemia cells. *Blood* 2006; **108**: 645-652
  - 49 **Qian DZ**, Kato Y, Shabbeer S, Wei Y, Verheul HM, Salumbides B, Sanni T, Atadja P, Pili R. Targeting tumor angiogenesis with histone deacetylase inhibitors: the hydroxamic acid derivative LBH589. *Clin Cancer Res* 2006; **12**: 634-642
  - 50 **Piacentini P**, Donadelli M, Costanzo C, Moore PS, Palmieri M, Scarpa A. Trichostatin A enhances the response of chemotherapeutic agents in inhibiting pancreatic cancer cell proliferation. *Virchows Arch* 2006; **448**: 797-804
  - 51 **Richards DA**, Boehm KA, Waterhouse DM, Wagener DJ, Krishnamurthi SS, Rosemurgy A, Grove W, Macdonald K, Gulyas S, Clark M, Dasse KD. Gemcitabine plus CI-994 offers no advantage over gemcitabine alone in the treatment of patients with advanced pancreatic cancer: results of a phase II randomized, double-blind, placebo-controlled, multicenter study. *Ann Oncol* 2006; **17**: 1096-1102
  - 52 **Xu S**, Furukawa T, Kanai N, Sunamura M, Horii A. Abrogation of DUSP6 by hypermethylation in human pancreatic cancer. *J Hum Genet* 2005; **50**: 159-167
  - 53 **Yamada N**, Hamada T, Goto M, Tsutsumida H, Higashi M, Nomoto M, Yonezawa S. MUC2 expression is regulated by histone H3 modification and DNA methylation in pancreatic cancer. *Int J Cancer* 2006; **119**: 1850-1857
  - 54 **Neureiter D**, Zopf S, Leu T, Dietze O, Hauser-Kronberger C, Hahn EG, Herold C, Ocker M. Apoptosis, proliferation and differentiation patterns are influenced by Zebularine and SAHA in pancreatic cancer models. *Scand J Gastroenterol* 2007; **42**: 103-116
  - 55 **Bai J**, Demirjian A, Sui J, Marasco W, Callery MP. Histone deacetylase inhibitor trichostatin A and proteasome inhibitor PS-341 synergistically induce apoptosis in pancreatic cancer cells. *Biochem Biophys Res Commun* 2006; **348**: 1245-1253
  - 56 **Bruns CJ**, Koehl GE, Guba M, Yezhelyev M, Steinbauer M, Seeliger H, Schwend A, Hoehn A, Jauch KW, Geissler EK. Rapamycin-induced endothelial cell death and tumor vessel thrombosis potentiate cytotoxic therapy against pancreatic cancer. *Clin Cancer Res* 2004; **10**: 2109-2119
  - 57 **Bruell D**, Bruns CJ, Yezhelyev M, Huhn M, Muller J, Ischenko I, Fischer R, Finnern R, Jauch KW, Barth S. Recombinant anti-EGFR immunotoxin 425(scFv)-ETA' demonstrates anti-tumor activity against disseminated human pancreatic cancer in nude mice. *Int J Mol Med* 2005; **15**: 305-313

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## Negundoside, an iridiod glycoside from leaves of *Vitex negundo*, protects human liver cells against calcium-mediated toxicity induced by carbon tetrachloride

Sheikh A Tasduq, Peerzada J Kaiser, Bishan D Gupta, Vijay K Gupta, Rakesh K Johri

Sheikh A Tasduq, Peerzada J Kaiser, Bishan D Gupta, Vijay K Gupta, Rakesh K Johri, Indian Institute of Integrative Medicine, CSIR, Jammu-Tawi-180001, Jammu and Kashmir, India

**Author contributions:** Tasduq SA designed the study, performed the experiments, analyzed the data, drafted the manuscript and contributed in use of new reagents/analytic tools; Kaiser PS was equally responsible as the first author for performing the experiments and helping in data setting, statistics, arrangement of figures and manuscript drafting; Gupta BD performed the chemistry experiments; Gupta VK worked on the data analysis, application of statistics and manuscript correction; Johri RK was the group leader and was responsible for checking the hypothesis of research study and final corrections of the manuscript and acquired funding for the study.

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Correspondence to: Sheikh A Tasduq, PhD, Scientist, Experimental Toxicology Lab, Division of Pharmacology, Indian Institute of Integrative Medicine, CSIR, Canal Road, Jammu 180001, Jammu and Kashmir, India. [tasduq11@gmail.com](mailto:tasduq11@gmail.com)  
Telephone: +91-191-2569000-10 Fax: +91-191-2569333

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### Abstract

**AIM:** To evaluate the protective effect of 2'-p-hydroxy benzoylmussaenosidic acid [negundoside (NG)], against carbon tetrachloride (CCl<sub>4</sub>)-induced toxicity in HuH-7 cells.

**METHODS:** CCl<sub>4</sub> is a well characterized hepatotoxin, and inducer of cytochrome P450 2E1 (CYP2E1)-mediated oxidative stress. In addition, lipid peroxidation and accumulation of intracellular calcium are important steps in the pathway involved in CCl<sub>4</sub> toxicity. Liver cells (HuH-7) were treated with CCl<sub>4</sub>, and the mechanism of the cytoprotective effect of NG was assessed. Silymarin, a known hepatoprotective drug, was used as control.

**RESULTS:** NG protected HuH-7 cells against CCl<sub>4</sub> toxicity and loss of viability without modulating CYP2E1 activity. Prevention of CCl<sub>4</sub> toxicity was associated with a reduction in oxidative damage as reflected by decreased generation of reactive oxygen species (ROS), a decrease in lipid peroxidation and accumulation of intracellular Ca<sup>2+</sup> levels and maintenance of intracellular glutathione homeostasis. Decreased mitochondrial membrane

potential (MMP), induction of caspases mediated DNA fragmentation and cell cycle arrest, as a result of CCl<sub>4</sub> treatment, were also blocked by NG. The protection afforded by NG seemed to be mediated by activation of cyclic adenosine monophosphate (cAMP) synthesis and inhibition of phospholipases (cPLA2).

**CONCLUSION:** NG exerts a protective effect on CYP2E1-dependent CCl<sub>4</sub> toxicity *via* inhibition of lipid peroxidation, followed by an improved intracellular calcium homeostasis and inhibition of Ca<sup>2+</sup>-dependent proteases.

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**Key words:** Negundoside; Silymarin; HuH-7; Carbon tetrachloride; CYP 2E1; Oxidative stress; Calcium; Toxicity

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### INTRODUCTION

Natural products from plant sources have extensive past and present use in treatment of diverse diseases and serve as compounds of interest both in their natural form and as templates for synthetic modification. The importance of natural products in modern medicine has been well recognized. More than 20 new drugs, launched world over between 2000 and 2005, originate from natural products. Scrutiny of medical indications by source of compounds has demonstrated that natural products and related drugs are used to treat 87% of all categorized human diseases (infectious and non-

infectious)<sup>[1]</sup>.

*Vitex negundo* (verbenaceae) is an important source of such natural drugs. It is a reputed medicinal herb and its parts have been employed as a traditional cure in Asian systems of medicine (Indian, Chinese, Malaysian) for a variety of disease conditions. A number of pharmacological activities have been attributed to *V. negundo*, such as: analgesic and anti-inflammatory activity<sup>[2]</sup>, enzymes inhibition<sup>[3]</sup>, nitric oxide scavenging activity<sup>[4]</sup>, snake venom neutralization activity<sup>[5]</sup>, antifeeding activity<sup>[6]</sup>, antiradical and antilipoperoxidative<sup>[7]</sup>, CNS activity<sup>[8]</sup>, hepatoprotective activity<sup>[9]</sup>, anti-bacterial activity<sup>[10]</sup>, anti-fungal<sup>[11]</sup>, larvicidal activity<sup>[12]</sup>, antiandrogenic effects<sup>[13]</sup>, mosquito repellent activity<sup>[14]</sup>.

In the recent past, some of our work and work as reported by others on botanical products from *V. negundo* have shown a promising hepatoprotective activity<sup>[9,15]</sup>. This activity has been evaluated against various hepatotoxic agents including carbon tetrachloride (CCl<sub>4</sub>). CCl<sub>4</sub> is a well established and widely used hepatotoxin and the principle cause of CCl<sub>4</sub>-induced liver injury is proposed to be lipid peroxidation by free radical derivatives of CCl<sub>4</sub>. CCl<sub>4</sub> is activated by NADH-CYP 450 2E1 system of the liver endoplasmic reticulum and converted into trimethyl CCl<sub>3</sub> radicals (*via* reductive dehalogenation) and, under aerobic conditions, in the more reactive trichloromethyl peroxy radical CCl<sub>3</sub>OO<sup>\*</sup>. Formation of the radicals CCl<sub>3</sub><sup>\*</sup> and CCl<sub>3</sub>OO<sup>\*</sup> causes oxidative stress. The CYP 2E1-mediated metabolism results in generation of reactive oxygen species, which further contributes to the development of cellular injury<sup>[16]</sup>. Also, considerable evidence suggests that CCl<sub>4</sub> modifies the expression levels of several pro-apoptotic and anti-apoptotic growth factors and receptors<sup>[17]</sup> especially during chronic administration. CCl<sub>4</sub> has been shown to be a carcinogen and has been classified as a group 2B carcinogen by inducing gene conversion, homozygosity and intra-chromosomal recombinations<sup>[18]</sup>.

It was, therefore, our interest to investigate, in-depth, the mechanism of modulation of CCl<sub>4</sub>-induced toxic manifestations with 2'-p-hydroxybenzoylmussaenosidic acid [negundoside (NG)] (a purified irridoid glycoside from leaves of *Vitex negundo*), particularly inhibition of downstream CYP 2E1 cascade of pro-apoptotic events with reference to the following: (1) role of CYP 450 2E1 activation on calcium-mediated oxidative stress; (2) involvement of calcium in phospholipase A2 (PLA2) and cyclic adenosine monophosphate (cAMP) regulation; (3) effect of activated cPLA2 on mitochondrial depolarization, inducing cytochrome C release resulting in caspase mediated apoptosis.

## MATERIALS AND METHODS

### Chemicals

DMEM F12 medium, Fetal calf serum, trypsin-EDTA solution, 2', 7'-dichlorofluoresceine diacetate (DCF-DA), rhodamine-123 (Rh-123), propidium iodide (PI), DNase-free RNase, proteinase K, 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Hoechst 33258,

cyclosporine A, penicillin, streptomycin, L-glutamine, pyruvic acid, camptothecin, malondialdehyde (MDA) and other biochemicals were purchased from Sigma Chemicals Co. (St. Louis, Mo). Caspase-3 (ApoAlert caspases assay kits) were from B.D. Clontech, USA. Phospholipase A2 (PLA2) and cyclic adenosine triphosphatase (cAMP) were measured by commercially available kits from Cyman company, USA, and R&D systems, USA respectively. Protein concentration was measured using the BCA Protein Assay Kit from Pierce (Rockford, IL, USA).

### Collection and identification of test material

Aerial parts of the plant *Vitex negundo* Linn were collected locally during August to October. Plant material was identified and authenticated by examination of the morphological characteristics by taxonomist of the Institute. A voucher specimen has been deposited in Indian Institute of Integrative Medicine (I.I.I.M.) Jammu Herbarium under collection No. 17814.

### Extraction procedure for preparation of NG

The shade dried and powdered leaves (1 kg) of *V. negundo* were soaked in ethanol (5 L) and kept overnight. The percolate was filtered and concentrated under reduced pressure at below 50°C. The extraction procedure was repeated three times more using 3 L of ethanol each time. The combined ethanol extract was stirred with water (300 mL) for 1 h and filtered through Celite. The aqueous extract was concentrated at 50°C and finally dried in vacuum desiccators.

### Isolation of NG

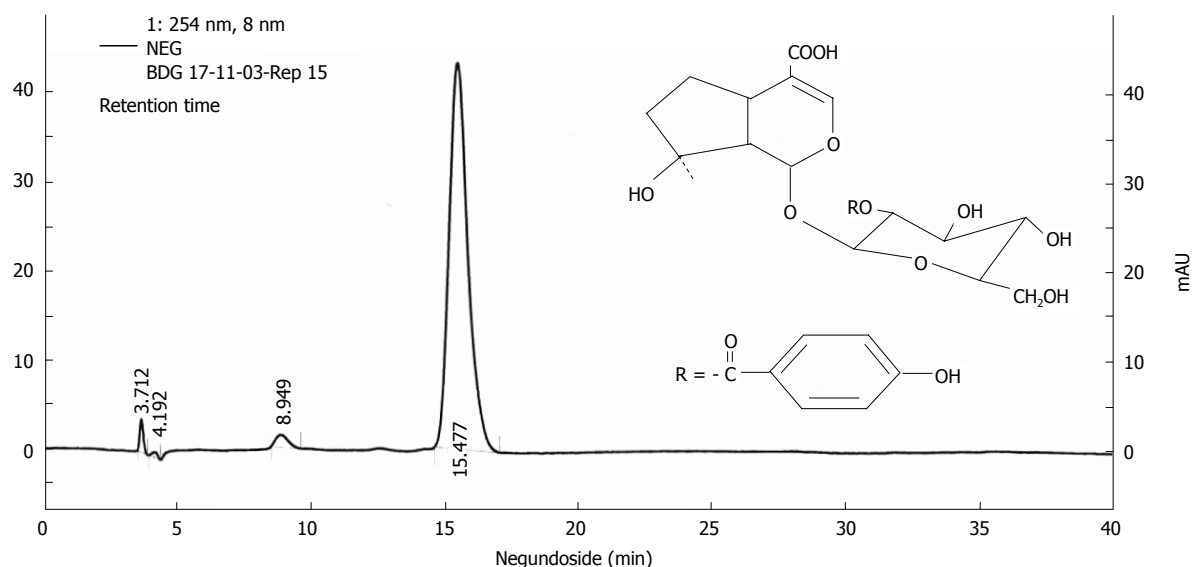
The ethanol extract (50 g) of *V. negundo* was adsorbed over silica gel (100 g) to make slurry which was packed over a column of silica gel (1 kg) packed in chloroform. Elution was done with chloroform followed by mixture of chloroform and methanol. Elution with 10% methanol in chloroform gave agnuside followed by mixture of agnuside and negundoside and then negundoside. The compounds were characterized on the basis of <sup>1</sup>HNMR, <sup>13</sup>CNMR mass spectral data (data not shown) and standardized by HPLC (Figure 1).

### Cell culture

The study was carried out using as a model a human hepatoma HuH-7 cells line (ATCC-USA), a generous gift from Dr. Vijai Kumar, International Centre for Genetic Engineering and Biotechnology (ICGEB), New Delhi, India. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal calf serum (FCS), supplemented with 100 Units/mL penicillin, 100 mg/L streptomycin in a humidified atmosphere in 5% CO<sub>2</sub> at 37°C, and were sub-cultured at 1:5 ratio once a week.

### Cell treatment

FCS was reduced to 3% for the experiments. Cells were plated at a density of 3 × 10<sup>4</sup> cells/cm<sup>2</sup> and maintained in culture medium for 12 h. Stock solutions of CCl<sub>4</sub>, NG and silymarin were prepared fresh to avoid



**Figure 1** Finger print profile and chemical structure of NG. The HPLC profile of NG was performed by employing Shimadzu HPLC system consisting of a diode array detector and C18 column (5  $\mu$ m, 250 mm x 4.0 mm I.D.) by UV detection at 254 nm. NG was resolved on a mobile phase consisting of methanol: 2% acetonitrile (30:70) and delivered at a flow rate of 0.6 mL/min. The chromatogram is representative of one of three independent analyses.

oxidation. For  $\text{CCl}_4$  toxicity experiments, test substances, were added to the cell cultures an hour prior to  $\text{CCl}_4$  treatment. Cells or supernatant were then collected for determination of various parameters.

### Cytotoxicity assays

Cells were seeded onto 24-well plates, and after the corresponding treatment, the medium was removed and cell viability was evaluated by assaying for the ability of functional mitochondria to catalyze the reduction of MTT to form formazan salt by mitochondrial dehydrogenases, as described<sup>[19]</sup> and determined by ELISA reader at 565 nm (Multiskan Spectrum; Thermo Electron Corporation, USA).

### Cellular and nuclear morphology

The cellular and nuclear morphology was observed under the light microscope (Nikon Eclipse TE2000U), at 40  $\times$  magnification, or under fluorescent microscopy, using Hoechst 33258 staining method as described<sup>[20]</sup> with certain modifications. Briefly, untreated and treated HuH-7 cells in 6 well plates were harvested by trypsinization, centrifuged at 100  $\times g$  for 5 min and washed twice with PBS. Cells were gently suspended in 100  $\mu$ L PBS and fixed in 400  $\mu$ L cold acetic acid: methanol (v/v = 1:3) overnight at 4°C. Cells were washed again in 1 mL of fixing solution, suspended in the residual volume of about 50  $\mu$ L, spread on a clean slide and dried overnight at room temperature. One milliliter of staining solution (Hoechst 33258, 10 mg/L in 0.01 mol/L citric acid and 0.45 mol/L disodium phosphate containing 0.05% Tween 20) was poured on each slide and stained for 30 min under subdued light at room temperature. Slides were washed under gentle flow of tap water, rinsed in distilled water followed by in PBS. While wet, 50  $\mu$ L of mounting fluid (PBS:glycerol, 1:1) was poured over the center of slide and covered with glass cover slip. The

slides were sealed with nail polish and observed for any nuclear morphological alterations and apoptotic bodies under inverted fluorescence microscope (Nikon Eclipse TE2000U, magnification 40X) using UV excitation.

### Anti-hemolytic activity

Anti-hemolytic activity of NG and silymarin was studied as described<sup>[21]</sup>. Briefly, blood was collected from healthy volunteers and centrifuged (3000 r/min) with an equal volume of sterilized Alsever solution (2% dextrose, 0.8% sodium citrate, 0.05% citric acid, and 0.42% sodium chloride in water) to obtain the packed cells. The cells were washed with isosaline (0.85%, pH 7.4) and diluted with phosphate buffer (0.15 mol/L, pH 7.4). RBCs ( $10^8$  cells/mL) were incubated with triton  $\times$  100 (1 g/L) to induce 100% cell lysis, in absence and presence of test materials at 37°C for 1 h. The  $A$  of the supernatant was determined at 540 nm.

### CYP2E1 catalytic activity assay

CYP2E1 activity was determined by assaying *p*-nitrophenol hydroxylation in rat liver microsomes prepared by calcium precipitation method as described earlier<sup>[22]</sup>. In brief, 1 mg microsomes in presence and absence of test material caused hydroxylation of 100 mmol/L aniline hydrochloride in presence of 30 mmol/L cumene hydroperoxide in 0.1 mol/L Tris buffer, pH 7.5. Liberated *p*-aminophenol was treated with 1 mL of 1 mol/L  $\text{Na}_2\text{CO}_3$  and 1 mL of 2% phenol solution in 0.5 mol/L NaOH. The samples were allowed to stand at room temperature for 30 min and read at 630 nm.

### Lipid peroxidation analysis

Cells were plated onto 60 mm Petri dishes, and at the end of the treatment they were washed twice in cold PBS and harvested using rubber policeman in 1 mL PBS. To the resulting cell suspension, we added 2 mL



of TCA-TBA reagent (15% TCA + 0.375% TBA in 5 mol/L HCl) in glass tubes. Tubes were kept in a boiling water bath (100°C) for 1 h, and then cooled to room temp. The contents were centrifuged at  $1500 \times g$  for 10 min. Absorbance of the supernatants was read at 535 nm against blank. Malonyldialdehyde (MDA) was used to draw the standard curve. The results were expressed as nmoles MDA/mg protein.

In other set of experiments, 1 g/L of rat liver microsomes in 0.15 mol/L NaCl were incubated with 0.1 mmol/L  $\text{FeSO}_4$  and 35 mmol/L  $\text{H}_2\text{O}_2$  in the presence and absence of test materials to stimulate lipid peroxidation. Generation of MDA was determined by assaying for thiobarbituric acid-reactive substances (TBARS) as described<sup>[22]</sup>.

### Determination of glutathione levels

Cells were seeded onto 60 mm Petri dishes and collected after the corresponding treatments. The total GSH content (reduced form) of samples was assayed as described<sup>[23]</sup>. Briefly, after treatments, cells were washed with ice cold PBS containing 10 mmol/L EDTA and centrifuged at  $1500 \times g$ . The cell pellets were resuspended in PBS/EDTA solution with 35% perchloric acid. The cell suspensions were kept on ice for 10 min and vortexed in between 5 times. Cell suspension was centrifuged at 13000 r/min for 10 min at 4°C. The supernatant was transferred to fresh tubes and the pH adjusted to 7 with triethanolamine, 1 mol/L,  $\text{K}_2\text{CO}_3$  1.65 mol/L and EDTA 30 mmol/L. The contents were centrifuged at 13000 r/min for 10 min. To the 50  $\mu\text{L}$  of the supernatant, we added 1850  $\mu\text{L}$  of PBS/EDTA solution and 100  $\mu\text{L}$  of o-phthalaldehyde (1 g/L in methanol). The samples were incubated in the dark for 20 min and read at 350 nm (excitation) and 420 nm (emission) with a fluorescent spectrofluorometer (Perkin Elmer; LS-55).

### Determination of cytochrome C by HPLC

Cytochrome C was determined as described<sup>[24]</sup>. Briefly, the reaction medium (2.5 mL) containing mitochondria (1 mg protein/mL) was 0.2 mol/L sucrose, 1 mmol/L  $\text{KH}_2\text{PO}_4$ , 5 mmol/L succinate, 2  $\mu\text{mol/L}$  rotenone, 10 mmol/L Tris-MOPS, pH 7.3 at 25°C. Mitochondria were preincubated with test materials [NG, silymarin and cyclosporin A (CsA; 0.2  $\mu\text{mol/L}$ )] for 10 min, and then in presence of  $\text{CCl}_4$  (2 mmol/L) for 30 min. After incubation, aliquots of mitochondrial suspensions were centrifuged at  $11000 \times g$  for 8 min to obtain the supernatant. Finally, the supernatant (50  $\mu\text{L}$ ) was introduced into an HPLC system equipped with a reverse-phase C4 Cosmosil column (150 mm  $\times$  4.6 mm, 5- $\mu\text{m}$  particle size, equipped with a UV-visible detector (393 nm). The column (Spelco; Supercosil LC-304; 24  $\mu\text{m} \times 4.6 \mu\text{m} \times 5 \mu\text{m}$ ) was eluted with a linear gradient of acetonitrile-water (solvents modified with 0.1 mL/L trifluoroacetic acid); the gradient started at 20% acetonitrile and changed to 60% during 12 min and the flow rate was 1.0 mL/min. The column was then

washed with the 60% acetonitrile for 5 min followed by reequilibration for 5 min in the 20% acetonitrile.

### Preparation of rat liver mitochondria

Mitochondria from livers of rats (Wistar) were prepared as described<sup>[25]</sup>. In brief, rat livers were washed once with physiological saline, dissected and washed twice in cold isolation solution (200 mmol/L D-mannitol, 70 mmol/L sucrose, 2 mmol/L HEPES, 0.5 g/L BSA). Dissected livers were minced with two volumes of isolation solution and homogenized (IKA homogenizer-WERK, Ultra Turrax, T 25 B). The homogenate was diluted with 7 volumes of isolation buffer and centrifuged for 10 min at  $560 \times g$ . The supernatant collected was again centrifuged for 15 min at  $7000 \times g$ . The mitochondrial pellet was collected and resuspended in 2.5 volumes of isolation solution and stored at -80°C until use.

### Measurement of intracellular calcium

Intracellular calcium levels were determined with the fluorescent calcium indicator fura2-AM by ratiometric fluorimetry as described<sup>[26]</sup>. HuH-7 cells were detached from the plate using HBSS buffer (118 mmol/L NaCl, 4.6 mmol/L KCl, 10 mmol/L glucose, 20 mmol/L Hepes, pH 7.2) containing 0.02% EDTA, resuspended in HBSS with 1 mmol/L  $\text{CaCl}_2$ , and incubated with 1  $\mu\text{mol/L}$  fura2-AM in the dark for 1 h at 37°C. Cells were washed with HBSS/ $\text{CaCl}_2$ , and resuspended in HBSS/ $\text{CaCl}_2$  at a density of  $1 \times 10^6$  cells/mL. EGTA (10 mmol/L) was added at the beginning of the experiment, followed by a 60 s-equilibration period<sup>[27]</sup>. Intracellular free calcium measurements were performed at 37°C using a ratiometric fluorescence spectrophotometer (Perkin-Elmer LS 50B). Intracellular  $\text{Ca}^{2+}$  concentration was estimated as described<sup>[28]</sup> based on the equation:  $[\text{Ca}^{2+}]_i = K_d[(R - R_{\min})/(R_{\max} - R)]F_{\min(380)}/F_{\max(380)}$ , where  $R$  is  $F_{340}/F_{380}$  ratio,  $R_{\min}$  and  $R_{\max}$  are the ratios with 50 mmol/L digitonin, and 50 mmol/L digitonin + 11 mmol/L  $\text{CaCl}_2$ , respectively.  $K_d$  represents the apparent dissociation constant of Fura-2 (224 nmol/L) and  $F_{\min(380)}/F_{\max(380)}$  are the fluorescence values of digitonized cells without or with 11 mmol/L  $\text{CaCl}_2$ , respectively.

### Flow cytometric analysis of mitochondrial membrane potential ( $\Delta\psi_m$ )

Changes in the mitochondrial membrane potential ( $\Delta\psi_m$ ) were examined by monitoring the cell fluorescence after staining with rhodamine 123 (Rh123) as described<sup>[29]</sup>. Rh123 is a membrane permeable fluorescent cationic dye that is selectively taken up by mitochondria directly proportional to the MMP<sup>[30]</sup>. The intensities from Rh123 and PI were determined using a BD-LSR flow cytometer equipped with electronic doublet discriminating capability.

### Intracellular measurement of reactive oxygen species (ROS)

The production of ROS was monitored with DCF-DA as the probe<sup>[31]</sup>. DCF-DA diffuses through the cell membrane and is enzymatically hydrolyzed by

intracellular esterases to nonfluorescent DCF-H, which is then rapidly oxidized to the highly fluorescent DCF in presence of ROS. Treated and non-treated HuH-7 cells were incubated for 1 h with DCF-DA (2  $\mu\text{mol/L}$ ) and, after the washing of cells, the production of free radicals were assessed *in situ* as the enhancement of fluorescence at excitation wavelength of 500 nm and emission wavelength of 520 nm and was measured in a fluorescent plate reader (Perkin Elmer LS 55, USA).

#### Measurement of intracellular $\text{H}_2\text{O}_2$

Intracellular  $\text{H}_2\text{O}_2$  levels were analyzed using 123 dihydorhodamine (123-DHR) as specific fluorescent dye probe as described by Katiyar *et al* with modifications for HuH-7 cells<sup>[32]</sup>. Briefly, non-treated and treated HuH-7 were washed twice with PBS and incubated in the culture medium without FCS and loaded with 123-DHR (5  $\mu\text{mol/L}$ ). Cells were further incubated for 45 min to irreversibly oxidize and convert DHR to fluorescent compound rhodamine 123 and fluorescence was read with aid of spectrofluorometer (Perkin Elmer LS 55, USA) with excitation wavelength 485 nm and emission wavelength of 530 nm. The cells for fluorescent-based assays were grown onto sterile black fluorescent plates (96 well format; Nunc, Denmark).

#### Cell cycle analysis (apoptosis)

Cell cycle was analysed as described by Yang *et al*<sup>[33]</sup>. Briefly, non-treated and treated HuH-7 cells were harvested by trypsinization, centrifuged at  $1500 \times g$  for 5 min, washed with PBS, and fixed in 70% ethanol at 4°C overnight. Fixed cells were washed twice with PBS and incubated in PBS containing 1.5 mg/L RNase A for 1 h at 37°C, followed by staining with 5  $\mu\text{L}$  PI (1 mmol/L stock) for 20 min on ice. The cells were analyzed for DNA content using BD-LSR flow cytometer equipped with electronic doublet discrimination capability using blue (488 nm) excitation from argon laser. Data were collected in list mode on 10 000 events for FL2-A vs FL2-W.

#### DNA laddering

DNA fragmentation was analysed as described by Yang *et al*<sup>[33]</sup>. Briefly, HuH-7 cells treated or untreated were harvested and centrifuged at  $1500 \times g$  for 5 min. After washing twice with PBS/ethylenediamine-N, N, N', N'-tetraacetic acid (EDTA). Cells were incubated in a lysis buffer [0.5 mL/L Triton X-100, 10 mmol/L EDTA, 0.4 g/L proteinase K, and 10 mmol/L Tris-HCl, pH 7.4] at 56°C for 1 h. Cell lysate was treated with 0.4 g/L RNase at 37°C for 30 min. The genomic DNAs were purified by phenol/chloroform extraction and ethanol precipitation, and resuspended in a Tris-EDTA buffer, DNA fragments were stained with ethidium bromide and visualized by 2.5% agarose electrophoresis.

#### Enzymic assay of caspase 3-activity

Caspase activation was measured using a caspase 3 fluorometric assay kit (BD Apoalert caspase 3 fluorescent assay kit). HuH-7 cells, treated or untreated were harvested, and centrifuged (approximately 1 mg protein)

at  $400 \times g$  for 5 min. The cell pellets were re-suspended in 50  $\mu\text{L}$  of chilled cell lysis buffer and incubated on ice for 10 min, and the lysates were centrifuged at  $15000 \times g$  for 10 min at 4°C to precipitate cellular debris. A total of 50  $\mu\text{L}$  of cell lysates was incubated with 50  $\mu\text{L}$  of reaction buffer/DTT mix. DEVD-CHO was used as an inhibitor of caspase 3 in an induced sample. Five  $\mu\text{L}$  of 1 mmol/L caspase-3 substrate (DEVD-AFC at a final concentration of 50  $\mu\text{mol/L}$ ) was added to each sample. The samples were incubated at 37°C for 1 h and read on a spectrofluorometer (Perkin Elmer LS 55) with excitation wavelength 400 nm and emission wavelength of 505 nm.

#### Cytosolic phospholipase $\text{A}_2$ assay

cPLA<sub>2</sub> activity was measured in cell lysates, using cPLA<sub>2</sub> assay kit (Cayman Chemical Company cPLA<sub>2</sub> assay kit) as per the instructions of the supplier. The kit involves the principle that cPLA<sub>2</sub> exhibits specificity towards arachidonic acid. Arachidonoyl thio-PC is used as a synthetic substrate to detect the phospholipase activity. Hydrolysis of the arachidonoyl thioester bond at the *sn*-2 position by cPLA<sub>2</sub> releases free thiol which are detected by DTNB (5, 5'-dithiobis-2-dinitrobenzoic acid).

#### Adenosine 3', 5'-cyclic monophosphate (cAMP) assay

cAMP activity was measured in the cell lysates, using cAMP assay kit (R&D Systems, Inc, cAMP assay kit) as per the instructions of the supplier. The kit is based on the competitive binding technique in which cAMP present in a sample competes with a fixed amount of horseradish peroxidase (HRP)-labeled cAMP for sites on a mouse monoclonal antibody. During the incubation, the monoclonal antibody becomes bound to the goat anti-mouse antibody coated onto the microplate. Following a wash to remove excess conjugate and unbound sample, a substrate solution is added to the wells to determine the bound activity. The colour developed was stopped and the absorbance read at 450 nm. The intensity of the colour is inversely proportional to the concentration of cAMP in the sample.

#### 2, 2'-azino-di-[3-ethylbenzthiazoline sulphate] oxidation inhibition assay (ABTS assay)

The assay was performed using antioxidant assay kit from Cayman Chemical Company, as per the instructions by the manufacturer. The assay was used to access the ability of the test materials (NG, Silymarin *etc*) to inhibit the oxidation of ABTS to ABTS<sup>+</sup> by metmyoglobin. The amount of ABTS<sup>+</sup> produced in absence and presence of test material was monitored by reading absorbance at 750 nm. Suppression of the absorbance at 750 nm in presence of test material is proportional to their antioxidant activity. The capacity of the antioxidant activity (inhibition of ABTS<sup>+</sup> formation) in the test sample was compared with that of Trolox, a water-soluble tocopherol analogue.

#### 1,1-diphenyl-2-picrylhydrazyl (DPPH) discolouration assay

This assay was performed as described by Gonzalez

*et al*<sup>[34]</sup>. In brief, for the assay, various concentrations of test compound were added to 3 mL of DPPH solution (20 mg/L methanol) and incubated for 5 min at 25°C. The absorbance was measured at 517 nm. A 100% decoloration was established using methanol-water 2:1 and the percentage of DPPH decoloration was calculated.

### Superoxide anion scavenging activity

*In vitro* effect of NG on superoxide anion radical generation was studied as described previously<sup>[35]</sup>. Two reaction systems used were: (a) enzymic; (b) non-enzymic. System (a) comprised of 100  $\mu$ mol/L xanthine, 600  $\mu$ mol/L nitroblue tetrazolium (NBT) and 0.07 U/mL xanthine oxidase in 50 mmol/L sodium carbonate pH 9.2, incubated for 10 min in presence and absence of test materials and *A* read at 560 nm. System (b) comprised of 10  $\mu$ mol/L phenazine methosulphate, 78  $\mu$ mol/L NADH, 25  $\mu$ mol/L NBT, incubated for 2 min in presence and absence of test materials and *A* read at 560 nm.

### Protein estimation

Protein concentration was measured with BCA Protein Assay Kit from Pierce (Rockford, IL, USA) as per the instructions of the manufacturer.

### Statistical analysis

Results are expressed as mean  $\pm$  SD. Comparisons were made between control and treated groups unless otherwise indicated using unpaired Student's *t*-test and *P* values < 0.05 were considered statistically significant.

## RESULTS

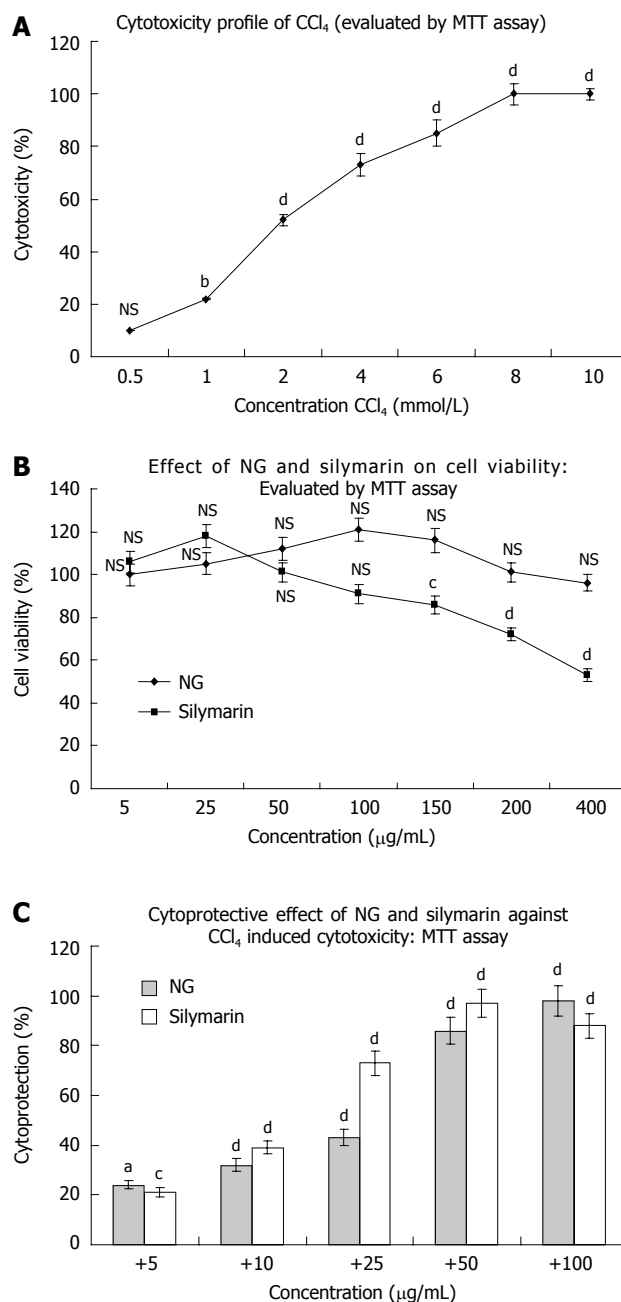
### Standardization of NG

For better scientific and clinical acceptability and proper global positioning of plant based products, it has become implicit to determine their chemical profile data on the basis of purity of compound. In this respect we have developed the HPLC protocol for NG (Figure 1). HPLC profile confirmed that NG was isolated as < 95% pure.

### Cytoprotective and membrane stabilizing effect of NG and silymarin against CCl<sub>4</sub>-induced cytotoxicity in HuH-7 cells

CCl<sub>4</sub> produced a concentration dependent loss of viability in HuH-7 cells as evaluated by MTT assay. At 24 h of incubation, the IC<sub>50</sub> value of CCl<sub>4</sub> was found to be 2 mmol/L approximately (1.958 mmol/L; Figure 2A). This concentration was used to generate oxidative stress to study the cytoprotective effect of NG and silymarin in further experimentations. NG alone was not toxic under the assay conditions up to a concentration of 400 mg/L. However, silymarin at concentrations above 50 mg/L produced loss of cell viability, with an estimated IC<sub>50</sub> value of 413.38 mg/L (Figure 2B).

To characterize the protective effect of NG and silymarin on CCl<sub>4</sub> induced cytotoxicity in HuH-7 cells,



**Figure 2** Cytotoxicity profile of CCl<sub>4</sub> and protective effect of NG and silymarin on CCl<sub>4</sub>-induced inhibition of cellular proliferation in HuH-7 cells. For cell proliferation assay, HuH-7 cells grown in 24-well culture plate were incubated with indicated concentrations of test materials. Cell proliferation was assessed by MTT reduction assay. **A, B:** Represents inhibition of cell proliferation by CCl<sub>4</sub> and test materials (NG and silymarin); **C:** Represents protection of NG and silymarin, against CCl<sub>4</sub> induced inhibition of cell proliferation. HuH-7 cells were treated with various concentrations of NG and silymarin (5 to 100 mg/L) 1 h before treatment with CCl<sub>4</sub> for 24 h and the cell proliferation was determined by MTT reduction assay. Control wells received medium containing DMSO (< 0.2 mL/L). The % cell cytotoxicity, % viability and % cytoprotection was calculated as, % Cytotoxicity = (Control - Test)/Control  $\times$  100, % Cell viability = % Cytotoxicity - 100, % Cytoprotection = 100 - (Treated - Control)/(CCl<sub>4</sub> - Control)  $\times$  100. Data are mean  $\pm$  SD (*n* = 8) and representative of one of three similar experiments and statistically significant *P* values: <sup>a</sup>*P* < 0.01; <sup>b</sup>*P* < 0.001; <sup>c</sup>*P* < 0.02; <sup>d</sup>*P* < 0.05; NS: Non-significant, CCl<sub>4</sub> treated vs control cells; CCl<sub>4</sub> + LIV-1/silymarin vs CCl<sub>4</sub> treated cells.

dose-response experiments were conducted using various concentrations of NG and silymarin. NG showed a significant dose dependent protective effect

against  $\text{CCl}_4$  induced loss of cell viability. The PC50 (50% protective concentration) for NG was estimated to be 24.46 mg/L and 15.30 mg/L for silymarin (Figure 2C).

Results obtained from MTT assay were in total correlation with the extent of cell death as confirmed by morphological changes observed under light microscope and Hoechst 33258 staining under fluorescence microscopy (Figure 3). Treatment with  $\text{CCl}_4$  caused HuH-7 cells to lose their normal structure with signs of cell swelling, most of the cells were detached and monolayer was disturbed (Figure 3 I A compared with Figure 3 I B). These structural changes were prevented to a large extent by 30 and 100  $\mu\text{g/mL}$  of NG and were comparable with the protection offered by silymarin at 50 mg/L (Figure 3 I C-F).

Nuclei of untreated HuH-7 cells appeared prominently round in shape (Figure 3 II A). After exposure with  $\text{CCl}_4$ , cells showed morphological alterations and condensation of nuclei (Figure 3 II B). The prominent changes were accompanied by an increase in apoptotic bodies and increase in cellular debris. All these alterations were prevented by co-exposure with NG and silymarin in a dose dependent manner (Figure 3 II C-E).

The above mentioned cytoprotective results were further correlated with the membrane stabilizing effect of NG and silymarin against Triton X 100 (1 g/L)-induced membrane disruption in human RBCs. NG showed an effect in the range of 4% to 91% in a concentration dependent manner (5 mg/L to 100 mg/L) against a protective effect of 6% to 88% at the same concentration, shown by silymarin (Figure 4).

#### **Effect of NG and silymarin on CYP2E1 catalytic activity**

To study the mechanism by which NG was preventing  $\text{CCl}_4$ -induced toxicity in HuH-7 cells, the possible interference of NG and silymarin on CYP2E1 activity (aniline hydroxylation) was studied. Hepatic microsomes from Wistar rats were used as a source to assay *in vitro* effect of NG and silymarin on CYP2E1 levels (Figure 5A). In another set of experiments, the CYP2E1 protein levels were analyzed to assay the protective effect of NG and silymarin against oxidation of 2.5 mmol/L ethanol (Figure 5B).

NG alone showed no significant effect on aniline hydroxylation levels at any concentrations used (5 to 100 mg/L), whereas silymarin produced an inhibitory effect in the range of 2.6% to 26.2% at the same concentrations (Figure 5A). Isoniazid (used as a positive control for inhibition of CYP2E1) showed a dose dependent inhibition, with IC<sub>50</sub> at 500  $\mu\text{mol/L}$  (data not shown).

Treatment of microsomes with 2.5 mmol/L ethanol caused an increase of 63% in aniline hydroxylation levels. Co-treatment of NG with ethanol showed no inhibitory effect on aniline hydroxylation levels induced by ethanol. However, silymarin caused inhibitions of 8.4%, 41%, 76% and 149% at 10 mg/L, 25 mg/L, 50 mg/L and 100 mg/L, respectively in aniline hydroxylation levels compared to microsomes treated with ethanol alone (Figure 5B).

The results (Figure 5 A and B) show that NG did not

inhibit CYP2E1 activity, suggesting that NG is most probably acting as an antioxidant, and not as a CYP2E1 inhibitor and silymarin acts both as an antioxidant, and an inhibitor of CYP2E1.

#### **Effect of NG and silymarin on $\text{FeSO}_4 + \text{H}_2\text{O}_2$ stimulated lipid peroxidation (LPO) in rat liver microsomes and on $\text{CCl}_4$ induced LPO in HuH-7 cells**

$\text{FeSO}_4 + \text{H}_2\text{O}_2$  increased LPO in rat liver microsomes by 6.8-fold. Incubation with NG (10 to 100 mg/L) decreased in a range of 3% to 69%, this increase in LPO levels (Figure 6A). Silymarin at the equivalent concentrations showed an enhanced inhibitory effect of 21% to 111%. Treatment of HuH-7 cells with  $\text{CCl}_4$ , increased LPO levels up to 4.6-fold. NG offered a protective effect against this increase by 10.4%, 21%, 39%, 54% and 131% at 5 mg/L, 10 mg/L, 25 mg/L, 50 mg/L and 100 mg/L, respectively. At similar concentrations, silymarin produced respective inhibitory effects of 17%, 32%, 64%, 146% and 164% (Figure 6B).

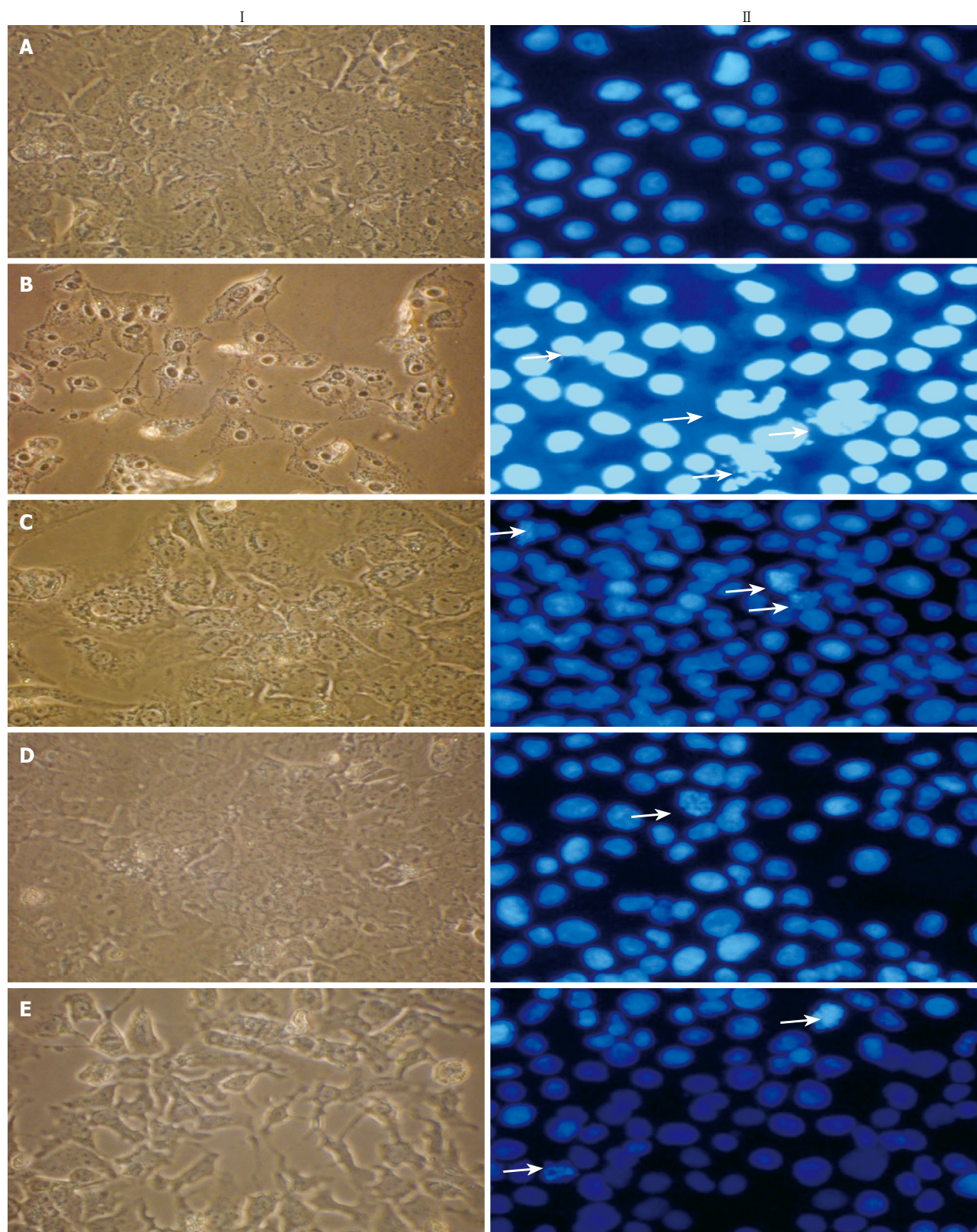
#### **Effect of NG and silymarin on the ROS generation induced by $\text{CCl}_4$**

Oxidative stress was studied by fluorescence spectrophotometrical analysis of the levels of ROS, using DCF-DA and DHR as the probes. Figure 7 shows the mean values of DCF and DHR fluorescence for cell populations with various treatments. Treatment with  $\text{CCl}_4$ , increased by 2.1 (DCF) and 2.2-fold (DHR) the production of ROS in HuH-7 cells in comparison with no addition control. NG and silymarin (10 to 100 mg/L) reduced the increase in the ROS levels produced by  $\text{CCl}_4$  in a significant and dose dependent manner. NG at 10 mg/L decreased DCF fluorescence intensity by 32% and DHR by 52%. At 50 mg/L, DCF and DHR intensities were decreased by 76% and 105%, respectively. At 100 mg/L, both the fluorescence intensities were down by 104% and 143%.  $\text{H}_2\text{O}_2$  (500  $\mu\text{mol/L}$ ) was used as positive control for ROS generation.

#### **Effect of NG and silymarin on intracellular $\text{Ca}^{2+}$ and caspase 3 levels induced by $\text{CCl}_4$**

Intracellular  $\text{Ca}^{2+}$  and caspase 3 levels were increased significantly by  $\text{CCl}_4$  treatment, which reflects the requirement of  $\text{Ca}^{2+}$  and caspase 3 in the overall toxicity pathway of  $\text{CCl}_4$  (Figures 8 and 9).  $\text{CCl}_4$  caused an increase of 4.8-fold in  $\text{Ca}^{2+}$  and 2.3-fold in caspase 3 levels in HuH-7 cells. This abnormal rise in  $\text{Ca}^{2+}$  levels were decreased by NG by 15%, 72% and 106% and caspase 3 levels were decreased by 16%, 92% and 139% at 10 mg/L, 50 mg/L and 100 mg/L, respectively. Silymarin also showed a dose dependent inhibitory effect on  $\text{Ca}^{2+}$  and caspase 3 levels increased by  $\text{CCl}_4$ . The effect was 28%, 114% and 207% in  $\text{Ca}^{2+}$  levels at 10 mg/L, 50 mg/L and 100 mg/L, and 34% and 160% in caspase 3 levels at 10 mg/L and 50 mg/L, respectively. However at 100 mg/L, silymarin showed a less significant effect (28% decrease). Thus, silymarin showed a higher  $\text{Ca}^{2+}$  inhibitory effect compared to NG, but less caspase 3 inhibition at concentrations

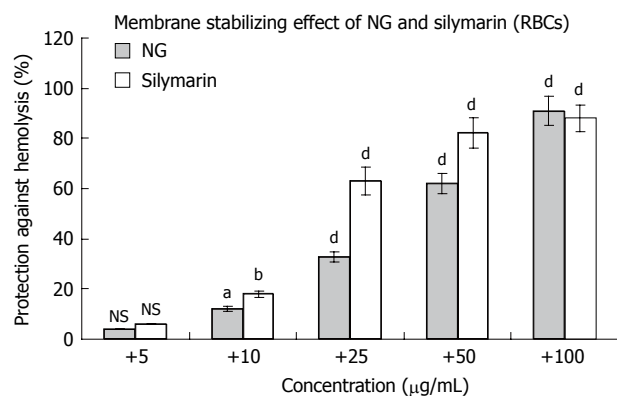




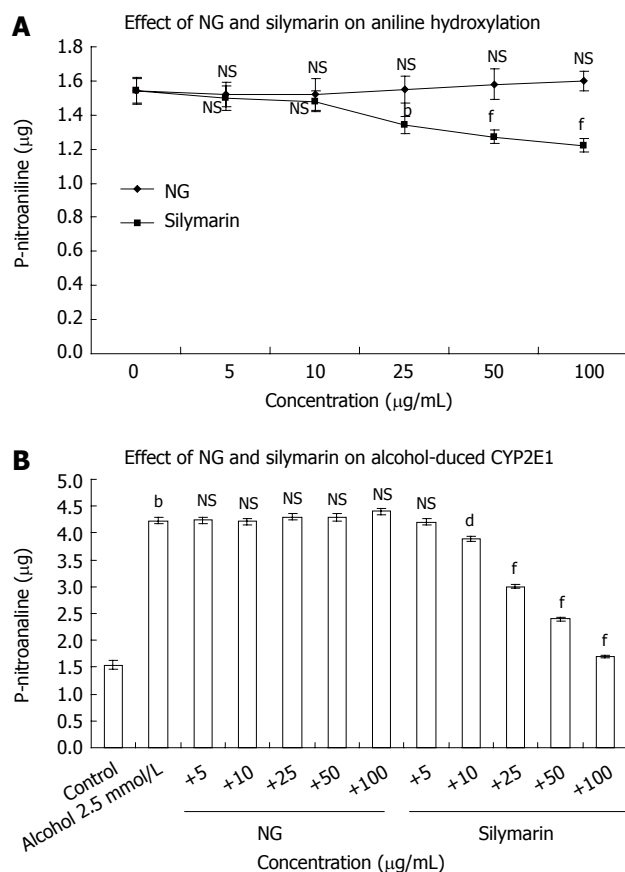
**Figure 3** Effect of NG and silymarin against  $\text{CCl}_4$ -induced altered cellular and nuclear morphology of HuH-7 cells. NG and silymarin rescued  $\text{CCl}_4$ -induced cellular (I) and nuclear morphological (II) changes. Cellular morphology was observed by normal phase contrast microscopy, while as nuclear morphology was evaluated by Hoechst 33258 staining of HuH-7 cells and observed under fluorescence microscopy as described in Materials and Methods. These methods detected influences of  $\text{CCl}_4$  on cellular and nuclear changes. **A:** Untreated control cells show normal cellular characteristics and rounded nuclei; **B:** Cells treated with  $\text{CCl}_4$  (2 mmol/L) for 24 h show altered membrane structure and condensed chromatin/nuclei, apoptotic (arrows) and scattered apoptotic bodies; **C:** Cells incubated with NG (30 mg/L); **D:** Cells incubated with NG (100 mg/L); **E:** Cells incubated with silymarin (50 mg/L), 1 h before the treatment with  $\text{CCl}_4$  showed protection against  $\text{CCl}_4$ -mediated cellular and nuclear alterations.

above 50 mg/L. Cyclosporine (10  $\mu\text{mol/L}$ ) was used as positive control for  $\text{Ca}^{2+}$  inhibition and camptothecin

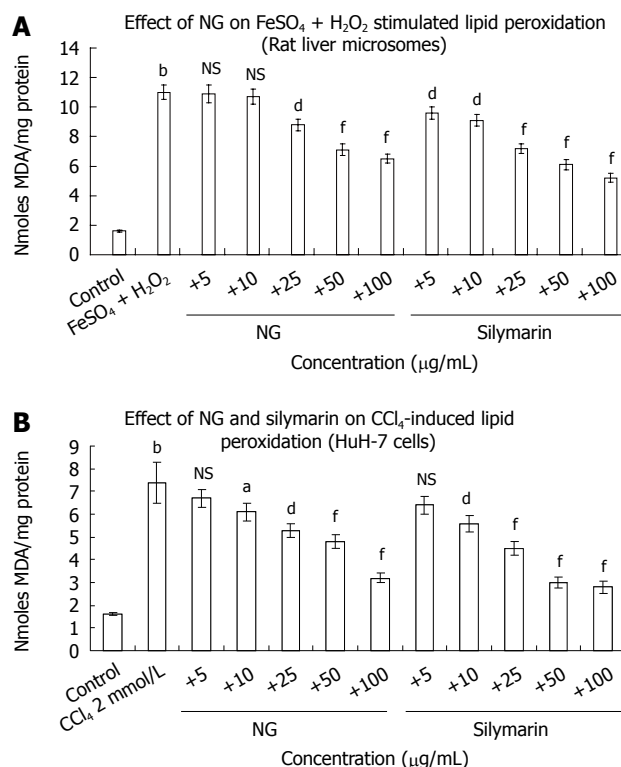
(inducer, 4  $\mu\text{mol/L}$ ) and DEVD-CHO (inhibitor, 20  $\mu\text{mol/L}$ ) were used as positive controls.



**Figure 4** Membrane stabilizing effect of NG and silymarin on human RBCs. RBC suspensions were pre-incubated with or without (control) test materials and triton (1 g/L) in phosphate buffered saline as described in Materials and Methods section. Data are mean  $\pm$  SD ( $n = 3$ ) and representative of one of three similar experiments and statistically significant  $P$  values: <sup>b</sup> $P < 0.01$ ; <sup>a</sup> $P < 0.05$ ; <sup>d</sup> $P < 0.001$ ; NS = Non-significant. Triton treated vs control cells; triton + NG/silymarin vs triton treated cells.



**Figure 5** Effect of NG and silymarin *in vitro* on CYP2E1 catalytic activity. **A:** Rat liver microsomes were incubated in the absence or presence of different concentrations (5 to 100 mg/L) of test materials. CYP2E1 activity was assayed by following the hydroxylation of aniline hydrochloride in presence of cumene hydroperoxide as described in Materials and Methods section; **B:** Concentration dependent protection by NG and silymarin against alcohol (2.5 mmol/L)-induced aniline hydroxylase levels in rat liver microsomes. The microsomes were pre-incubated with medium containing various concentrations (5 to 100 mg/L) of test materials for 5 min before addition of alcohol and aniline hydroxylase levels were determined. Data are expressed as mean  $\pm$  SEM and are from a representative experiments repeated twice and conducted in triplicate.  $P$  values: <sup>b</sup> $P < 0.001$  vs the corresponding alcohol-treated microsomes; <sup>d</sup> $P < 0.001$  and <sup>f</sup> $P < 0.001$  vs alcohol-treated cells in presence of test materials; NS: Non-significant.



**Figure 6** Protective effect of NG and silymarin against stimulated lipid peroxidation. **A:** Anti-lipid peroxidative effect of NG and silymarin *in vitro*. Liver microsomes (1 mg protein/mL, 0.15 mol/L NaCl, pH 7.0) were incubated for 20 min at 37°C in the absence (control) and presence of 100 mmol/L FeSO<sub>4</sub> + 50 mmol/L H<sub>2</sub>O<sub>2</sub> (stimulated). In identically set-up Fe<sup>2+</sup>/H<sub>2</sub>O<sub>2</sub>-stimulated incubations, NG and silymarin (20 mg/L to 100 mg/L, in 30% DMSO) were added (test C). Control incubations received vehicle only; **B:** HuH-7 cells were pre-incubated for 1 h with medium containing test materials (NG and silymarin) at different concentrations (5 mg/L to 100 mg/L). The cells were further incubated in absence or presence of 2 mmol/L CCl<sub>4</sub> (stimulated) for further 24 h. The cells were harvested by scraping and assayed for the production of MDA using TBARS assay, as described under materials and methods section. Reaction was terminated by the addition of 2.0 mL TCA-TBA reagent (15% TCA, 0.375% TBA in 5mol/L HCl) and LPO content determined as nmol MDA formed/mg protein. Data are expressed as mean  $\pm$  SD and are from representative experiments repeated twice and conducted in triplicate. Statistical significance, <sup>b</sup> $P < 0.01$  vs the untreated control. <sup>d</sup> $P < 0.01$ ; <sup>a</sup> $P < 0.05$ ; <sup>f</sup> $P < 0.001$  and NS: Non-significant vs stimulated (FeSO<sub>4</sub> + H<sub>2</sub>O<sub>2</sub> and CCl<sub>4</sub> treatments).

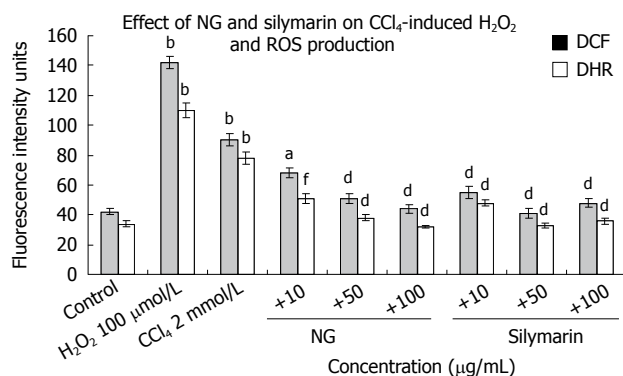
#### Effect of NG and silymarin on cytochrome C release from isolated rat liver mitochondria induced by CCl<sub>4</sub>

Isolated rat liver mitochondria were used to study the effect of NG and silymarin on cytochrome C release induced by CCl<sub>4</sub> (Figure 10). CCl<sub>4</sub> caused an increase of 2.1-fold in cytochrome C levels, which was inhibited by 75% and 105% at 50 mg/L and 100 mg/L of NG treatment, respectively. Silymarin showed an effect in the range of 27% at 10 mg/L, 135% at 50 mg/L. However at a higher concentration (100 mg/L), silymarin showed a biphasic effect, with a slight increase in cytochrome C levels compared to 50 mg/L (36% increase). Cyclosporine 5 µmol/L was used as a positive control for cytochrome C inhibition.

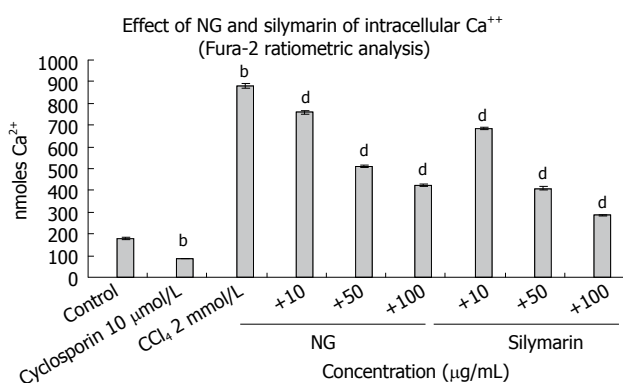
#### Effect of NG and silymarin on mitochondrial membrane permeability transition onset by CCl<sub>4</sub> in HuH-7 cells

Oxidative damage to mitochondria and the onset of MMP transition seems to play an important role in CCl<sub>4</sub>-





**Figure 7** Effect of NG and silymarin on CCl<sub>4</sub>-induced ROS production. HuH-7 cells were pre-incubated for 1 h with medium containing test materials (NG and silymarin) at different concentrations (10 to 100 mg/L). The cells were kept for further incubation in absence or presence of 2 mmol/L CCl<sub>4</sub> (stimulated). After 24 h of incubation maintaining the specific treatments, the cells were incubated with serum-free medium containing DCF-DA and 123-DHR and ROS levels were studied as mentioned in material and methods section. H<sub>2</sub>O<sub>2</sub> (100 μmol/L) was used as positive control for ROS generation. Data are expressed as mean ± SD and are from representative experiments repeated twice and conducted in triplicate. Statistical significance: <sup>b</sup>*P* < 0.001 vs the untreated control; <sup>d</sup>*P* < 0.001, <sup>a</sup>*P* < 0.05; <sup>i</sup>*P* < 0.01 vs CCl<sub>4</sub>-treated cells.

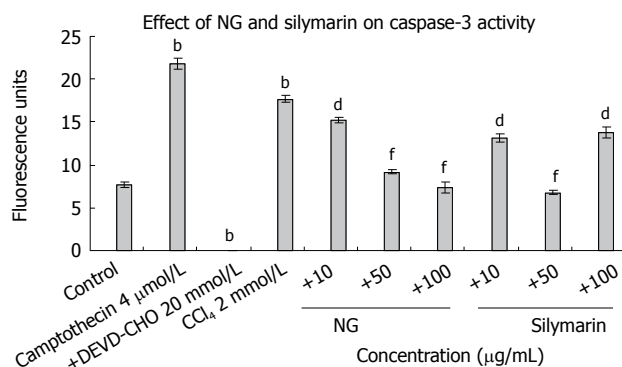


**Figure 8** Effect of NG and silymarin against CCl<sub>4</sub>-induced cytosolic free Ca<sup>2+</sup> concentrations. HuH-7 cells pre-incubated for 1 h with medium containing test materials (NG and silymarin) at different concentrations (10 to 100 mg/L) were exposed to CCl<sub>4</sub> (2 mmol/L) for indicated time period, washed and loaded with Fura-2 AM as described in materials and methods section. Cyclosporine (10 μmol/L) was used as positive control. Data are expressed as mean ± SD, and are from a representative experiments repeated twice and conducted in triplicate. Statistical significance: <sup>b</sup>*P* < 0.001 vs the untreated control, <sup>d</sup>*P* < 0.001 vs CCl<sub>4</sub> treated cells.

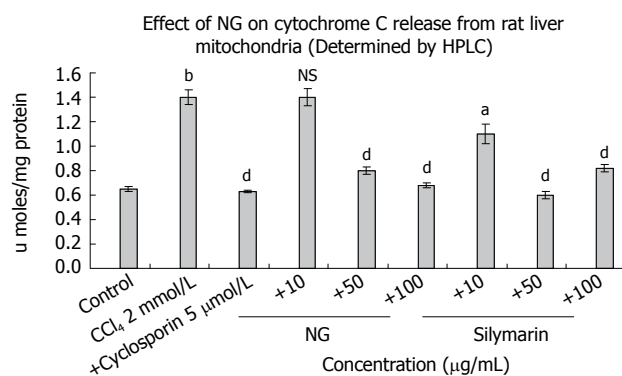
induced toxicity in HuH-7 cells. MMP transitions were analyzed by flow cytometry after staining with Rh123 (Figure 11). Untreated HuH-7 cells were strong in Rh123 fluorescence intensity, suggestive of intact viable cells. A very small percentage of cells (8%) were showing low Rh123 fluorescence, reflective of damaged cells. CCl<sub>4</sub> caused a 6-fold increase in percentage of cells with low Rh123 fluorescence. Incubation in the presence of 50 mg/L and 100 mg/L of NG and silymarin, respectively significantly protected HuH-7 cells from decline in MMP produced by CCl<sub>4</sub>.

#### Effect of NG and silymarin on CCl<sub>4</sub>-induced apoptosis in HuH-7 cells

Apoptosis was studied by internucleosomal DNA fragmentation analysis and cell cycle analysis by flow



**Figure 9** Effect of NG and silymarin against caspase 3-mediated apoptosis. CCl<sub>4</sub> treatment increases caspase 3 activity in HuH-7 cells. HuH-7 cells were treated with CCl<sub>4</sub> in presence or absence of test materials (NG and silymarin) and caspase 3 activity was measured as described in Materials and Methods section. Data are expressed as mean ± SD and are from representative experiments repeated twice and conducted in triplicate. Statistical significance: <sup>b</sup>*P* < 0.001 vs the untreated control; <sup>d</sup>*P* < 0.01; <sup>i</sup>*P* < 0.001 vs CCl<sub>4</sub>-treated cells.



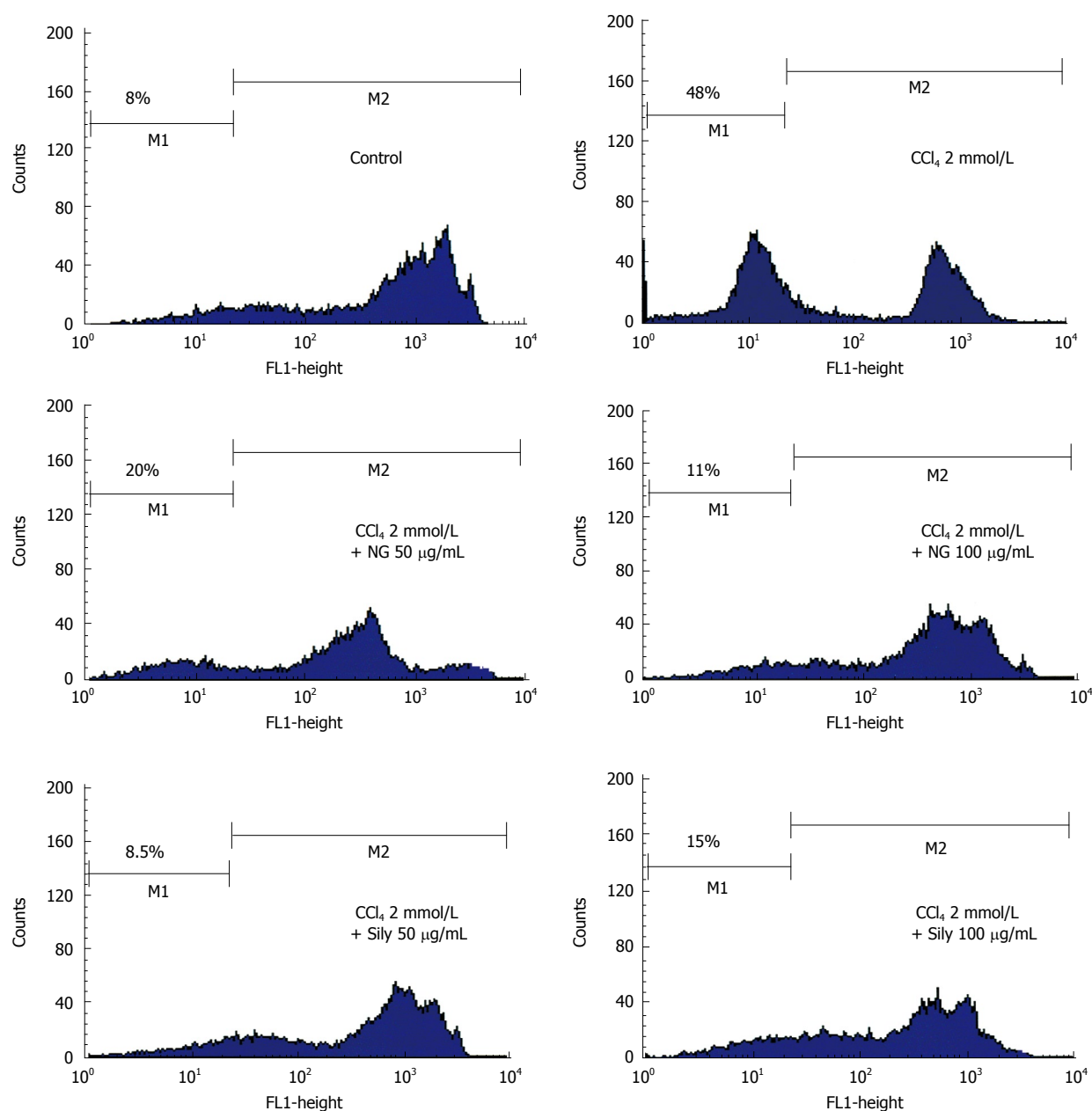
**Figure 10** Effect of NG and silymarin against CCl<sub>4</sub>-induced cytochrome C release from isolated rat liver mitochondria. CCl<sub>4</sub> treatment causes cytochrome C release from rat liver mitochondria. Mitochondria were treated with CCl<sub>4</sub> in presence or absence of test materials (NG and silymarin) and cytochrome C levels were measured as described in Materials and Methods section. Data are expressed as mean ± SD and are from a representative experiments repeated twice and conducted in triplicate. Statistical significance: <sup>b</sup>*P* < 0.001 vs the untreated control; <sup>a</sup>*P* < 0.02; <sup>d</sup>*P* < 0.001 and NS = non-significant vs CCl<sub>4</sub> treated mitochondria.

cytometry. A DNA ladder formation was found with cells treated with CCl<sub>4</sub> (Figure 12). Treatment with NG and silymarin protected HuH-7 cells against CCl<sub>4</sub> induced DNA fragmentation.

In cell cycle analysis, treatment with CCl<sub>4</sub> markedly increased proportion of apoptotic cells significantly (49%). NG and silymarin had an obvious anti-apoptosis effect. As shown in Figure 13, the co-treatment with 25 mg/L, 50 mg/L and 100 mg/L of NG markedly reduced the percentage of the apoptotic cells to 27%, 14% and 11%, respectively. Silymarin showed a reduction in apoptotic cells by 21% and 9% at 25 mg/L and 50 mg/L, respectively. At 100 mg/L, however, silymarin had an effect of 18% (Figure 13).

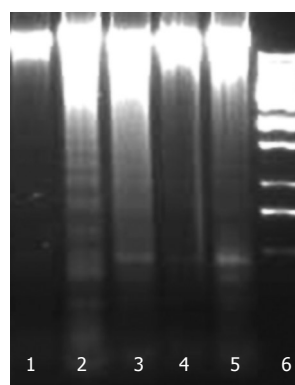
#### Effect of NG and silymarin on CCl<sub>4</sub>-induced alterations in cAMP and cPLA<sub>2</sub> levels in HuH-7 cells

To test the effect of CCl<sub>4</sub>-induced oxidative stress on cAMP levels in HuH-7 cells, we evaluated cAMP with



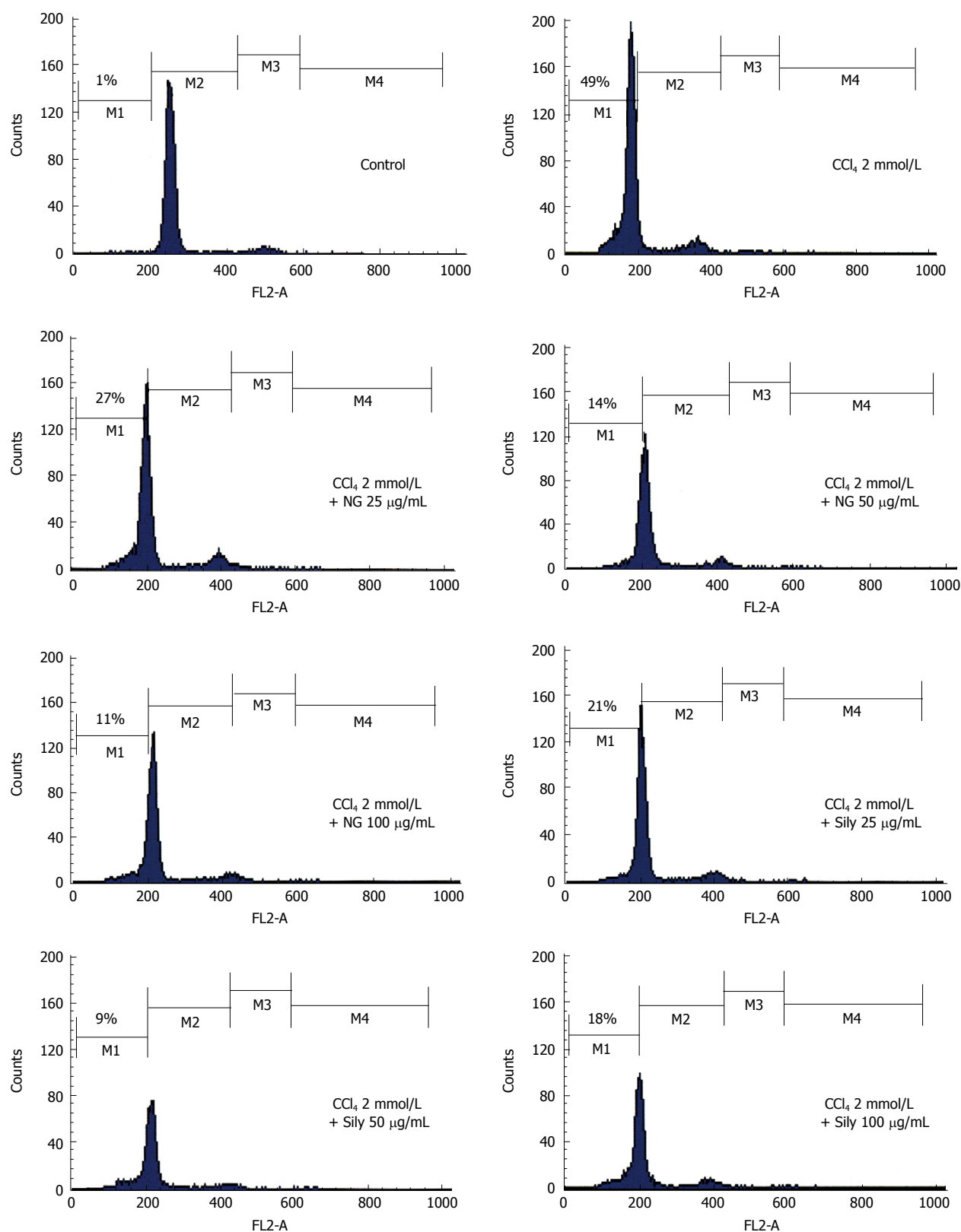
**Figure 11** Effect of NG and silymarin against  $\text{CCl}_4$ -induced loss of mitochondrial membrane potential ( $\Delta\psi\text{m}$ ). HuH-7 cells were pre-incubated for 1 h with test materials (NG and silymarin) at mentioned concentrations. The cells were further incubated for 24 h with  $\text{CCl}_4$  (2 mmol/L). Thereafter, cells were stained with Rhodamine-123 and analysed by flow cytometry as described in Materials and Methods section. Representative histograms are shown and the percentage of cells in depolarized zone (M1 zone) are shown.

and without  $\text{CCl}_4$  and then in the presence of  $\text{CCl}_4$  with NG and silymarin. As hypothesized, cAMP levels were significantly reduced by  $\text{CCl}_4$ . Concomitant treatment of HuH-7 cells with NG significantly increased the levels of cAMP. Similar results were evident with treatment with silymarin. Forskolin (100  $\mu\text{mol/L}$ ) was used as a positive inducer of cAMP (Figure 14). On the contrary, phospholipase A2 levels were significantly increased with the  $\text{CCl}_4$  treatment (2.3-fold). NG effectively reduced this increase by 44% at 10 mg/L and 304% at 50 mg/L. Silymarin reduced these levels by 97% at 10 mg/L and 136% at 50 mg/L. Bee venom (1 mg/L) was used as positive control to induce cPLA2 levels (Figure 15).



**Figure 12** Effect of NG and silymarin against  $\text{CCl}_4$ -induced DNA fragmentation. HuH-7 cells were pre-incubated for 1 h with test materials (NG and silymarin) at mentioned concentrations. The cells were further incubated for 24 h with  $\text{CCl}_4$  (2 mmol/L). Thereafter, genomic DNA was extracted from cells and subjected to gel electrophoresis as mentioned in Materials and Methods section. Lanes: 1: Control; 2:  $\text{CCl}_4$  2 mmol/L; 3:  $\text{CCl}_4$  2 mmol/L + NG 30 mg/L; 4:  $\text{CCl}_4$  2 mmol/L + NG 100 mg/L; 5:  $\text{CCl}_4$  2 mmol/L + Silymarin 50 mg/L; 6: Ladder.



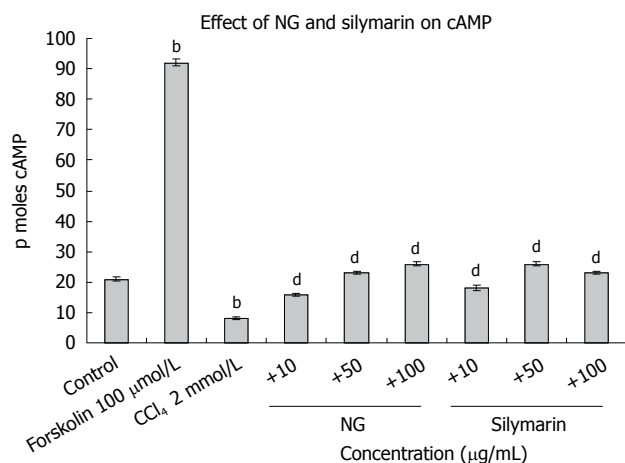


**Figure 13** Effect of NG and silymarin against CCl<sub>4</sub>-induced cell cycle arrest. HuH-7 cells were pre-incubated for 1 h with test materials (NG and silymarin) at mentioned concentrations. The cells were further incubated for 24 h with CCl<sub>4</sub> (2 mmol/L). Thereafter, cells were harvested by trypsinization, fixed with ethanol, stained with PI, and analyzed using flow cytometry. Representative histograms are shown, and the percentage of cells in the sub G<sub>0</sub>/G<sub>1</sub> fraction (M1 zone, hypodiploid area) are shown.

#### **Effect of NG and silymarin on CCl<sub>4</sub>-induced depletion of GSH levels in HuH-7 cells**

Figure 16 depicts the effect of CCl<sub>4</sub> on GSH levels and restorative effect of NG and silymarin in a dose-

response manner. Treatment of HuH-7 cells with CCl<sub>4</sub>-depleted the GSH content by 2 folds. Co-exposure with NG and silymarin effectively restored the depleted levels of GSH in a dose response manner. Restorative



**Figure 14** Effect of NG and silymarin against CCl<sub>4</sub>-depleted cAMP levels. Pre-incubated, (1 hour at mentioned concentrations of NG and silymarin) HuH-7 cells were exposed to 2 mmol/L CCl<sub>4</sub> for 24 h. cAMP levels were determined in the cell culture supernatants as described in Materials and Methods. Forskolin (100 µmol/L) was used as positive control. Data are expressed as mean ± SD and are from representative experiments repeated twice and conducted in triplicate. Statistical significance: <sup>b</sup>*P* < 0.001 vs the untreated control. <sup>d</sup>*P* < 0.001 vs CCl<sub>4</sub>-treated cells.

effect of NG was in the range of 17% to 147% at 5 mg/L to 100 mg/L, respectively. Silymarin showed an effect in the range of 23% to 152% at a concentration of 5 to 50 mg/L. However at higher concentration (100 mg/L), there was a slight decrease in this effect. BSO (100 µmol/L) was used as a positive inhibitor of GSH.

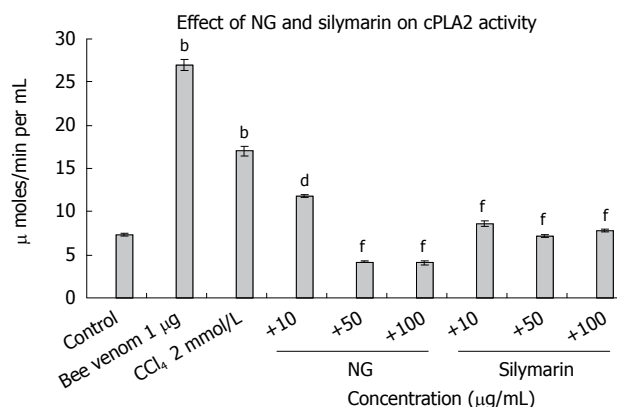
#### *In vitro* antioxidant activity of NG and silymarin

Figure 17 is representative of anti-oxidant activity of NG and silymarin. NG showed a strong activity, with I.C. 50 values of 17.31 mg/L for DPPH, 22.75 mg/L for enzymatic reaction, 13.49 mg/L for non-enzymatic reaction and 8.71 mg/L for ABTS assay. The IC 50 values of silymarin for the same assays were 34.07, 24.35, 21.10 and 12.36 mg/L respectively.

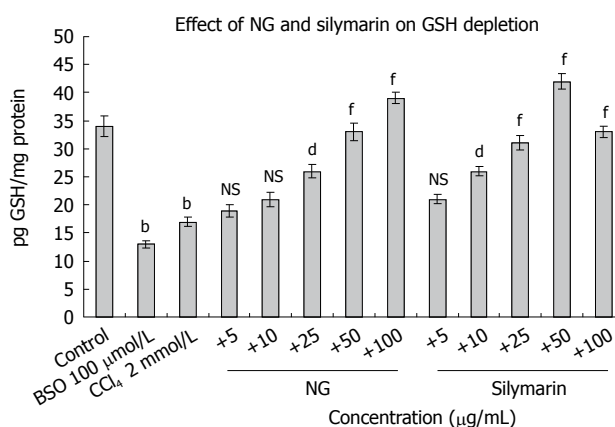
## DISCUSSION

Liver cells exposed to various chemicals/drugs (pro-oxidants) appear to be a useful *in vitro* model to characterize the biochemical and toxicological properties of such entities, and the possible protection provided by added agents<sup>[36]</sup>. The main goal of this work was to investigate the influence of an irridoid glycoside compound negundoside (NG) on CYP2E1-mediated toxicity in HuH-7 cells induced by CCl<sub>4</sub>. Overall, the results of the present study indicate that NG is effective in protecting against the toxicity and the loss of viability induced by CCl<sub>4</sub>.

The main mechanism by which CCl<sub>4</sub> is known to mediate its toxic effects is through oxidative stress and oxidative damage due to an increased production of ROS<sup>[37]</sup>. Induction of CYP2E1 by CCl<sub>4</sub> is one of the main pathways by which CCl<sub>4</sub> increases ROS production and generates a state of oxidative stress in the liver<sup>[38,39]</sup>. Since CYP2E1 is a key contributor to injury produced



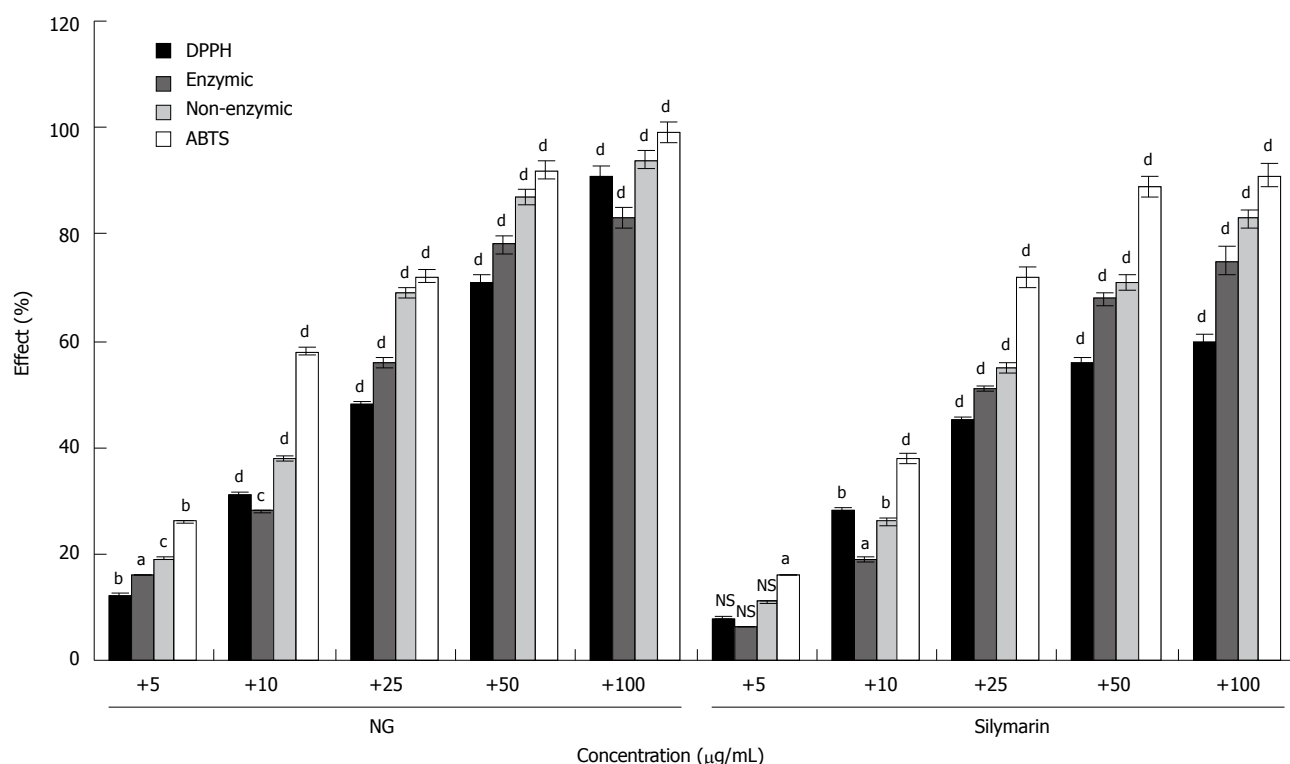
**Figure 15** Effect of NG and silymarin against CCl<sub>4</sub>-induced cPLA2 levels. Pre-incubated, (1 h with mentioned concentrations of NG and silymarin) HuH-7 cells were exposed to 2 mmol/L CCl<sub>4</sub> for 24 h. cPLA2 levels were determined as described in Materials and Methods. Bee venom (1 µg) was used as positive control. Data are expressed as mean ± SD and are from representative experiments repeated twice and conducted in triplicate. Statistical significance: <sup>b</sup>*P* < 0.001 vs the untreated control; <sup>d</sup>*P* < 0.01; <sup>f</sup>*P* < 0.001 vs CCl<sub>4</sub>-treated cells.



**Figure 16** Effect of NG and silymarin on CCl<sub>4</sub>-induced decrease in GSH levels. HuH-7 cells were pre-incubated with medium containing test materials (NG and silymarin) for 1 h. The cultures were then further incubated in presence and absence of CCl<sub>4</sub> for 24 h. The cells were harvested by scraping and GSH levels were determined as described under Materials and Methods section. BSO (100 µmol/L) was used as positive control. Data are expressed as mean ± SD and are from representative experiments repeated twice and conducted in triplicate. Statistical significance: <sup>b</sup>*P* < 0.001 vs the untreated control; <sup>d</sup>*P* < 0.01; <sup>f</sup>*P* < 0.001; NS = non-significant vs CCl<sub>4</sub>-treated cells.

by CCl<sub>4</sub>, one possible mechanism involved in the prevention of this toxicity by NG could have been an inhibition of CYP2E1 catalytic activity. Results in this study indicate that NG does not affect *p*-nitrophenol metabolism by CYP2E1 in liver microsomes under the experimental conditions (Figure 5 A and B); therefore, the mechanism by which NG affords its protection is not by inhibition of CYP2E1 activity. This is in confirmation to earlier reports in which many plant derived products like, *Scutellariae radix*<sup>[40]</sup>, *Humulus lupulus*<sup>[41]</sup>, green tea compounds<sup>[42]</sup> have also been shown to be hepatoprotective in other systems without any effect on CYP2E1 catalytic activity.

Bio-metals, such as iron are powerful catalysts of

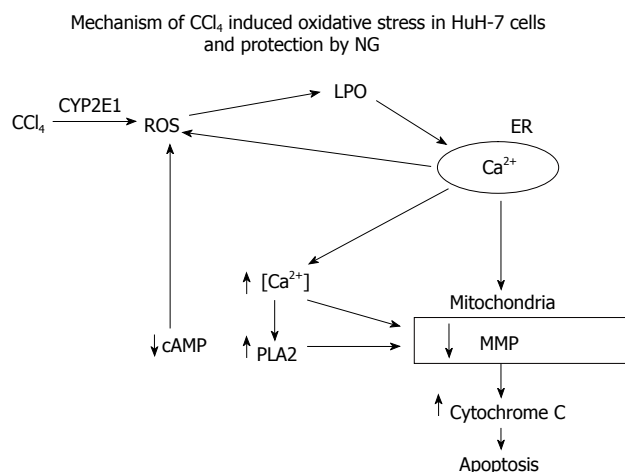


**Figure 17** *In vitro* effect of NG and silymarin on free radicals generation. Free radicals scavenging effect of NG and silymarin was studied against DPPH radicals (stable hydroxyl radical), enzymic, non-enzymic (superoxide radicals) and ABTS radical (stable hydroxyl radical). Percentage anti-oxidant activity (% effect) was determined as described in Materials and Methods section. Values are mean from five independent determinations. <sup>b</sup>*P* < 0.001 versus control. Control O.D. system DPPH, 0.650, system Enzymatic, 0.250 and system Non-enzymatic, 0.300 and system ABTS, 0.750. Data are expressed as mean ± SD and are from a representative experiments repeated twice and conducted in triplicate. Statistical significance: <sup>a</sup>*P* < 0.05; <sup>b</sup>*P* < 0.01; <sup>c</sup>*P* < 0.02; <sup>d</sup>*P* < 0.001; NS = non-significant vs respective controls.

free radical formation and lipid peroxidation processes, and polyunsaturated fatty acids in cellular membranes (microsomes) provide basic substrates for these reactions<sup>[43]</sup>. Scavenging or preventing formation of lipid radicals may prevent damage when cellular antioxidant defense mechanism is strengthened or iron overload is sequestered by exogenous treatment with cytoprotective drugs as NG. As lipid peroxidation (LPO) has been shown to play an important role in the ensuing toxicities in CYP2E1-induced conditions<sup>[44,45]</sup>, in this respect, NG strongly inhibited lipid peroxidation promoted by H<sub>2</sub>O<sub>2</sub>+Fe in microsomes and CCl<sub>4</sub> in HuH-7 cells (Figure 6 A and B). It has been reported that glycosides, such as NG, are potent cytoprotective agents against oxidative stress induced cytotoxicity<sup>[46]</sup>. Therefore, one major mechanism underlying the effectiveness of NG in protecting against the CCl<sub>4</sub>-induced LPO in HuH-7 cells may involve its capability to prevent lipid peroxidation chain reactions as a consequence of scavenging free radicals or chelating iron.

Intracellular calcium has been suggested to play a critical role in the oxidative damage of liver cells. Earlier, it has been reported that treatment of liver cells with CCl<sub>4</sub> increases calcium levels and produce cellular toxicity through calcium dependent pathways. Elevated levels of calcium initiates a cascade of signaling events leading to activation of calcium dependent degradative enzymes as phospholipases A2, endonucleases, or proteases<sup>[47]</sup>. Our results are in corroboration with this

and showed that CCl<sub>4</sub>-induced cell death in HuH-7 cells was mediated by release of intracellular calcium with subsequent activation of caspase 3 and cPLA2 (Figures 9 and 15) and simultaneous inhibition of cAMP levels (Figure 14). Increased intracellular calcium, activation of PLA2 and inhibition of cAMP were almost parallel to toxicity. Oxidative stress-mediated LPO is suggested to be the initiator of intracellular calcium release<sup>[25]</sup>, which later influences down stream apoptotic signaling processes. cAMP levels are known to be regulated by catalytic activity of adenylate cyclase and phosphodiesterase. Increasing concentration of intracellular cAMP has been directly associated with inhibition of phosphodiesterase, reduced release of ROS and inhibition of chemotaxis, degranulation and cell death<sup>[48]</sup>. NG restored the calcium and cAMP to normal levels, inhibited lipid peroxidation, activated cPLA2 levels were inhibited and cytotoxicity was reversed without altering CYP2E1 levels. Therefore we hypothesize that NG inhibits CCl<sub>4</sub>-induced oxidative stress and, hence LPO, which increases intracellular calcium and PLA2 activation and converge on mitochondria, inducing mitochondrial damage. All these downstream events of CYP2E1 mediated toxicity were effectively inhibited by NG, thus demonstrating its strong anti-oxidant capacity. We also suggest that inhibition of intracellular calcium release mediated cPLA2 activation, increase in cAMP levels, and restoration of MMP are the key factors in cytoprotection afforded by NG. Recently, it has been



**Figure 18** Proposed sequence of events and mechanism involved in the toxicity of CCl<sub>4</sub> and cytoprotection offered by NG. CCl<sub>4</sub> is activated by CYP 450 2E1 system and converted into trimethyl CCl<sub>3</sub> radicals inducing oxidative stress (increased ROS inducing membrane lipid peroxidation) and disturbed cellular Ca<sup>2+</sup> homeostasis. Increase in intracellular Ca<sup>2+</sup> concentrations leads to activation of phospholipase A2 and a decline in cAMP levels. All these signaling events converge onto the mitochondrial-initiating mitochondrial pore transition and ultimately to cellular injury. The highly increased levels of ROS is, in part, the consequence of the increase in Ca<sup>2+</sup>, and also as a result of mitochondrial permeabilization resulting in activation of Ca<sup>2+</sup>-dependent proteases. NG exerts a protective effect via inhibition of oxidative stress, maintenance of disrupted intracellular calcium homeostasis and inactivation of Ca<sup>2+</sup>-dependent proteases.

suggested that calcium levels do not play a direct role in toxicity<sup>[26]</sup>, but that activation of PLA2, promotion of the mitochondrial permeability transition and loss of mitochondrial function, which are secondary manifestations of increased calcium levels, form a general pathway involved in the toxicity: all these events were restored to normal by NG.

As the main antioxidant inside mammalian cells, GSH plays a pivotal role in preventing oxidative stress and mitochondrial damage caused by numerous toxins<sup>[49]</sup>. Therefore, the effect of CCl<sub>4</sub> in the absence or presence of NG on GSH content was evaluated. CCl<sub>4</sub> treatment drastically depleted intracellular GSH in HuH-7 cells, an effect prevented in the presence of NG (Figure 16). Accordingly, the maintenance of intracellular GSH levels by NG may help in protecting against the oxidative toxicity induced by CCl<sub>4</sub> in HuH-7 cells and avoid cell degeneration and death. Previously as well, depletion of GSH has been shown to enhance CYP2E1 resulting in CYP2E1-derived ROS leading to toxicity<sup>[50]</sup>.

Decreased MMP has been proposed to be a key mechanism by which CYP2E1-dependent LPO causes loss in cell viability. Mitochondria are a main source for generating ROS and, hence, a target for damage by oxidative stress<sup>[51]</sup>. In this respect, CCl<sub>4</sub> treatment caused a decrease in the MMP in HuH-7 cells, and this effect was prevented by NG as well as by silymarin (Figure 11). These results suggest that NG and silymarin may protect the cells by preventing oxidant-induced MMP transition leading to pathogenesis of necrotic or apoptotic cell death<sup>[52]</sup>. It has been proposed earlier that mitochondrial injury derived from oxidative damage can lead not only to necrosis by depleting ATP, but also to apoptotic cell

death by inducing the release of mitochondrial factors such as cytochrome C, which activates the caspase cascade<sup>[53, 54]</sup>.

Regardless of its precise mechanism of action, numerous studies in various animal models and in humans describe protective effects of NG against oxidative stress-related disease states<sup>[2-15]</sup>. This enhances its potential usefulness as a preventive agent toward oxidative damage involved in the development of liver injury caused by oxidative stress. Since NG acts as a very potent membrane stabilizer, it is also suggested that NG, may be acting as amphipathic substance, localizing near the membrane surface, trapping any radicals generated in the lipid environment of the membranes as well as in the cytosol. Such localization is suggested from the fact that CYP2E1 is found in the microsomes, and mitochondria appear to be a target for the CYP2E1-mediated damage in the presence of hepatotoxins such as CCl<sub>4</sub>, which was effectively inhibited by NG. Moreover, NG is well tolerated without adverse health effects by humans even after oral administration at high doses as evident from its use in Asian traditional medicine practices for various ailments.

In conclusion, this report shows that NG can protect against CCl<sub>4</sub>-induced toxicity and oxidative stress. The mechanism of protection involves decreased production of ROS and lipid peroxidation when the CYP2E1 mediated oxidative stress was produced in HuH-7 cells with pro-oxidant as CCl<sub>4</sub>. The main mechanism involved in the cytoprotection of NG seems to be its ability to protect the mitochondria against depletion in its membrane potential, an event that is very critical in the loss of cell viability as a consequence of oxidative stress. This mechanism has been postulated in the Figure 18. NG has been shown to prevent CCl<sub>4</sub>-induced liver injury, which may be, in part, due to the protection against CYP2E1-dependent oxidative stress as demonstrated in this study. NG supplementation could also prove to be protective against numerous toxicants that involve induction of oxidative stress through increased generation of ROS.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

*Vitex negundo* is a reputed medicinal herb of Indian sub-continent. All plant parts are considered important in Ayurvedic system of medicine for various indications.

### Research frontiers

Several pharmacological studies validate the medicinal claims of *Vitex negundo*. Diverse chemical constituents have been reported from various parts of this plant which are considered responsible for its varied pharmacological activities. The negundoside seems to be a potential constituent that exerts a protective effect on CYP2E1-dependent toxicity caused by carbon tetrachloride (CCl<sub>4</sub>) via inhibition of lipid peroxidation, followed by an improved intracellular calcium homeostasis and inhibition of Ca<sup>2+</sup>-dependent proteases.

### Innovation and breakthrough

The present investigation shows that negundoside is a potent phytopharmaceutical that acts in a novel way in inhibiting liver toxicity by interfering in the key events that are the main causative factors leading to liver



dysfunction.

### Applications

Negundoside exerts a protective action on CYP2E1-dependent oxidative stress and toxicity that may contribute to preventing chemically-induced liver injury, and may be useful in preventing toxicity by various other hepatotoxins as well.

### Peer review

The authors investigated the anti-apoptotic effect of negundoside (NG), being extracted from leaves of *Vitex negundo*, on cultured human hepatoma cell line, HuH-7G2. The authors performed a large amount of experiments, and found that NG inhibited ROS formation, lipid peroxidation, intracellular calcium elevation, GSH depletion, elevated anti-oxidant activity, declined MMP, cytochrome C release, and finally carbon tetrachloride-mediated apoptosis. The manuscript addressed the authors' hypothesis with sufficient data.

## REFERENCES

- Chin YW, Balunas MJ, Chai HB, Kinghorn AD. Drug discovery from natural sources. *AAPS J* 2006; **8**: E239-E253
- Dharmasiri MG, Jayakody JR, Galhena G, Liyanage SS, Ratnasooriya WD. Anti-inflammatory and analgesic activities of mature fresh leaves of *Vitex negundo*. *J Ethnopharmacol* 2003; **87**: 199-206
- Azhar-Ul-Haq, Malik A, Khan MT, Anwar-Ul-Haq, Khan SB, Ahmad A, Choudhary MI. Tyrosinase inhibitory lignans from the methanol extract of the roots of *Vitex negundo* Linn. and their structure-activity relationship. *Phytomedicine* 2006; **13**: 255-260
- Jagetia GC, Baliga MS. The evaluation of nitric oxide scavenging activity of certain Indian medicinal plants in vitro: a preliminary study. *J Med Food* 2004; **7**: 343-348
- Alam MI, Gomes A. Snake venom neutralization by Indian medicinal plants (*Vitex negundo* and *Embllica officinalis*) root extracts. *J Ethnopharmacol* 2003; **86**: 75-80
- Chandramu C, Manohar RD, Krupadanam DG, Dashavantha RV. Isolation, characterization and biological activity of betulinic acid and ursolic acid from *Vitex negundo* L. *Phytother Res* 2003; **17**: 129-134
- J Munasinghe TC, Seneviratne CK, Thabrew MI, Abeysekera AM. Antiradical and antilipoperoxidative effects of some plant extracts used by Sri Lankan traditional medical practitioners for cardioprotection. *Phytother Res* 2001; **15**: 519-523
- Gupta M, Mazumder UK, Bhawal SR. CNS activity of *Vitex negundo* Linn. in mice. *Indian J Exp Biol* 1999; **37**: 143-146
- Avadhoot Y, Rana AC. Hepatoprotective effect of *Vitex negundo* against carbon tetrachloride-induced liver damage. *Arch Pharm Res* 1991; **14**: 96-98
- Perumal Samy R, Ignacimuthu S, Sen A. Screening of 34 Indian medicinal plants for antibacterial properties. *J Ethnopharmacol* 1998; **62**: 173-1782
- Damayanti M, Susheela K, Sharma GJ. Effect of plant extracts and systemic fungicide on the pineapple fruit-rotting fungus, *Ceratocystis paradoxa*. *Cytobios* 1996; **86**: 155-165
- Pushpalatha E, Muthukrishnan J. Larvicidal activity of a few plant extracts against *Culex quinquefasciatus* and *Anopheles stephensi*. *Indian J Malariol* 1995; **32**: 14-23
- Bhargava SK. Antiandrogenic effects of a flavonoid-rich fraction of *Vitex negundo* seeds: a histological and biochemical study in dogs. *J Ethnopharmacol* 1989; **27**: 327-339
- Hebbalkar DS, Hebbalkar GD, Sharma RN, Joshi VS, Bhat VS. Mosquito repellent activity of oils from *Vitex negundo* Linn. leaves. *Indian J Med Res* 1992; **95**: 200-203
- Prabhakar A, Gupta BD, Suri KA, Satti NK, Malhotra S, Gupta KK, Sharma VK, Johri RK, Jaggi BS, Chandan BK, Shankar L, Bedi KL, Suri OP, Qazi GN. Hepatoprotective activity of 2'-p-hydroxybenzoylmussaenodidic acid. United States patent, US Patent 7, 259, 148, 2007; **259**: 148
- Dey A, Caro AA, Cederbaum AI. S-adenosyl methionine protects ob/ob mice from CYP2E1-mediated liver injury. *Am J Physiol Gastrointest Liver Physiol* 2007; **293**: G91-G103
- Kume Y, Ikeda H, Inoue M, Tejima K, Tomiya T, Nishikawa T, Watanabe N, Ichikawa T, Kaneko M, Okubo S, Yokota H, Omata M, Fujiwara K, Yatomi Y. Hepatic stellate cell damage may lead to decreased plasma ADAMTS13 activity in rats. *FEBS Lett* 2007; **581**: 1631-1634
- Beddowes EJ, Faux SP, Chipman JK. Chloroform, carbon tetrachloride and glutathione depletion induce secondary genotoxicity in liver cells via oxidative stress. *Toxicology* 2003; **187**: 101-115
- Jimenez-Lopez JM, Cederbaum AI. Green tea polyphenol epigallocatechin-3-gallate protects HepG2 cells against CYP2E1-dependent toxicity. *Free Radic Biol Med* 2004; **36**: 359-370
- Lee CS, Kim YJ, Han ES. Glycyrrhizin protection against 3-morpholinostyrene-induced mitochondrial dysfunction and cell death in lung epithelial cells. *Life Sci* 2007; **80**: 1759-1767
- Gandhidasan R, Thamarachelvan, Baburaj S. Anti inflammatory action of *Lannea coromandelica* by HRBC membrane stabilization. *Fitoterapia* 1991; **62**: 81-83
- Tasduq SA, Kaiser P, Sharma SC, Johri RK. Potentiation of isoniazid-induced liver toxicity by rifampicin in a combinational therapy of antitubercular drugs (rifampicin, isoniazid and pyrazinamide) in Wistar rats: A toxicity profile study. *Hepatol Res* 2007; **37**: 845-853
- Tietze F. Enzymic method for quantitative determination of nanogram amounts of total and oxidized glutathione: applications to mammalian blood and other tissues. *Anal Biochem* 1969; **27**: 502-522
- Nakagawa Y, Suzuki T, Kamimura H, Nagai F. Role of mitochondrial membrane permeability transition in N-nitrosodifluoramine-induced cell injury in rat hepatocytes. *Eur J Pharmacol* 2006; **529**: 33-39
- Kawamura-Sato K, Hirama Y, Agata N, Ito H, Torii K, Takeno A, Hasegawa T, Shimomura Y, Ohta M. Quantitative analysis of cereulide, an emetic toxin of *Bacillus cereus*, by using rat liver mitochondria. *Microbiol Immunol* 2005; **49**: 25-30
- Caro AA, Cederbaum AI. Role of intracellular calcium and phospholipase A2 in arachidonic acid-induced toxicity in liver cells overexpressing CYP2E1. *Arch Biochem Biophys* 2007; **457**: 252-263
- Rameh LE, Rhee SG, Spokes K, Kazlauskas A, Cantley LC, Cantley LG. Phosphoinositide 3-kinase regulates phospholipase Cgamma-mediated calcium signaling. *J Biol Chem* 1998; **273**: 23750-23757
- Grynkiewicz G, Poenie M, Tsien RY. A new generation of Ca<sup>2+</sup> indicators with greatly improved fluorescence properties. *J Biol Chem* 1985; **260**: 3440-3450
- Caro AA, Cederbaum AI. Ca<sup>2+</sup>-dependent and independent mitochondrial damage in HepG2 cells that overexpress CYP2E1. *Arch Biochem Biophys* 2002; **408**: 162-170
- Emaus RK, Grunwald R, Lemasters JJ. Rhodamine 123 as a probe of transmembrane potential in isolated rat-liver mitochondria: spectral and metabolic properties. *Biochim Biophys Acta* 1986; **850**: 436-448
- Rothe G, Valet G. Flow cytometric analysis of respiratory burst activity in phagocytes with hydroethidine and 2',7'-dichlorofluorescein. *J Leukoc Biol* 1990; **47**: 440-448
- Katiyar SK, Afaq F, Azizuddin K, Mukhtar H. Inhibition of UVB-induced oxidative stress-mediated phosphorylation of mitogen-activated protein kinase signaling pathways in cultured human epidermal keratinocytes by green tea polyphenol (-)-epigallocatechin-3-gallate. *Toxicol Appl Pharmacol* 2001; **176**: 110-117
- Yang D, Yaguchi T, Yamamoto H, Nishizaki T. Intracellularly transported adenosine induces apoptosis in HuH-7 human hepatoma cells by downregulating c-FLIP expression causing caspase-3/-8 activation. *Biochem Pharmacol* 2007; **73**: 1665-1675
- Gonzalez-Avila M, Arriaga-Alba M, de la Garza M, del

- Carmen HernandezPretelin M, Dominguez-Ortiz MA, Fattel-Fazenda S, Villa-Trevino S. Antigenotoxic, antimutagenic and ROS scavenging activities of a Rheo discolor ethanolic crude extract. *Toxicol In Vitro* 2003; **17**: 77-83
- 35 **Tasduq SA**, Kaiser P, Gupta DK, Kapahi BK, Maheshwari HS, Jyotsna S, Johri RK. Protective effect of a 50% hydroalcoholic fruit extract of *Emblica officinalis* against anti-tuberculosis drugs induced liver toxicity. *Phytother Res* 2005; **19**: 193-197
- 36 **Cao J**, Jiang LP, Liu Y, Yang G, Yao XF, Zhong LF. Curcumin-induced genotoxicity and antigenotoxicity in HepG2 cells. *Toxicol* 2007; **49**: 1219-1222
- 37 **Arteel GE**. Oxidants and antioxidants in alcohol-induced liver disease. *Gastroenterology* 2003; **124**: 778-790
- 38 **Morimoto M**, Hagbjork AL, Nanji AA, Ingelman-Sundberg M, Lindros KO, Fu PC, Albano E, French SW. Role of cytochrome P4502E1 in alcoholic liver disease pathogenesis. *Alcohol* 1993; **10**: 459-464
- 39 **Tsukamoto H**. Cyp2e1 and ALD. *Hepatology* 2000; **32**: 154-156
- 40 **Kim JY**, Lee S, Kim DH, Kim BR, Park R, Lee BM. Effects of flavonoids isolated from *Scutellariae radix* on cytochrome P-450 activities in human liver microsomes. *J Toxicol Environ Health A* 2002; **65**: 373-381
- 41 **Henderson MC**, Miranda CL, Stevens JF, Deinzer ML, Buhler DR. In vitro inhibition of human P450 enzymes by prenylated flavonoids from hops, *Humulus lupulus*. *Xenobiotica* 2000; **30**: 235-251
- 42 **Obermeier MT**, White RE, Yang CS. Effects of bioflavonoids on hepatic P450 activities. *Xenobiotica* 1995; **25**: 575-584
- 43 **Wilhelm J**. Metabolic aspects of membrane lipid peroxidation. *Acta Univ Carol Med Monogr* 1990; **137**: 1-53
- 44 **Caro AA**, Cederbaum AI. Synergistic toxicity of iron and arachidonic acid in HepG2 cells overexpressing CYP2E1. *Mol Pharmacol* 2001; **60**: 742-752
- 45 **Chen Q**, Galleano M, Cederbaum AI. Cytotoxicity and apoptosis produced by arachidonic acid in Hep G2 cells overexpressing human cytochrome P4502E1. *J Biol Chem* 1997; **272**: 14532-14541
- 46 **Moon MK**, Choi BM, Oh GS, Pae HO, Kim JD, Oh H, Oh CS, Kim DH, Rho YD, Shin MK, Lee HS, Chung HT. Catalposide protects Neuro 2A cells from hydrogen peroxide-induced cytotoxicity via the expression of heme oxygenase-1. *Toxicol Lett* 2003; **145**: 46-54
- 47 **Manibusan MK**, Odin M, Eastmond DA. Postulated carbon tetrachloride mode of action: a review. *J Environ Sci Health C Environ Carcinog Ecotoxicol Rev* 2007; **25**: 185-209
- 48 **Matsushashi T**, Otaka M, Odashima M, Jin M, Komatsu K, Konishi N, Wada I, Sato T, Horikawa Y, Ohba R, Oyake J, Hatakeyama N, Watanabe S. Specific type IV phosphodiesterase inhibitor ameliorates thioacetamide-induced liver injury in rats. *J Gastroenterol Hepatol* 2005; **20**: 135-140
- 49 **Balasubramaniyan V**, Shukla R, Murugaiyan G, Bhonde RR, Nalini N. Mouse recombinant leptin protects human hepatoma HepG2 against apoptosis, TNF-alpha response and oxidative stress induced by the hepatotoxin-ethanol. *Biochim Biophys Acta* 2007; **1770**: 1136-1144
- 50 **Zhuge J**, Cederbaum AI. Depletion of S-adenosyl-l-methionine with cycloleucine potentiates cytochrome P450 2E1 toxicity in primary rat hepatocytes. *Arch Biochem Biophys* 2007; **466**: 177-185
- 51 **Wu D**, Cederbaum AI. Cyclosporine A protects against arachidonic acid toxicity in rat hepatocytes: role of CYP2E1 and mitochondria. *Hepatology* 2002; **35**: 1420-1430
- 52 **Lemasters JJ**, Nieminen AL, Qian T, Trost LC, Elmore SP, Nishimura Y, Crowe RA, Cascio WE, Bradham CA, Brenner DA, Herman B. The mitochondrial permeability transition in cell death: a common mechanism in necrosis, apoptosis and autophagy. *Biochim Biophys Acta* 1998; **1366**: 177-196
- 53 **Lemasters JJ**, Qian T, Bradham CA, Brenner DA, Cascio WE, Trost LC, Nishimura Y, Nieminen AL, Herman B. Mitochondrial dysfunction in the pathogenesis of necrotic and apoptotic cell death. *J Bioenerg Biomembr* 1999; **31**: 305-319
- 54 **Hoek JB**, Cahill A, Pastorino JG. Alcohol and mitochondria: a dysfunctional relationship. *Gastroenterology* 2002; **122**: 2049-2063

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BASIC RESEARCH

## Hepatitis B virus DNA is more powerful than HBeAg in predicting peripheral T-lymphocyte subpopulations in chronic HBV-infected individuals with normal liver function tests

Jing You, Hucha Sriplung, Alan Geater, Virasakdi Chongsuvivatwong, Lin Zhuang, Hong-Ying Chen, Jun-Hua Huang, Bao-Zhang Tang

Jing You, Hucha Sriplung, Alan Geater, Virasakdi Chongsuvivatwong, Epidemiology Unit, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand

Lin Zhuang, Department of Hepatology, Third Municipal People's Hospital of Kunming, Kunming 650041, Yunnan Province, China

Hong-Ying Chen, Bao-Zhang Tang, Department of Infectious Diseases, First Affiliated Hospital of Kunming Medical University, Kunming 650032, Yunnan Province, China

Jun-Hua Huang, Department of Infectious Diseases, Yunnan General Hospital of the Chinese People's Armed Police Forces, Kunming 650111, Yunnan Province, China

**Author contributions:** You J, Sriplung H, Geater A and Chongsuvivatwong V conceptualized the study; You J and staff of the research group assisted with the data collection; You J was responsible for data management and data analysis; You J was responsible for interpretation of data; Sriplung H, Geater A and Chongsuvivatwong V provided advice and review; You J wrote the manuscript; All authors read and approved the final manuscript.

**Correspondence to:** Jing You, Epidemiology Unit, Faculty of Medicine, Prince of Songkla University, Hat Yai, Songkhla 90110, Thailand. [jingyoukm@126.com](mailto:jingyoukm@126.com)

Telephone: +66-84-6320906 Fax: +66-74-212900

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### Abstract

**AIM:** To investigate the peripheral T-lymphocyte subpopulation profile, and its correlations with hepatitis B virus (HBV) replication level in chronic HBV-infected (CHI) individuals with normal liver function tests (LFTs).

**METHODS:** Frequencies of T-lymphocyte subpopulations in peripheral blood were measured by flow cytometry in 216 CHI individuals. HBV markers were detected with ELISA. Serum HBV DNA load was assessed with quantitative real-time PCR. Information of age at HBV infection, and maternal HBV infection status was collected. ANOVA linear trend test and linear regression were used in statistical analysis.

**RESULTS:** CHI individuals had significantly decreased relative frequencies of CD3<sup>+</sup>, CD4<sup>+</sup> subpopulations

and CD4<sup>+</sup>/CD8<sup>+</sup> ratio, and increased CD8<sup>+</sup> subset percentage compared with uninfected individuals (all  $P < 0.001$ ). There was a significant linear relationship between the load of HBV DNA and the parameters of T-lymphocyte subpopulations (ANOVA linear trend test  $P < 0.01$ ). The parameters were also significantly worse among individuals whose mothers were known to be HBV carriers, and those having gained infection before the age of 8 years. In multiple regressions, after adjustment for age at HBV infection and status of maternal HBV infection, log copies of HBV DNA maintained its highly significant predictive coefficient on T-lymphocyte subpopulations, whereas the effect of HBeAg was not significant.

**CONCLUSION:** HBV DNA correlates with modification in the relative T-lymphocyte subpopulation frequencies. High viral load is more powerful than HBeAg in predicting the impaired balance of T-cell subsets.

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**Key words:** Hepatitis B virus; Chronic hepatitis B virus infection; Hepatitis B virus DNA; T-lymphocyte subpopulation; Immune function

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### INTRODUCTION

Hepatitis B virus (HBV) infection is a global public

health problem. It is estimated that approximately 2 billion people have serological evidence of past or present HBV infection and more than 350 million individuals worldwide are chronically infected with HBV<sup>[1]</sup>. In infected adolescents or adults, 5%-10% will develop into a chronic carrier state, whereas in infected neonates up to 90% develop chronicity<sup>[1-2]</sup>. HBV infection is especially prevalent in African and Asian countries such as Korea, Japan, Taiwan and mainland China because most patients with chronic HBV infection have acquired the infection perinatally from carrier mothers<sup>[3]</sup>. China has the highest prevalence of HBV, with over one-third of the world's total estimated HBV carriers. Out of the chronic HBV-infected patients, 70%-80% could have persistent normal liver function for many years or a lifetime<sup>[1-2]</sup>. Further persistent viral infection can, however, lead to subclinical hepatitis and chronic active hepatitis, even liver cirrhosis and the development of hepatocellular carcinoma<sup>[1-2]</sup>.

The pathogenesis of persistent viral infection and hepatitis B is complex. Generally, it is not HBV itself that damages hepatocytes directly, but the result of function disorder of cell-mediated immunity<sup>[4-6]</sup>. The cellular immune response to HBV is thought to be responsible for viral clearance, and disease pathogenesis during infection. The T-cell response to HBV is vigorous, polyclonal, and multispecific in acutely infected patients who successfully clear the virus, and it is relatively weak and narrowly focused in chronically infected patients<sup>[7-8]</sup>. The outcome of HBV infection would depend upon the balance between development of immunity (leading to virus elimination) and tolerance (leading to chronic viral persistence). HBeAg may play an important role in the interaction of the virus with the immune system. Secreted HBeAg has been proposed to have an immunoregulatory function in uterus by establishing T-cell tolerance to HBeAg and HBcAg that may predispose neonates born to HBV-infected mothers to develop persistent HBV infection<sup>[9]</sup>. Recent studies have further demonstrated an immunomodulatory role of HBeAg in antigen presentation and recognition by CD4<sup>+</sup> cells<sup>[10]</sup>.

It is essential to study the HBV replication status and its effects on cellular immune function in normal LFTs chronic HBV-infected (CHI) individuals. Firstly, they are the majority of chronic HBV-infected individuals; secondly, the understanding of the immune response upon HBV infection is useful to develop appropriate therapeutic strategies for controlling viral hepatitis and disease progression, as well as to improve current knowledge regarding persistent HBV infection prognosis. However, the correlations between HBV-specific T-cell response, and HBV viral load and HBeAg expression in CHI individuals are complicated. So are the effects of age at first infection and maternal HBV infection status. The aim of the work reported herein was to evaluate the peripheral blood T-lymphocyte subpopulation profile, and its correlations with HBV replication level, and to determine further which active marker of HBV active replication, HBV DNA or HBeAg is more powerful in predicting peripheral T-lymphocyte subpopulation in CHI individuals.

## MATERIALS AND METHODS

### *Enrollment of study subjects*

Two hundred and sixteen consecutive CHI individuals with normal LFTs were recruited from the Department of Infectious Diseases and of Hepatology of the First Affiliated Hospital of Kunming Medical University, the Third Municipal People's Hospital of Kunming and the Yunnan General Hospital of The Chinese People's Armed Police Forces, between January 2004 and May 2007.

The following criteria were fulfilled by all individuals:

(1) steady positivity for HBsAg in their serum for at least 12 mo and persistently normal liver function tests; and (2) exclusion of other concomitant causes of liver disease (hepatitis C, D and HIV infection and alcohol consumption of more than 60 g/day) and relatively rare liver disease (autoimmune hepatitis and metabolic liver disease) and treated with immunosuppressive therapy or antiviral therapy for HBV-infection within the recent 12 mo before entry. None of the patients was a drug user, or exposed to hepatotoxin. Informed consent was obtained from each study subject. The study protocol conformed to the guidelines of Declaration of Helsinki and was approved by ethics committees of the Faculty of Medicine of Prince of Songkla University and the First Affiliated Hospital of Kunming Medical University.

One hundred individuals who were free of HBsAg were identified from individuals coming to the outpatient service for a health check-up; 61 of the participants were male, 39 were female; mean age, 33.24 (SD, 10.28) years. These served as the control group for comparison of T-lymphocyte subpopulation with those who had HBV infection.

### *Serological liver function tests and hepatitis B virus markers evaluation*

Serum alanine amino-transferase (ALT), aspartate transaminase (AST) and total bilirubin (TBil) were tested with routine automated techniques (upper limit of normal: 40 U/L, 40 U/L and 17.1  $\mu$ mol/mL, respectively) (AU2700, Japan). HBV markers (HBsAg, HBsAb, HBeAg, HBeAb, HBcAb, and anti-HBcAb IgM) were measured at a virological laboratory with enzyme-linked immunosorbent assay (ELISA) (Anthos 2010, Austria). The experimental methods followed those specified within the reagent kit (Sino-American Biotech Co., Ltd) package insert.

### *Quantitative measurement HBV DNA (viraemia)*

Serum HBV DNA load in individuals was assessed by the real-time fluorescent quantitative polymerase chain reaction method (Real-Time-PCR) using a Lightcycler PCR system (FQD-33A, Bioer) with a lower limit of detection of approximately 1000 viral genome copies/mL. The handling procedures were performed in strict accordance with the reagent kit (Shenzhen PG Biotech Co., Ltd.) package insert. The primer was provided in the kit, the reaction volume was 40  $\mu$ L, and the reaction condition was 37°C for 5 min, 94°C for 1 min then 40 cycles as 95°C for 5 s and 60°C for 30 s.



**Peripheral blood T lymphocyte subsets measurement**

The key components of cellular immunity are T-lymphocyte and its subpopulations. CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> cells are major functional subgroups of T cells, and play an important role in response to HBV infection, which can reflect the situations of cellular immune function and immunoregulation, and are usually regarded as a valuable index to forecast the changes of patients' immunity<sup>[4-5]</sup>. These indices were chosen in our study for evaluating cellular immune function status of normal LFTs CHI individuals.

Blood samples were collected in heparinized vacutainer tubes. Whole blood samples were analyzed with a Multi-Q-Prep processor (Coulter, USA) and thereafter Epics-XL flow cytometry (FCM) (Coulter, USA). Lymphocytes were analyzed using a gate set on forward scatter versus side scatter, and a three color flow cytometry to combination reagent of CD3, CD4 and CD8. Anti-human monoclonal antibodies CD3-PE-CY5/CD4-FITC/CD8-PE were purchased from Immunotech, Ltd, USA. The detection was analyzed with the CELLQuest software (Coulter, USA) for each sample. The results were expressed as the percentages of CD3<sup>+</sup>, CD3<sup>+</sup>/CD4<sup>+</sup> (short for CD4<sup>+</sup> below) and CD3<sup>+</sup>/CD8<sup>+</sup> (short for CD8<sup>+</sup> below) cells found to be positive for the marker antigen in the total T-cell population. The handling procedures were performed in strict accordance with the instructions within the reagent kit package insert.

**Maternal HBV infection status (MH)**

All mothers of the subjects were reviewed in medical records for previous HBV infection and most of those who were infected could be identified. In addition, all of them were invited to undergo HBV-marker tests. For those with a positive result, a second set of tests was conducted 3 mo after the first test to confirm chronic HBV carrier status. If the mother had died, the cause of death was investigated based on medical records and history taking whether it was from HBV-related liver diseases such as chronic hepatitis B, HBV-related liver cirrhosis or hepatocellular carcinoma. If so, the MH was classified as positive.

**Age at HBV infection**

In the recent three decades in China, all children have been obligated to be tested for HBV markers when they first enter kindergarten and elementary schools. Subsequent obligatory tests are made when they apply for university or for a job. The results of these tests were obtained from medical records and interview. Based on this setting, we classified the age of first positive test as before 8 years, between 8-20 years and after 20 years.

**Statistical analysis**

Initial calculation came up with a sample size of 50 subjects with HBV DNA positive and the same number of HBV DNA negative group. This could provide the study with a statistical power of 80% at the 0.025 level

of significance to detect a difference in T-cell variation values of 33 *versus* 38. However, to cover the problem of being potentially confounded by other variables and to have enough subjects for stratifying levels of HBV DNA load to examine dose-response relationship, we ultimately recruited 216 CHI individuals and 100 controls.

Descriptive statistics were used to examine the age, gender, serum HBV viral load, HBeAg status, age at HBV-infection and maternal HBV infection status. The levels of T-lymphocyte subpopulation in normal individuals (HBsAg-negative) were summarized as means and standard deviation to serve as a control reference. Effects of various independent demographic, clinical and serological variables on T-cell profile were analyzed only among HBsAg-positive individuals. In univariate analysis, breakdown of these profiles by individual independent variables was carried out. Independent *t* test was done for 2-level independent variables and one-way ANOVA for more than 2-level variables. The relationship of HBV replication level and peripheral T-lymphocyte subpopulation was analyzed by correlation analysis and ANOVA linear trend test. Finally, multiple linear regression models were employed in multivariate analysis to assess the independent effects of variables on peripheral blood T lymphocytes. Variables yielding a *P* value  $\leq 0.2$  in the univariate analysis were included in the multivariate analysis, and the models were refined by backward elimination guided by the change in log likelihood of successive models. A final *P* value of less than 0.05 was considered statistically significant. Computations were carried out with the aid of R software version 2.5.1<sup>[11]</sup>.

**RESULTS****Demographic characteristics and clinical features of CHI individuals**

Demographic, serological, and clinical characteristics of the CHI individuals are summarized in Table 1. They were predominated by male (57.9%). One hundred and twenty four (57.4%) were less than 30 years old.

Of the CHI individuals, 37% got the infection before the age of 8 years. Almost three quarters had detectable serum levels of HBV DNA. Among these, the majority (68.4%, 93/136) had over 10<sup>7</sup> copies per milliliter. Just over half of them were HBeAg positive (56.5%).

Around 60% of the individuals' mothers were HBV positive. Among these individuals, nearly half had young age of infection and five-sixths had detectable serum levels of HBV DNA, of whom the majority (79.2%) had high viral load. Over 75% were HBeAg positive, whereas non-MH individuals were characterized by high age of infection, low viral load and low positivity of HBeAg.

Of those who had young age at infection, 80% (64/80) were HBeAg positive, and the majority (69/80) had detectable serum levels of HBV DNA, of whom nearly 74% (51/69) had high viral load.

Table 1 Characteristics of chronic HBV-infected individuals with normal liver function tests

Characteristics	All individuals ( <i>n</i> = 216)	Maternal HBV-infection status (MH)		<i>P</i>
		Individuals with MH ( <i>n</i> = 129)	Individuals without MH ( <i>n</i> = 87)	
Sex (male/female)	125/91	75/54	50/37	0.922 <sup>1</sup>
Mean age (yr)	31.53 ± 11.23	29.11 ± 11.44	35.13 ± 9.94	< 0.001 <sup>2</sup>
Age of HBV-infection (yr) (%)				< 0.001 <sup>1</sup>
< 8	80 (37.0)	62 (48.1)	18 (20.7)	
8-20	56 (25.9)	36 (27.9)	20 (23.0)	
> 20	58 (26.9)	21 (16.3)	37 (42.5)	
Unknown	22 (10.2)	10 (7.8)	12 (13.8)	
HBV DNA positive (%)	136 (63.0)	106 (82.2)	30 (34.5)	< 0.001 <sup>1</sup>
Serum HBV DNA (copies/mL) (%)				< 0.001 <sup>1</sup>
≤ 1.0 × 10 <sup>3</sup>	80 (37.0)	23 (17.8)	57 (65.5)	
1.0 × 10 <sup>3</sup> -1.0 × 10 <sup>5</sup>	14 (6.5)	5 (3.9)	9 (10.3)	
1.0 × 10 <sup>5</sup> -1.0 × 10 <sup>7</sup>	29 (13.4)	17 (13.2)	12 (13.8)	
> 1.0 × 10 <sup>7</sup>	93 (43.1)	84 (65.1)	9 (10.3)	
HBV DNA load (log, copies/mL)	5.90 ± 2.61	7.13 ± 2.36	4.07 ± 1.74	< 0.001 <sup>2</sup>
HBeAg positive (%)	122 (56.5)	97 (75.2)	25 (28.7)	< 0.001 <sup>1</sup>

<sup>1</sup>Chi-square test *P* value; <sup>2</sup>Student *t* test *P* value; HBV: Hepatitis B virus; MH: Maternal HBV-infection status.

Table 2 Peripheral T-cell subsets in normal control and CHI individuals broken down by various factors (mean ± SD)

Groups	<i>n</i>	CD3 <sup>+</sup> (%)	CD4 <sup>+</sup> (%)	CD8 <sup>+</sup> (%)	CD4 <sup>+</sup> /CD8 <sup>+</sup> ratio
HBV status <sup>1</sup>					
Negative	100	71.07 ± 4.76	38.94 ± 3.39	24.02 ± 4.35	1.67 ± 0.33
Positive	216	57.35 ± 13.81	32.97 ± 7.00	33.08 ± 7.99	1.07 ± 0.41
Maternal HBV-infection status <sup>1</sup>					
Negative	87	64.67 ± 10.74	35.75 ± 6.08	28.71 ± 5.56	1.29 ± 0.35
Positive	129	52.42 ± 13.49	31.10 ± 6.98	36.03 ± 8.04	0.93 ± 0.38
Age at HBV-infection (yr)					
< 8	80	66.35 ± 8.19	30.78 ± 7.03	35.36 ± 7.12	0.91 ± 0.30
8-20	56	66.46 ± 9.33	31.65 ± 5.06	35.80 ± 7.79	0.93 ± 0.28
> 20	58	69.35 ± 9.85	36.37 ± 7.31 <sup>df</sup>	28.22 ± 7.11 <sup>dh</sup>	1.37 ± 0.46 <sup>dh</sup>
Unknown	22	69.35 ± 9.85	35.35 ± 6.62 <sup>a</sup>	30.69 ± 7.48 <sup>bt</sup>	1.24 ± 0.46 <sup>af</sup>
HBV DNA load (copies/mL) <sup>2</sup>					
≤ 1.0 × 10 <sup>3</sup>	80	65.84 ± 9.39	37.11 ± 6.29	28.12 ± 5.65	1.38 ± 0.40
1.0 × 10 <sup>3</sup> -1.0 × 10 <sup>5</sup>	14	65.36 ± 5.15	34.70 ± 2.79	28.66 ± 6.21	1.28 ± 0.38
1.0 × 10 <sup>5</sup> -1.0 × 10 <sup>7</sup>	29	66.20 ± 9.99	33.66 ± 6.39	32.40 ± 6.54	1.06 ± 0.24
> 1.0 × 10 <sup>7</sup>	93	46.09 ± 10.52	28.94 ± 5.95	38.23 ± 7.21	0.79 ± 0.22
HBeAg status <sup>1</sup>					
Negative	94	64.45 ± 10.44	35.81 ± 6.69	29.05 ± 6.43	1.30 ± 0.42
Positive	122	51.89 ± 13.63	30.78 ± 6.46	36.19 ± 7.69	0.89 ± 0.31

<sup>1</sup>*P* < 0.001 for all comparisons of +ve vs -ve for each measure and each T-cell parameter; <sup>2</sup>*P* < 0.01 for ANOVA linear trend test;

<sup>a</sup>*P* < 0.05, <sup>b</sup>*P* < 0.01, <sup>d</sup>*P* < 0.001 vs < 8 yr group; <sup>f</sup>*P* < 0.01, <sup>h</sup>*P* < 0.001 vs 8-20 yr group.

### Peripheral T lymphocyte subpopulation composition in CHI individuals with normal LFTs

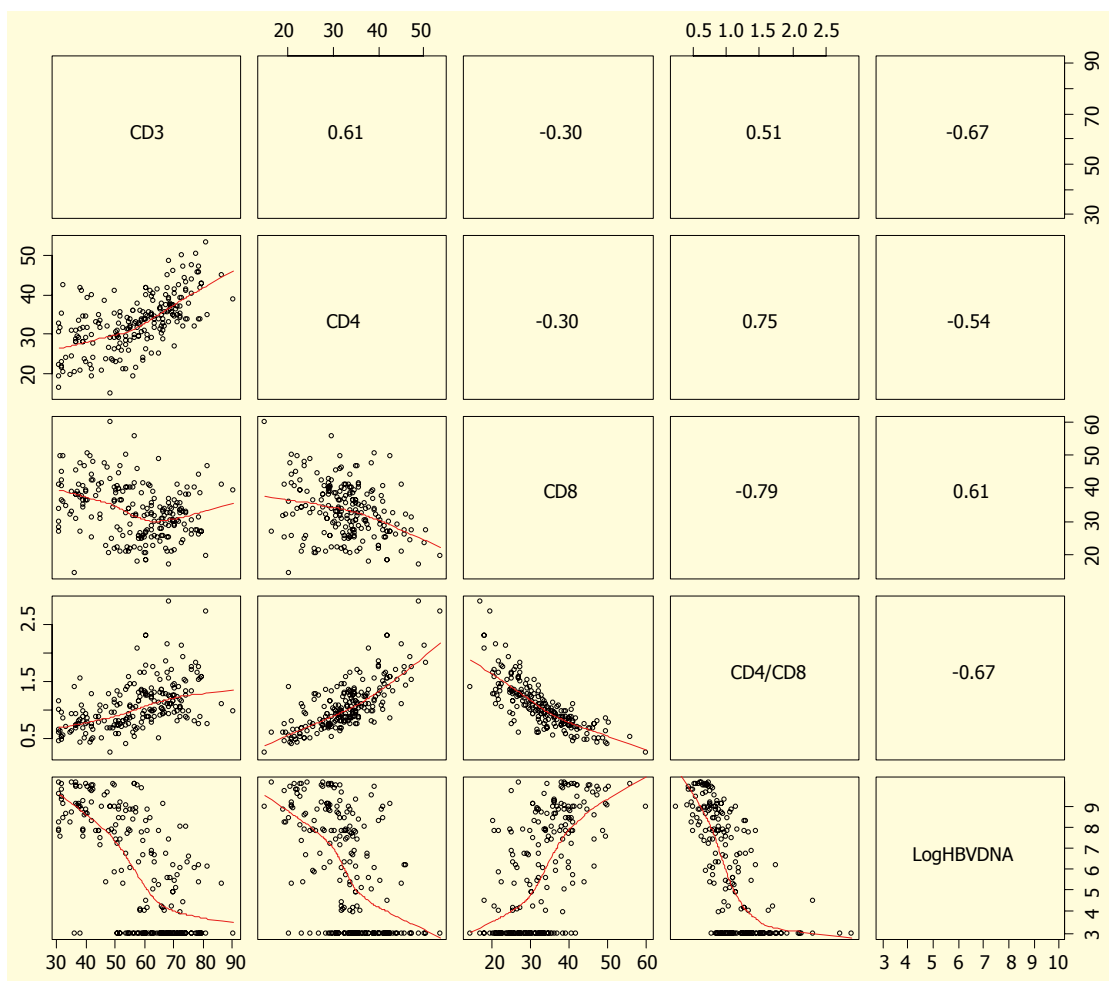
CHI individuals had significantly decreased relative frequencies of CD3<sup>+</sup> and CD4<sup>+</sup> subpopulations and CD4<sup>+</sup>/CD8<sup>+</sup> ratio, and increased CD8<sup>+</sup> subset percentage compared with the control group. Univariate analyses showed that the impaired balance of T-cell subsets was significantly associated with high viral load, presence of serum HBeAg expression, history of maternal HBV-infection and low age at HBV-infection (Table 2). Linear dose-response relationship between the level of T-lymphocyte subpopulation and log copies of HBV DNA was also highly significant (linear trend test *P* value < 0.01). Correlation between T-lymphocyte subpopulations and viral load is also shown in Figure 1

(*r* = -0.67, -0.54, 0.61, -0.67, respectively, for CD3<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup> and CD4<sup>+</sup>/CD8<sup>+</sup> ratio; all *P* < 0.0001) and Figure 2.

### Linear regression predicting peripheral blood T-lymphocyte subpopulation from relevant parameters

In Table 3, linear regression models are separately summarized for CD3<sup>+</sup>, CD4<sup>+</sup> and CD8<sup>+</sup> cells and CD4<sup>+</sup>/CD8<sup>+</sup> ratio, which are the dependent variables. After adjustment for all independent variables listed in the table, serum level of HBV viral load was the only significant predictor for each outcome variable, whereas the effects of HBeAg and other variables were not significant.

Figure 3 shows the relationship between T-lym-



**Figure 1** Correlation between peripheral T-cell subsets and serum HBV viral load. The numbers in the boxes refer to correlation coefficients. There is a negative correlation between the CD3<sup>+</sup> and CD4<sup>+</sup> cells and CD4<sup>+</sup>/CD8<sup>+</sup> ratio and serum viral load in CHI individuals with normal LFTs ( $r = -0.67, -0.54, -0.67$ ;  $P < 0.0001$ ), and a positive correlation between the levels of CD8<sup>+</sup> cells and viral load ( $r = 0.61$ ,  $P < 0.0001$ ).

**Table 3** Multiple linear regression predicting peripheral blood T lymphocyte subpopulation ( $n = 216$ )

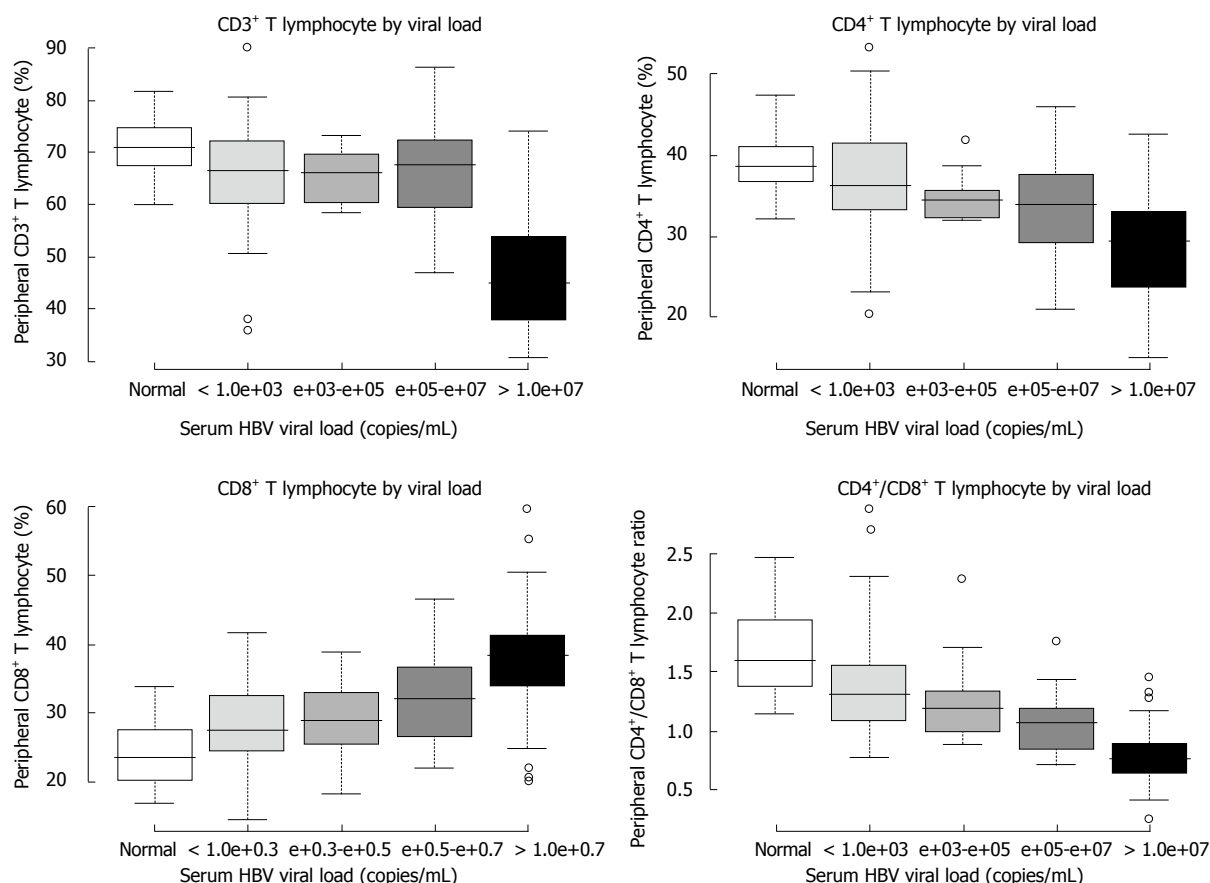
	CD3 <sup>+</sup> T lymphocyte			CD4 <sup>+</sup> T lymphocyte			CD8 <sup>+</sup> T lymphocyte			CD4 <sup>+</sup> /CD8 <sup>+</sup> ratio		
	$\beta$	SE	P	$\beta$	SE	P	$\beta$	SE	P	$\beta$	SE	P
Intercept	79.54	3.03	-	40.09	1.78	-	25.78	1.88	-	1.5	0.09	-
Serum HBV load (log, copies/mL) <sup>1</sup>	-3.65	0.43	< 0.0001	-1.38	0.25	< 0.0001	1.36	0.26	< 0.0001	-0.08	0.01	< 0.0001
HBeAg negative	0.05	1.98	0.98	0.61	1.16	0.6	0.5	1.22	0.69	-0.02	0.06	0.74
Age at HBV-infection(yr) <sup>2</sup>			0.06			0.63			0.19			0.02
8-20	-1.53	1.8		0.27	1.05		1.52	1.11		-0.03	0.05	
> 20	-4.77	2.08		1.2	1.22		-1.28	1.28		0.15	0.06	
Unknown	1.28	2.55		1.64	1.49		-0.77	1.58		0.13	0.08	
Maternal HBV-infection status	2.17	1.74	0.21	0.31	1.02	0.77	-2.45	1.08	0.02	0.06	0.05	0.27

$\beta$ : Coefficients from the model; SE: Standard error. <sup>1</sup>Continuous variable; <sup>2</sup>control group, < 8 yr of age at HBV infection.

phocyte subpopulations and viral load stratified by age at HBV infection. There was no significant difference of T-cell subsets among groups of age at HBV infection after adjustment for serum level of HBV viral load. A similar pattern is also seen in the figures that show the relationship between T-lymphocyte subpopulations and viral load stratified by maternal HBV carrier status and by HBeAg status in CHI individuals with normal LFTs respectively.

## DISCUSSION

This study demonstrated an impaired balance of the T-cell subsets related to an increased proportion of CD8<sup>+</sup> T-lymphocytes and decreased proportion of CD4<sup>+</sup> T-lymphocytes and CD4<sup>+</sup>/CD8<sup>+</sup> ratio in CHI individuals who had normal liver function tests. The level of the T-cell impairment had a linear dose-response relationship with the load of HBV DNA. The study also



**Figure 2** Peripheral T-lymphocyte subpopulations by serum HBV viral load. Composition of T-cell subpopulations from peripheral blood of patients with various serum HBV viral loads. Results are expressed as percentage of cells for each phenotype. Top of the box represents the 75th percentile, the bottom of the box represents the 25th percentile, and the solid line in the middle of the box represents the median. Whiskers above and below the box indicate the 90th and 10th percentiles, while circles represent outliers. Linear dose-response relationship between the level of T-lymphocyte subpopulations and copies of HBV DNA was highly significant (linear trend test,  $P$  value < 0.001). On the figure, the marks "< 1.0e+03", "e+03-e+05", "e+05-e+07" and "> 1.0e+07" denote "< 10<sup>3</sup>", "10<sup>3</sup>-10<sup>5</sup>", "10<sup>5</sup>-10<sup>7</sup>" and "> 10<sup>7</sup>", respectively.

illustrated the strong independent effects of HBV DNA, which eliminate the effects of maternal carrier status, younger age of infection and HBeAg positivity.

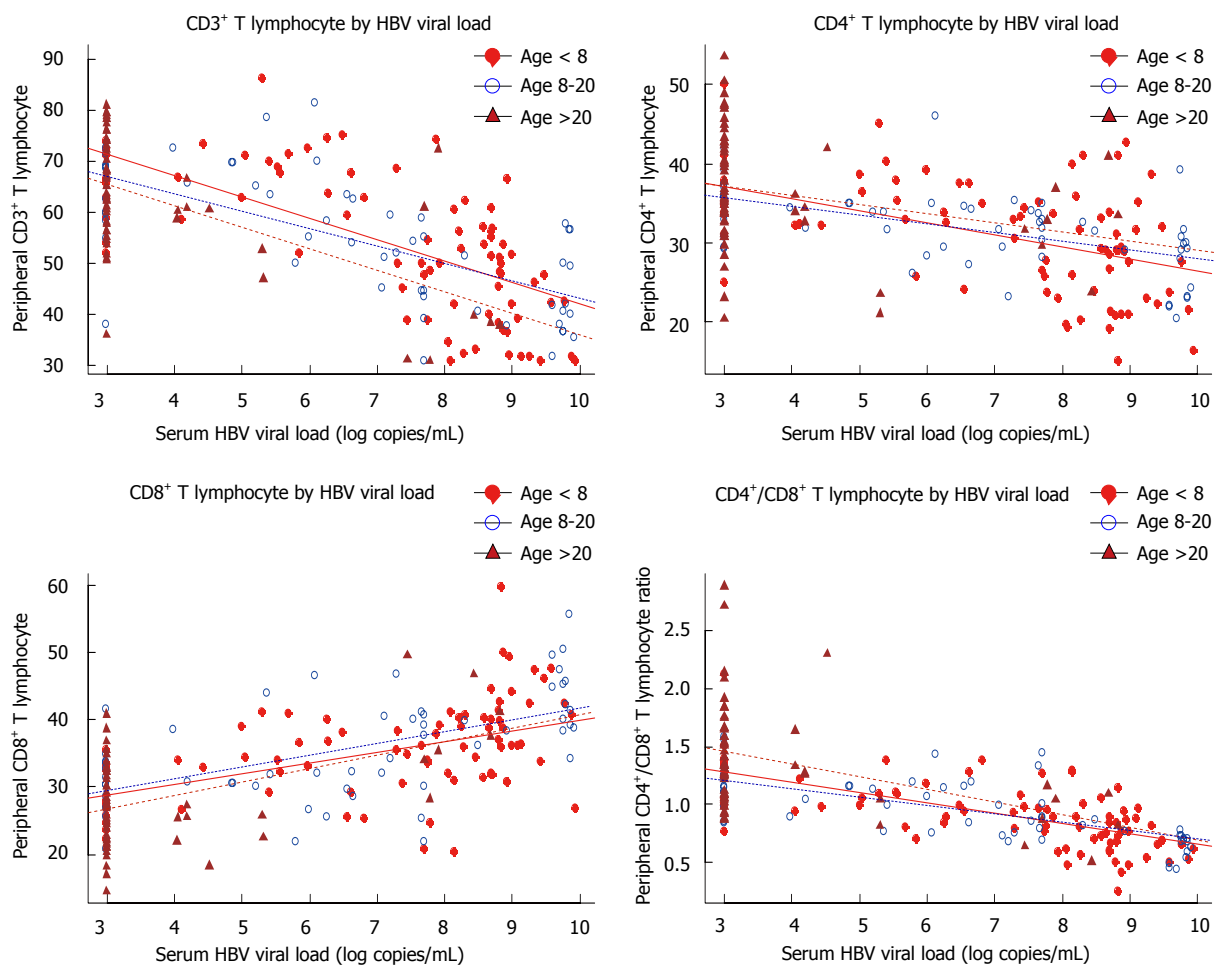
Our findings indicate that normal LFTs chronic HBV-infected individuals have an impaired balance of T-cell profile. The same finding also has been proved by previous researches in patients with chronic hepatitis B (CHB) that the chronicity of HBV infection is caused by a deficiency in cellular immune function, and hepatocytic damage is mainly caused by immunological injury<sup>[12-21]</sup>. However, the mechanism has not been defined<sup>[5]</sup>. Recently, the results have been reported by Tian *et al*<sup>[22]</sup> that CD4<sup>+</sup> and CD8<sup>+</sup> T cells decreased in both 33 CHB patients and 21 asymptomatic HBV carriers. Thus, most evidence has come from research in experimental animals<sup>[23-25]</sup> and in CHB patients<sup>[26-30]</sup>.

Our results reveal that T-cell impairment was significantly associated with viral replication level. The substantial linear dose-response relationship and strong independent predictive ability of HBV DNA, but not of other variables, on T-cell subpopulations suggests a close proximity between them in the causal pathway. However, cross-sectional study nature of our data does not allow us to identify the temporal direction of the causal relationship between these two variables. Mizukoshi

*et al*<sup>[31]</sup> suggested that antiviral therapy of persistently infected patients appeared to increase the frequency of HBV-specific CD4<sup>+</sup> T cell responses during the first year of treatment. Boni *et al*<sup>[32-35]</sup> reported that antiviral treatment can overcome CD8<sup>+</sup> T cell hypo-responsiveness in subjects with chronic HBV infection, suggesting that the T cells are present, but suppressed. It was reported by Pham *et al*<sup>[36]</sup> in 21 CHB patients that the ratio of CD4<sup>+</sup>/CD8<sup>+</sup> liver-derived lymphocytes, and not of peripheral blood lymphocytes appeared to be related to the level of HBV replication, and it revealed a positive correlation with viral load. The evidence that efficient antiviral T cell response can be restored by mono-antiviral treatment in CHB patients concurrently with reduction of viremia, indicates the importance of viral load in the pathogenesis of T cell hypo-responsiveness in these patients.

The stronger independent effect of viral load on the T-cell impairment and viral factor (viral variants) might explain the disappearance of the effect of other variables in multivariate analysis. Among our research subjects, the majority were characterized by young age of first HBV infection, maternal carrier status and high viral load in serum, and also high HBeAg expression. As a matter of fact, in addition to HBV DNA, HBeAg is also a seromarker for high viral replication which plays





**Figure 3** Correlation between T-cell subsets and viral load stratified by age at HBV infection. Three separate regression lines (with different slopes) are drawn for different groups of age at HBV infection. The coefficients of the interaction term "HBV DNA: age-at-HBV-infection" are not statistically significant for each parameter of T lymphocyte subpopulations (all  $P > 0.05$ ). The  $P$  value indicates no significant influence of age at HBV infection on peripheral T-cell subpopulations.

a crucial role in chronicity of HBV infection and high viral load by inducing immunological tolerance to HBV in the fetus. The tolerating effect of HBeAg has been well characterized in mice<sup>[37-39]</sup> and likely contributes to the low level of core-specific T-cell responses present in HBeAg<sup>+</sup> chronic patients<sup>[4,5]</sup>. Clinical evidence supports the tolerogenic effect of HBeAg<sup>[4,40]</sup>. Also, viral mutations that abrogate or antagonize antigen recognition by virus-specific T cells have been reported in patients with chronic HBV infection<sup>[41-42]</sup>, although the results from univariate analysis in our study showed that dysfunction of T-cell was significantly related with HBeAg, which later disappeared in multivariate analysis. One possible reason is that some of the subjects were infected with pre-C stop codon mutation virus (pre-C/C mutant), which resulted in a loss of HBeAg. In these patients, therefore, viral replication may persist despite elimination of HBeAg and seroconversion to anti-HBe. While the loss of HBeAg appears irrelevant for the biology of the virus, it may play an important role in the interaction of the virus with the immune system. This may weaken the independent association between HBeAg and the T-cell impairment so that the sample size in our study cannot detect this magnitude of association. Those who had maternal carrier history usually got infection at a

younger age (Table 1) and a higher HBV viral load was detected in the majority of those who had infection at a younger age. This phenomenon suggests that infection from the mother and/or at younger age predisposes to tolerance to HBV infection and, thus, higher viral load.

The strength of this study lies in the large sample size of CHI individuals with normal LFTs and the measurements of T-lymphocyte subpopulations using modern advanced flow cytometric technology and viral load by the quantitative real-time PCR method. A limitation of this study is the unknown age at HBV-infection of 22 individuals, the specificity of T-lymphocyte subpopulations, and liver-derived T-lymphocyte were not explored concurrently. Although the strong relationship of T-lymphocyte subpopulations with viral load is illustrated, further studies are needed to confirm the causal relationship between them.

Our results, which suggest that high viral load contributes to the impaired balance of the T-cell subsets in normal LFTs CHI individuals, have practical implications for understanding of pathogenesis and controlling of persistent viral infection, disease progression and prognosis because these individuals are also at risk of persistent viral infection leading to sub-clinical hepatitis, and chronic active hepatitis, even

liver cirrhosis and the development of hepatocellular carcinoma<sup>[1-3]</sup>. Perhaps, we should take this contribution into account in designing interventional strategies such as anti-viral therapeutic and/or immunotherapeutic strategies to prevent the progression and long-term consequences, which have been proved effective in CHB patients. Further clinical studies are needed to explore this possibility not only in CHB patients but also in normal LFTs chronic HBV-infected individuals.

In conclusion, we found that a strong independent predictive effect of HBV DNA load on T-lymphocyte subpopulations suggests a close proximity in the causal pathway between HBV viral load and the T-cell impairment. This information is of great interest because, first, it will be possible to predict the variation of T-lymphocyte subpopulations in peripheral blood in the future by measuring serum viral load level in chronic HBV-infected individuals with normal LFTs and second, this parameter can be monitored in blood easily and cheaply. Therefore, the measurement of viral load in serum of individuals suffering from chronic HBV infection could represent a simple parameter for the evaluation of cellular immune function status.

## COMMENTS

### Background

Hepatitis B virus (HBV) infection is a serious public health problem worldwide and a major cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. HBV infection is especially prevalent in African and Asian countries because most patients with chronic HBV infection have acquired the infection perinatally from carrier mothers. The pathogenesis of persistent viral infection is very complex and has not been clarified until now. Generally, it is not HBV itself that damages hepatocytes directly, but the results of disorder of cell-mediated immunity. The outcome of HBV infection would depend on the balance between development of immunity (leading to virus elimination) and tolerance (leading to chronic viral persistence).

### Research frontiers

Outcome of HBV infection, and the pathogenesis of liver disease are determined by immune-mediated host-virus interaction, which have been difficult to fully elucidate because the host range of HBV is limited to man and chimpanzees. The pathogenesis of liver disease and interaction between virus and host remain the research hotspots in this field.

### Innovations and breakthroughs

The pathogenesis and correlation of cellular immune disorder and HBV viral replication level remain unknown. In the present study, peripheral T-lymphocyte subpopulations of chronic HBV-infected (CHI) individuals who had normal liver function tests in big sample size were measured using advanced flow cytometry technology and HBV viral load with sensitive quantitative real-time-PCR method. The results suggest that the impaired balance of T-cell subpopulations was significantly associated with viral replication level. The substantial linear dose-response relationship and strong independent predictive effect of viral load on T-lymphocyte subpopulations suggests a close proximity of the causal pathway between them, and indicates the importance of viral load in the pathogenesis of T-cell impairment in these patients.

### Applications

The results, which suggest that high viral load contributes to the impaired balance of the T-cell subsets in normal liver function tests (LFTs) CHI individuals, have practical implications for understanding of pathogenesis and controlling of persistent viral infection and disease progression and prognosis because these individuals are also at risk of persistent viral infection leading to subclinical hepatitis and chronic active hepatitis, even liver cirrhosis and the development of hepatocellular carcinoma. In addition, it is possible to predict the variation of T-lymphocyte subpopulations in peripheral blood in the future by measuring serum viral load level in chronic HBV-infected patients.

## Peer review

The article is clearly written and demonstrates that high viral load is more powerful than HBeAg in predicting the impaired balance of T-cell subsets.

## REFERENCES

- 1 **World Health Organization, Department of Communicable diseases surveillance and response.** Hepatitis B. WHO Fact Sheets. Available from: URL: <http://www.who.int>. Accessed: August 28, 2007
- 2 **Pol S.** [Natural history of hepatitis B infection] *Presse Med* 2006; **35**: 308-316
- 3 **Chen CJ, Wang LY, Yu MW.** Epidemiology of hepatitis B virus infection in the Asia-Pacific region. *J Gastroenterol Hepatol* 2000; **15** Suppl: E3-E6
- 4 **Baumert TF, Thimme R, von Weizsacker F.** Pathogenesis of hepatitis B virus infection. *World J Gastroenterol* 2007; **13**: 82-90
- 5 **Bertoletti A, Gehring AJ.** The immune response during hepatitis B virus infection. *J Gen Virol* 2006; **87**: 1439-1449
- 6 **Liu DX.** A new hypothesis of pathogenetic mechanism of viral hepatitis B and C. *Med Hypotheses* 2001; **56**: 405-408
- 7 **Michalak TI, Hodgson PD, Churchill ND.** Posttranscriptional inhibition of class I major histocompatibility complex presentation on hepatocytes and lymphoid cells in chronic woodchuck hepatitis virus infection. *J Virol* 2000; **74**: 4483-4494
- 8 **Khettry U, Anand N, Gordon FD, Jenkins RL, Tahan SR, Loda M, Lewis WD.** Recurrent hepatitis B, hepatitis C, and combined hepatitis B and C in liver allografts: a comparative pathological study. *Hum Pathol* 2000; **31**: 101-108
- 9 **Milich DR, Jones JE, Hughes JL, Price J, Raney AK, McLachlan A.** Is a function of the secreted hepatitis B e antigen to induce immunologic tolerance in utero? *Proc Natl Acad Sci USA* 1990; **87**: 6599-6603
- 10 **Milich DR.** Do T cells "see" the hepatitis B core and e antigens differently? *Gastroenterology* 1999; **116**: 765-768
- 11 **R Development Core Team.** R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0. Available from: URL: <http://www.R-project.org>. Accessed: August, 2007
- 12 **Wang KX, Peng JL, Wang XF, Tian Y, Wang J, Li CP.** Detection of T lymphocyte subsets and mIL-2R on surface of PBMC in patients with hepatitis B. *World J Gastroenterol* 2003; **9**: 2017-2020
- 13 **Webster GJ, Reignat S, Maini MK, Whalley SA, Ogg GS, King A, Brown D, Amlot PL, Williams R, Vergani D, Dusheiko GM, Bertoletti A.** Incubation phase of acute hepatitis B in man: dynamic of cellular immune mechanisms. *Hepatology* 2000; **32**: 1117-1124
- 14 **Webster GJ, Reignat S, Brown D, Ogg GS, Jones L, Seneviratne SL, Williams R, Dusheiko G, Bertoletti A.** Longitudinal analysis of CD8+ T cells specific for structural and nonstructural hepatitis B virus proteins in patients with chronic hepatitis B: implications for immunotherapy. *J Virol* 2004; **78**: 5707-5719
- 15 **Sarin SK, Thakur V, Guptan RC, Saigal S, Malhotra V, Thyagarajan SP, Das BC.** Profile of hepatocellular carcinoma in India: an insight into the possible etiologic associations. *J Gastroenterol Hepatol* 2001; **16**: 666-673
- 16 **Shoenfeld Y, Aron-Maor A.** Vaccination and autoimmunity-'vaccinosis': a dangerous liaison? *J Autoimmun* 2000; **14**: 1-10
- 17 **Trobonjaca Z, Kroger A, Stober D, Leithauser F, Moller P, Hauser H, Schirmbeck R, Reimann J.** Activating immunity in the liver. II. IFN-beta attenuates NK cell-dependent liver injury triggered by liver NKT cell activation. *J Immunol* 2002; **168**: 3763-3770
- 18 **Rapicetta M, Ferrari C, Levrero M.** Viral determinants and host immune responses in the pathogenesis of HBV

- infection. *J Med Virol* 2002; **67**: 454-457
- 19 **Tanner MS**. Mechanisms of liver injury relevant to pediatric hepatology. *Crit Rev Clin Lab Sci* 2002; **39**: 1-61
  - 20 **Rivero M**, Crespo J, Fabrega E, Casafont F, Mayorga M, Gomez-Fleitas M, Pons-Romero F. Apoptosis mediated by the Fas system in the fulminant hepatitis by hepatitis B virus. *J Viral Hepat* 2002; **9**: 107-113
  - 21 **Okumura A**, Ishikawa T, Yoshioka K, Yuasa R, Fukuzawa Y, Kakumu S. Mutation at codon 130 in hepatitis B virus (HBV) core region increases markedly during acute exacerbation of hepatitis in chronic HBV carriers. *J Gastroenterol* 2001; **36**: 103-110
  - 22 **Tian Y**, Qiu ZF, Li TS. [Difference and significance of peripheral blood T-lymphocyte subsets in patients with chronic hepatitis B and asymptomatic HBV carriers] *Zhonghua Yixue Zazhi* 2005; **85**: 3354-3358
  - 23 **Chen M**, Sallberg M, Thung SN, Hughes J, Jones J, Milich DR. Nondeletional T-cell receptor transgenic mice: model for the CD4(+) T-cell repertoire in chronic hepatitis B virus infection. *J Virol* 2000; **74**: 7587-7599
  - 24 **Lin CM**, Wang FH. Selective modification of antigen-specific CD4(+) T cells by retroviral-mediated gene transfer and in vitro sensitization with dendritic cells. *Clin Immunol* 2002; **104**: 58-66
  - 25 **Chen M**, Sallberg M, Thung SN, Hughes J, Jones J, Milich DR. Modeling the T-helper cell response in acute and chronic hepatitis B virus infection using T-cell receptor transgenic mice. *Antiviral Res* 2001; **52**: 99-111
  - 26 **Thimme R**, Wieland S, Steiger C, Ghayeb J, Reimann KA, Purcell RH, Chisari FV. CD8(+) T cells mediate viral clearance and disease pathogenesis during acute hepatitis B virus infection. *J Virol* 2003; **77**: 68-76
  - 27 **Lau GK**, Suri D, Liang R, Rigopoulou EI, Thomas MG, Mullerova I, Nanji A, Yuen ST, Williams R, Naoumov NV. Resolution of chronic hepatitis B and anti-HBs seroconversion in humans by adoptive transfer of immunity to hepatitis B core antigen. *Gastroenterology* 2002; **122**: 614-624
  - 28 **Sing GK**, Li D, Chen X, Macnaughton T, Lichanska AM, Butterworth L, Ladham A, Cooksley G. A molecular comparison of T lymphocyte populations infiltrating the liver and circulating in the blood of patients with chronic hepatitis B: evidence for antigen-driven selection of a public complementarity-determining region 3 (CDR3) motif. *Hepatology* 2001; **33**: 1288-1298
  - 29 **Stoop JN**, van der Molen RG, Baan CC, van der Laan LJ, Kuipers EJ, Kusters JG, Janssen HL. Regulatory T cells contribute to the impaired immune response in patients with chronic hepatitis B virus infection. *Hepatology* 2005; **41**: 771-778
  - 30 **Franzese O**, Kennedy PT, Gehring AJ, Gotto J, Williams R, Maini MK, Bertolotti A. Modulation of the CD8+-T-cell response by CD4+ CD25+ regulatory T cells in patients with hepatitis B virus infection. *J Virol* 2005; **79**: 3322-3328
  - 31 **Mizukoshi E**, Sidney J, Livingston B, Ghany M, Hoofnagle JH, Sette A, Rehermann B. Cellular immune responses to the hepatitis B virus polymerase. *J Immunol* 2004; **173**: 5863-5871
  - 32 **Boni C**, Bertolotti A, Penna A, Cavalli A, Pilli M, Urbani S, Scognamiglio P, Boehme R, Panebianco R, Fiaccadori F, Ferrari C. Lamivudine treatment can restore T cell responsiveness in chronic hepatitis B. *J Clin Invest* 1998; **102**: 968-975
  - 33 **Boni C**, Penna A, Ogg GS, Bertolotti A, Pilli M, Cavallo C, Cavalli A, Urbani S, Boehme R, Panebianco R, Fiaccadori F, Ferrari C. Lamivudine treatment can overcome cytotoxic T-cell hyporesponsiveness in chronic hepatitis B: new perspectives for immune therapy. *Hepatology* 2001; **33**: 963-971
  - 34 **Boni C**, Penna A, Bertolotti A, Lamonaca V, Rapti I, Missale G, Pilli M, Urbani S, Cavalli A, Cerioni S, Panebianco R, Jenkins J, Ferrari C. Transient restoration of anti-viral T cell responses induced by lamivudine therapy in chronic hepatitis B. *J Hepatol* 2003; **39**: 595-605
  - 35 **Boni C**, Fiscaro P, Valdatta C, Amadei B, Di Vincenzo P, Giuberti T, Laccabue D, Zerbini A, Cavalli A, Missale G, Bertolotti A, Ferrari C. Characterization of hepatitis B virus (HBV)-specific T-cell dysfunction in chronic HBV infection. *J Virol* 2007; **81**: 4215-4225
  - 36 **Pham BN**, Mosnier JF, Walker F, Njapoum C, Bougy F, Degott C, Erlinger S, Cohen JH, Degos F. Flow cytometry CD4+/CD8+ ratio of liver-derived lymphocytes correlates with viral replication in chronic hepatitis B. *Clin Exp Immunol* 1994; **97**: 403-410
  - 37 **Milich D**, Liang TJ. Exploring the biological basis of hepatitis B e antigen in hepatitis B virus infection. *Hepatology* 2003; **38**: 1075-1086
  - 38 **Chen MT**, Billaud JN, Sallberg M, Guidotti LG, Chisari FV, Jones J, Hughes J, Milich DR. A function of the hepatitis B virus precore protein is to regulate the immune response to the core antigen. *Proc Natl Acad Sci USA* 2004; **101**: 14913-14918
  - 39 **Chen M**, Sallberg M, Hughes J, Jones J, Guidotti LG, Chisari FV, Billaud JN, Milich DR. Immune tolerance split between hepatitis B virus precore and core proteins. *J Virol* 2005; **79**: 3016-3027
  - 40 **Liu CJ**, Kao JH, Lai MY, Chen PJ, Chen DS. Precore/core promoter mutations and genotypes of hepatitis B virus in chronic hepatitis B patients with fulminant or subfulminant hepatitis. *J Med Virol* 2004; **72**: 545-550
  - 41 **Bertolotti A**, Costanzo A, Chisari FV, Levrero M, Artini M, Sette A, Penna A, Giuberti T, Fiaccadori F, Ferrari C. Cytotoxic T lymphocyte response to a wild type hepatitis B virus epitope in patients chronically infected by variant viruses carrying substitutions within the epitope. *J Exp Med* 1994; **180**: 933-943
  - 42 **Bertolotti A**, Sette A, Chisari FV, Penna A, Levrero M, De Carli M, Fiaccadori F, Ferrari C. Natural variants of cytotoxic epitopes are T-cell receptor antagonists for antiviral cytotoxic T cells. *Nature* 1994; **369**: 407-410

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## Ineffective oesophageal motility: Manometric subsets exhibit different symptom profiles

Horst Gunter Haack, Ross David Hansen, Allison Malcolm, John Edward Kellow

Horst Gunter Haack, Ross David Hansen, Allison Malcolm, John Edward Kellow, Department of Gastroenterology, Gastrointestinal Investigation Unit, Royal North Shore Hospital, University of Sydney, Australia

**Author contributions:** Haack HG performed research and data analysis; Hansen RD performed conceptual, data and statistical analysis and wrote the paper; Malcolm A discussed the conceptual aspects and the data analysis; Kellow JE designed conceptual aspects, discussed data and statistical analysis and wrote the paper.

**Correspondence to:** John E Kellow, MD, Department of Gastroenterology, Royal North Shore Hospital, St. Leonards, NSW 2065, Australia. [johnk@med.usyd.edu.au](mailto:johnk@med.usyd.edu.au)

Telephone: +61-2-99267355 Fax: +61-2-94363719

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### Abstract

**AIM:** To compare the demographic and clinical features of different manometric subsets of ineffective oesophageal motility (IOM; defined as  $\geq 30\%$  wet swallows with distal contractile amplitude  $< 30$  mmHg), and to determine whether the prevalence of gastro-oesophageal reflux differs between IOM subsets.

**METHODS:** Clinical characteristics of manometric subsets were determined in 100 IOM patients (73 female, median age 58 years) and compared to those of 100 age- and gender-matched patient controls with oesophageal symptoms, but normal manometry. Supine oesophageal manometry was performed with an eight-channel DentSleeve water-perfused catheter, and an ambulatory pH study assessed gastro-oesophageal reflux.

**RESULTS:** Patients in the IOM subset featuring a majority of low-amplitude simultaneous contractions (LASC) experienced less heartburn (prevalence 26%), but more dysphagia (57%) than those in the IOM subset featuring low-amplitude propagated contractions (LAP; heartburn 70%, dysphagia 24%; both  $P \leq 0.01$ ). LASC patients also experienced less heartburn and more dysphagia than patient controls (heartburn 68%, dysphagia 11%; both  $P < 0.001$ ). The prevalence of heartburn and dysphagia in IOM patients featuring a majority of non-transmitted sequences (NT) was 54% ( $P = 0.04$  vs LASC) and 36% ( $P < 0.01$  vs controls), respectively. No differences in

age and gender distribution, chest pain prevalence, acid exposure time (AET) and symptom/reflux association existed between IOM subsets, or between subsets and controls.

**CONCLUSION:** IOM patients with LASC exhibit a different symptom profile to those with LAP, but do not differ in gastro-oesophageal reflux prevalence. These findings raise the possibility of different pathophysiological mechanisms in IOM subsets, which warrants further investigation.

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**Key words:** Age; Dysphagia; Heartburn; Ineffective oesophageal motility; Oesophageal manometry; Simultaneous contractions

**Peer reviewer:** Yvan Vandenplas, Professor, Department of Pediatrics, AZ-VUB, Laarbeeklaan 101, Brussels 1090, Belgium

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### INTRODUCTION

The term ineffective oesophageal motility (IOM), the most common variant of oesophageal dysmotility, was introduced in 1997 to replace the term “non-specific oesophageal motility disorder”<sup>[1]</sup>, as the former term appears to better reflect the functional and clinical relevance of manometric alterations<sup>[2,3]</sup>. IOM is defined manometrically as  $\geq 30\%$  of swallow sequences with a contractile amplitude  $< 30$  mmHg in the distal oesophagus<sup>[4]</sup>. Such a definition, however, encompasses three abnormal contractile patterns, namely low-amplitude propagated contractions (LAP), low-amplitude simultaneous contractions (LASC), and non-transmitted contractions. IOM is, therefore, diagnosed if there is a combination of these abnormal contractile patterns.

It is not known if the predominant oesophageal symptom (heartburn, dysphagia, chest pain) experienced by IOM patients is associated with the predominance



of one of these ineffective manometric patterns. In addition, although gastro-oesophageal reflux disease (GORD) has been associated with IOM<sup>[5-7]</sup>, it is not known whether particular manometric subsets of IOM display a higher prevalence of GORD.

This study was thus aimed at (1) comparing the demographic and manometric features of IOM patients with different predominant symptoms (heartburn, dysphagia, chest pain); (2) comparing the demographic and clinical features of different manometric subsets of IOM; (3) comparing the clinical features of IOM subsets with those of patients with oesophageal symptoms, but normal oesophageal manometry; and (4) determining whether the prevalence of gastro-oesophageal reflux differs between the IOM manometric subsets.

## MATERIALS AND METHODS

### Patients

A total of 100 consecutive patients (73 females, mean  $\pm$  SD age  $56 \pm 18$  years) were studied. Patients with oesophageal symptoms were referred to the Gastrointestinal Investigation Unit of the Royal North Shore Hospital. All patients fulfilled the current manometric criteria for IOM:  $\geq 30\%$  of wet swallows with low-amplitude propagated sequences (distal contractile amplitude  $< 30$  mmHg), LASC, or non-transmitted contractions<sup>[4]</sup>. Swallow sequences were considered to be simultaneous when propagation velocity was  $> 8$  cm/s between two or more manometric channels, and were considered to be non-transmitted when contractile amplitude was  $\leq 10$  mmHg at any site. A group of 100 age- and gender-matched patients (73 females, age  $56 \pm 20$  years) referred with oesophageal symptoms, but exhibiting normal oesophageal manometry<sup>[8]</sup> served as a patient control group. Patients in the two groups had undergone upper gastrointestinal endoscopy and/or barium studies to exclude structural disease of the oesophagus, apart from the presence of reflux oesophagitis and sliding hiatus hernia. Patients with systemic diseases that could alter oesophageal motility, such as diabetes mellitus or scleroderma, were excluded. The study was approved by the Human Research Ethics Committee of the Northern Sydney Area Health Service.

### Symptom assessment

A standardized symptom assessment was completed by all patients. This assessment comprised the Rome II Integrative Questionnaire<sup>[9]</sup> with an additional evaluation of oesophageal symptoms. This additional evaluation determined the predominant (i.e. most troublesome) oesophageal symptom (heartburn, dysphagia, chest pain, or others)<sup>[10]</sup> and the time period since its first appearance. The usual intensity, frequency, and duration of all reported symptoms were assessed, and symptom severity scores for heartburn, dysphagia, and chest pain were calculated as the product of these intensity, frequency, and duration data.

### Oesophageal manometry

All patients were studied after an overnight fast. Oesophageal manometry was performed using an eight-channel DentSleeve water-perfused manometric catheter with an external diameter of 4.5 mm (DentSleeve Pty. Ltd., Belair, SA, Australia) and a computer-based data acquisition and charting system (Acquidata, Neomedix Systems, Warriewood, NSW, Australia). The catheter was introduced transnasally and swallowing was recorded via a port positioned in the pharynx, 25 cm above the proximal end of the hydraulic sleeve segment of the catheter. Oesophageal contractions were measured by ports located 5, 10, 15, and 20 cm above the proximal end of the sleeve and gastric pressure by a side hole 1 cm below the distal end of the 6 cm long sleeve. Each patient made approximately 10 swallows, each of 5 mL water of room temperature, in the seated position to acclimatize to the procedure. The patient was then placed in the supine position, and a minimum of 10 supine water swallows, each of 5 mL water, with at least 30 s in between swallows, were performed. A station pull-through technique was then used to accurately locate the position of the lower oesophageal sphincter (LOS). The LOS function was assessed by measuring mid-respiratory LOS resting pressure and swallow-induced LOS relaxation<sup>[8]</sup>.

### 24-h pH monitoring

A subset of patients (54 IOM, 72 patient controls with normal manometry) underwent an ambulatory oesophageal pH study. A probe with an antimony pH sensor (Flexilog, Oakfield Instruments Ltd., Eynsham, England) was introduced transnasally and the sensor was positioned 5 cm above the upper edge of the LOS, which was determined manometrically as described above. The probe was connected to a data logger (Digitrapper Mark II, Synectics, Sweden), which sampled pH at 5-s intervals for approximately 24 h. Mealtimes, symptom events, and supine periods were recorded in a patient diary. The computerized analysis (Esophagram, Synectics, Sweden) included the percentage of time pH was below 4 (Acid Exposure Time, AET)<sup>[11]</sup>, and the symptom association probability (SAP)<sup>[12]</sup> was calculated when possible.

### Data analysis

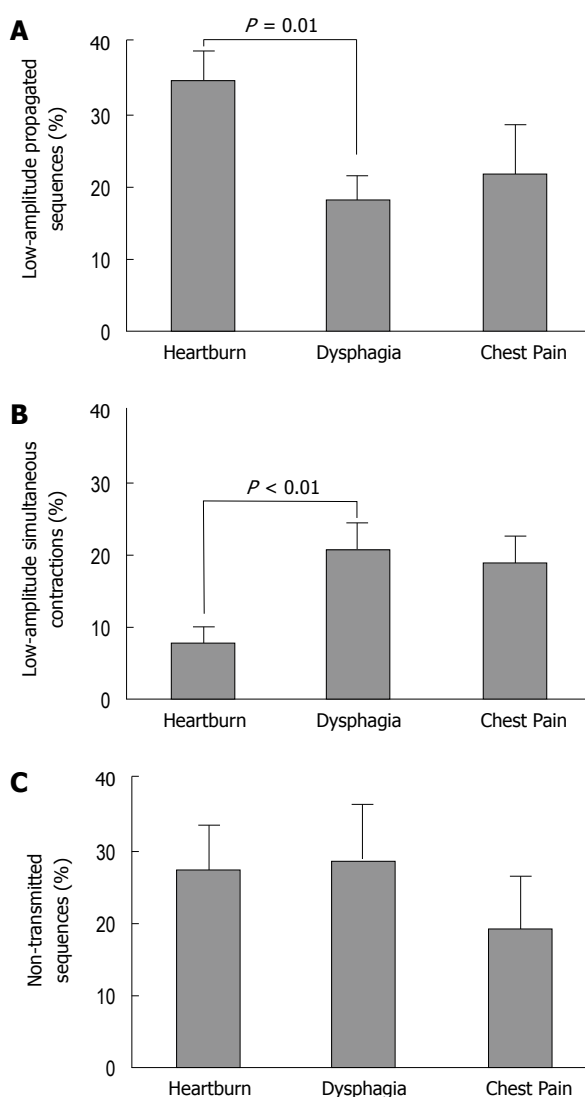
**Analysis based on predominant symptom:** Proportions of total abnormal swallows, and proportions of LAP, LASC, and non-transmitted sequences (NT) were calculated for each of the three main predominant symptoms (heartburn, dysphagia, chest pain). Differences in proportions were then determined between symptom subgroups.

**Analysis based on predominant contractile abnormality:** All IOM manometric studies were further categorized into the following three subsets according to the predominant contractile abnormality contributing to the 30% or more abnormal swallows: those exhibiting a majority of LAP, those exhibiting a majority of LASC,

**Table 1** Gender, age, time since onset of symptom, and proportion of total abnormal swallows in IOM patients according to the predominant oesophageal symptom<sup>1</sup>

	Gender (F:M)	Median age [yr (range)]	Median time since symptom onset (yr)	Total abnormal swallows (mean $\pm$ SD) (%)
Heartburn	37:16	57 (26-82)	6.7	69 $\pm$ 22
Dysphagia	28:8	63 (19-86)	2.5	68 $\pm$ 22
Chest pain	8:1	59 (31-76)	6.1	61 $\pm$ 25

<sup>1</sup>Two male patients reported "other" predominant symptoms (hiccoughs, halitosis).



**Figure 1** Proportions of low-amplitude propagated sequences (A), LASC (B), and NT (C) in the three predominant symptom subgroups. Proportions are expressed as (mean  $\pm$  SE) % of total swallows.

and those exhibiting a majority of NT. Differences in clinical features between these three subsets were then determined.

**Statistical analysis:** Results are presented as mean  $\pm$  SE, unless otherwise stated.  $\chi^2$  and Fisher's exact tests were used to determine differences in gender distribution

between symptom subgroups, and differences in symptom prevalence between manometric subsets. Age differences between subsets, and between IOM patients and patient controls, were determined via Kruskal-Wallis tests with post-hoc Mann-Whitney U comparisons. Similarly, differences between symptom subgroups in proportions of contractile abnormalities and in LOS pressure and AET were determined via one-way analysis of variance (ANOVA) with post-hoc Scheffe tests. Correlation and regression analysis was used to describe relationships between variables. All analyses were performed using the SPSS statistical program (Release 14, SPSS Inc., Chicago, IL), with  $P < 0.05$  considered significant.

## RESULTS

### Analysis based on the predominant symptom

**Symptom, gender, and age distribution:** The prevalence of predominant heartburn, predominant dysphagia, and predominant chest pain in IOM patients was 53%, 36%, and 9%, respectively. The prevalence of heartburn was significantly lower in IOM patients than in patient controls (68%,  $P = 0.03$ ), and the prevalence of dysphagia was significantly higher in IOM patients than in patient controls (11%,  $P < 0.001$ ).

There were no significant differences in gender and age distribution, and in time period since symptom onset, between the three symptom subgroups in IOM (Table 1).

**Proportions of abnormal swallows:** The mean proportion of LAP was significantly higher in patients with heartburn than in those with dysphagia, and the mean proportion of LASC was significantly higher in patients with dysphagia than in those with heartburn. The mean proportion of NT was similar across the three symptom subgroups (Figure 1; ANOVA and post-hoc tests). The proportion of total abnormal swallows did not differ between the three symptom subgroups (Table 1).

When all reported symptoms were considered (i.e. predominant symptom plus additional symptoms), the relationships between symptom severity scores and proportions of abnormal sequences were examined. There were positive correlations between dysphagia severity score and proportion of LASC ( $r = 0.2$ ,  $P = 0.05$ ), and between chest pain severity score and proportion of LAP ( $r = 0.22$ ,  $P < 0.05$ ).

**LOS mid-respiratory resting pressure:** Mean LOS pressure was significantly higher in patients with predominant dysphagia ( $12.5 \pm 1.2$  mmHg) than in those with heartburn ( $9.2 \pm 0.6$  mmHg;  $P = 0.03$ ). The mean value for chest pain patients was  $11.1 \pm 1.0$  mmHg.

**Ambulatory pH data:** The gender and age distribution of the patients who underwent a pH study closely reflected that of the total subject pool, and mean LOS pressures of subgroups were almost identical to those of the total subject pool. There were no significant

**Table 2** Gender and age distribution, and ambulatory oesophageal pH data<sup>1</sup>, for IOM manometric subsets and for patient controls who exhibited normal manometry

	Gender (F:M)	Median age [yr (range)]	AET <sup>2</sup> (%)	Abnormal AET <sup>3</sup> (%)	SAP+ <sup>4</sup> (%)
IOM-LAP	25:12	58 (26-77)	8.8 ± 1.8	58	52
IOM-LASC	17:7	60 (29-86)	14.7 ± 5.1	90	60
IOM-NT	31:8	61 (19-76)	11.6 ± 2.5	81	33
Patient controls	73:27	58 (19-91)	8.6 ± 0.8	69	51

<sup>1</sup>pH data was available in 54 IOM patients and 72 patient controls; <sup>2</sup>AET (% of time pH < 4), reported as mean ± SE; <sup>3</sup>% of patients with an AET > 4%;

<sup>4</sup>Expressed as % of patients with a significant SAP value.

differences between the three subgroups in terms of AET or proportion of patients with an abnormal AET, or proportion with significant SAP values.

### Analysis based on predominant contractile abnormality

**Gender and age distribution:** The gender and age distribution for the three IOM manometric subsets and the patient controls is shown in Table 2. There were no statistical differences in gender and age distribution between patient controls and any of the IOM manometric subsets, or between IOM subsets.

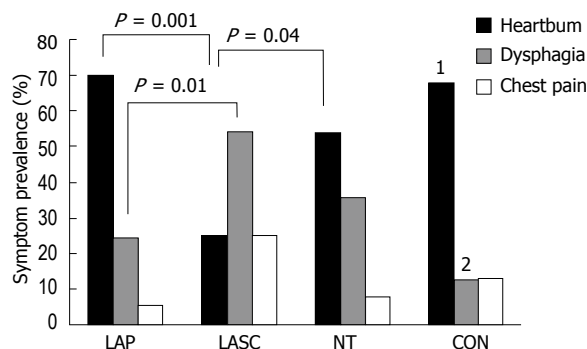
**Predominant symptoms:** Figure 2 shows the distribution of predominant symptoms for the three manometric subsets and for patient controls. Heartburn was highly prevalent (70%) in the LAP subset, but was significantly less common (26%) in the LASC subset. Conversely, dysphagia was more prevalent in LASC (57%) than in LAP (24%) ( $\chi^2$  tests). There were no significant differences between IOM subsets, however, in severity scores for heartburn, dysphagia or chest pain, or in time since the onset of the primary symptom.

**LOS mid-respiratory resting pressure:** There were no statistically significant differences between the mean LOS pressures of the IOM subsets: values for LAP, LASC, and NT were  $10.1 \pm 0.9$ ,  $11.3 \pm 1.4$ , and  $10.9 \pm 0.8$  mmHg, respectively. There was a trend ( $P = 0.05$ ) for the LAP subset to have a lower value than that of patient controls ( $12.0 \pm 0.5$  mmHg).

**Ambulatory pH data:** There were no significant differences in the prevalence of abnormal AET between IOM subsets. Analysis of mean AET values between the IOM subsets, and compared to patient controls, also revealed no significant differences (Table 2). Similarly, there were no differences in upright and supine AET values between subsets and groups. There were no significant SAP differences between the IOM subsets or in comparison to the control group (Table 2).

## DISCUSSION

Despite almost 10 years of usage of the category IOM, and recent studies evaluating IOM with combined intraluminal impedance and manometry<sup>[3]</sup>, the extent



**Figure 2** Prevalence of predominant symptoms in the three manometrically-determined IOM subsets, and in patient controls (CON) who exhibited normal manometry. <sup>1</sup>CON significantly higher than LASC ( $P < 0.001$ ); <sup>2</sup>CON significantly lower than LASC ( $P < 0.001$ ) and NT ( $P < 0.01$ ) ( $\chi^2$  tests).

to which the various contractile patterns of IOM differ according to the predominant symptom has remained largely unexplored. Our study in a large patient cohort shows that IOM is indeed a heterogeneous clinical entity with regard to the predominant symptom reported. We focused on the type of contractile abnormality, rather than solely considering the contractile amplitude. The novel finding was that two specific contractile patterns, namely LASC and LAP, were closely related to predominant dysphagia and predominant heartburn, respectively. Additional analyses provided further support for these findings in that as the proportion of LASC increased, the dysphagia severity score also increased. Consistent with these findings, there were notable differences in symptom distribution between IOM manometric subsets, categorized according to the most frequent type of contractile pattern in an individual patient. Differences were particularly prominent between patients with low-amplitude propagated sequences and those with LASC; the latter experienced more dysphagia and less heartburn than the former patients.

We did not find a difference in age distribution between manometric subsets. Others have noted that simultaneous contractions are more likely to occur in older than in younger patients<sup>[13,14]</sup>, especially in the presence of severe GORD<sup>[15]</sup>. The effect of age in relation to the occurrence of simultaneous contractions in healthy volunteers, however, remains unclear, as one study revealed no relationship between simultaneous contractions and age<sup>[16]</sup>, whereas a more recent study did find a direct correlation between the proportion of simultaneous contractions and age<sup>[17]</sup>. The most recent study revealed differences in muscle thickness between similarly-categorized IOM subsets, and a significant correlation between muscle thickness, and the occurrence of simultaneous contractions<sup>[14]</sup>. Whether muscle thickness actually plays a causative role in the pathophysiology of simultaneous contractions remains unclear, and this needs to be further evaluated. It is possible that the clinical profile of IOM patients with a majority of LASC is similar to that of patients with diffuse oesophageal spasm (DOS), a disorder that also features intermittent simultaneous (but moderate- to

high-amplitude) contractions<sup>[4]</sup>. Hence, treatments currently considered for DOS patients<sup>[18-20]</sup> may prove beneficial for selected IOM patients, and this should also be further evaluated.

In the subset of IOM patients with mainly non-transmitted swallow patterns, it is feasible that this pattern could represent an evolving achalasia-like dysmotility, although these patients exhibited only a modest prevalence of dysphagia. As long-term follow-up of IOM patients has scarcely been reported<sup>[21]</sup>, findings regarding disease progression remain inconclusive, and further studies are needed.

Neither the prevalence nor the severity of gastro-oesophageal acid reflux differed between IOM subsets. In addition, we found no significant differences in symptom/reflux association or heartburn severity score between IOM subsets, despite some differences in LOS resting pressure. A low LOS tone and transient LOS relaxation<sup>[22]</sup> are regarded as the main mechanisms of gastro-oesophageal reflux. As the LOS tone was lower in IOM patients with low-amplitude propagated sequences compared with patient controls, and heartburn prevalence was highest in this IOM subset, these patients could be expected to have the highest acid exposure. This was not the case, and our findings are consistent with those of Lemme *et al.*, who found that the proportion of low-amplitude swallows did not differ between IOM patients with erosive *versus* non-erosive GORD<sup>[23]</sup>. It is feasible that the presence of propagating (albeit low-amplitude) sequences equates to more efficient clearance<sup>[24]</sup> of refluxate in these patients, and novel approaches to stimulating clearance<sup>[25]</sup> may prove beneficial in this IOM subset. Another explanation for the high heartburn prevalence is that these patients exhibit oesophageal hypersensitivity<sup>[26]</sup>. Despite the lack of difference in acid exposure between IOM subsets, pooled analyses of all IOM patients showed that a low LOS tone was associated with both high heartburn severity scores and high AETs. These findings indicate the complex interactions between LOS characteristics, oesophageal body dysfunction and symptomatology in IOM patients with gastro-oesophageal reflux, which require further investigation.

The current study focused primarily on oesophageal body dysmotility in IOM. LOS swallow-induced relaxation is assessed via manometry, but does not feature in the diagnosis of IOM. Conchillo *et al.* have recently shown that, in addition to inadequate LOS relaxation, a shorter duration of LOS relaxation could contribute to abnormal bolus transit in IOM patients<sup>[27]</sup>. This might represent an additional manometric discriminator of dysmotility, and warrants further investigation.

In summary, we have examined subsets of IOM patients based on symptoms and manometrically-determined oesophageal body dysmotility. These subsets differ in regard to symptom profile, but do not differ in acid exposure or symptom/reflux association. Patients with LASC experience more dysphagia, but less heartburn, than IOM patients with low-amplitude, but propagated sequences. These findings raise the

possibility of different pathophysiological mechanisms in IOM subsets, and this warrants further investigation.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

The term ineffective oesophageal motility (IOM) encompasses a variety of symptoms and three types of abnormal oesophageal body peristalsis.

### Research frontiers

The pathogenesis of IOM remains unknown, particularly whether it is related to GORD or represents a primary oesophageal motor disorder.

### Innovations and breakthroughs

This study raises the possibility of different pathophysiological mechanisms in the different subsets of IOM.

### Applications

Further study of the IOM subsets using novel technologies such as impedance and topographic manometry is required.

### Terminology

IOM refers to low-amplitude, simultaneous, or non-transmitted oesophageal body contractions.

### Peer review

The authors compared the demographic and clinical features of different manometric subsets of ineffective oesophageal motility and determined whether the prevalence of gastro-oesophageal reflux differs between IOM subsets. This is an interesting and well-written study.

## REFERENCES

- 1 Leite LP, Johnston BT, Barrett J, Castell JA, Castell DO. Ineffective esophageal motility (IEM): the primary finding in patients with nonspecific esophageal motility disorder. *Dig Dis Sci* 1997; **42**: 1859-1865
- 2 Kahrilas PJ, Dodds WJ, Hogan WJ. Effect of peristaltic dysfunction on esophageal volume clearance. *Gastroenterology* 1988; **94**: 73-80
- 3 Tutuian R, Castell DO. Clarification of the esophageal function defect in patients with manometric ineffective esophageal motility: studies using combined impedance-manometry. *Clin Gastroenterol Hepatol* 2004; **2**: 230-236
- 4 Spechler SJ, Castell DO. Classification of oesophageal motility abnormalities. *Gut* 2001; **49**: 145-151
- 5 Ho SC, Chang CS, Wu CY, Chen GH. Ineffective esophageal motility is a primary motility disorder in gastroesophageal reflux disease. *Dig Dis Sci* 2002; **47**: 652-656
- 6 Fouad YM, Katz PO, Hatlebakk JG, Castell DO. Ineffective esophageal motility: the most common motility abnormality in patients with GERD-associated respiratory symptoms. *Am J Gastroenterol* 1999; **94**: 1464-1467
- 7 Diener U, Patti MG, Molena D, Fisichella PM, Way LW. Esophageal dysmotility and gastroesophageal reflux disease. *J Gastrointest Surg* 2001; **5**: 260-265
- 8 Kahrilas PJ, Clouse RE, Hogan WJ. American Gastroenterological Association technical review on the clinical use of esophageal manometry. *Gastroenterology* 1994; **107**: 1865-1884
- 9 Drossman DA, Corraziari EC, Talley NJ, Thompson WG, Whitehead WE. Rome II integrative questionnaire. In: Drossman DA, Corraziari EC, Talley NJ, Thompson WG, Whitehead WE. Rome II: the functional gastrointestinal disorders. McLean VA: Degnon Associates, 2000: 691-710
- 10 Bak YT, Lorang M, Evans PR, Kellow JE, Jones MP, Smith RC. Predictive value of symptom profiles in patients with suspected oesophageal dysmotility. *Scand J Gastroenterol*



- 1994; **29**: 392-397
- 11 **Kahrilas PJ**, Quigley EM. Clinical esophageal pH recording: a technical review for practice guideline development. *Gastroenterology* 1996; **110**: 1982-1996
- 12 **Weusten BL**, Roelofs JM, Akkermans LM, Van Berge-Henegouwen GP, Smout AJ. The symptom-association probability: an improved method for symptom analysis of 24-hour esophageal pH data. *Gastroenterology* 1994; **107**: 1741-1745
- 13 **Ribeiro AC**, Klingler PJ, Hinder RA, DeVault K. Esophageal manometry: a comparison of findings in younger and older patients. *Am J Gastroenterol* 1998; **93**: 706-710
- 14 **Kim JH**, Rhee PL, Park EH. Evaluation of esophageal muscle thickness in patients with ineffective esophageal motility using a high-frequency intraluminal ultrasound. *Gastroenterology* 2005; **128**: W1551
- 15 **Achem AC**, Achem SR, Stark ME, DeVault KR. Failure of esophageal peristalsis in older patients: association with esophageal acid exposure. *Am J Gastroenterol* 2003; **98**: 35-39
- 16 **Richter JE**, Wu WC, Johns DN, Blackwell JN, Nelson JL 3rd, Castell JA, Castell DO. Esophageal manometry in 95 healthy adult volunteers. Variability of pressures with age and frequency of "abnormal" contractions. *Dig Dis Sci* 1987; **32**: 583-592
- 17 **Grande L**, Lacima G, Ros E, Pera M, Ascaso C, Visa J, Pera C. Deterioration of esophageal motility with age: a manometric study of 79 healthy subjects. *Am J Gastroenterol* 1999; **94**: 1795-1801
- 18 **Storr M**, Allescher HD, Classen M. Current concepts on pathophysiology, diagnosis and treatment of diffuse oesophageal spasm. *Drugs* 2001; **61**: 579-591
- 19 **Storr M**, Allescher HD, Rosch T, Born P, Weigert N, Classen M. Treatment of symptomatic diffuse esophageal spasm by endoscopic injections of botulinum toxin: a prospective study with long-term follow-up. *Gastrointest Endosc* 2001; **54**: 754-759
- 20 **Miller LS**, Pullela SV, Parkman HP, Schiano TD, Cassidy MJ, Cohen S, Fisher RS. Treatment of chest pain in patients with noncardiac, nonreflux, nonachalasia spastic esophageal motor disorders using botulinum toxin injection into the gastroesophageal junction. *Am J Gastroenterol* 2002; **97**: 1640-1646
- 21 **Achem SR**, Crittenden J, Kolts B, Burton L. Long-term clinical and manometric follow-up of patients with nonspecific esophageal motor disorders. *Am J Gastroenterol* 1992; **87**: 825-830
- 22 **Sifrim D**, Tack J, Lerut T, Janssens J. Transient lower esophageal sphincter relaxations and esophageal body muscular contractile response in reflux esophagitis. *Dig Dis Sci* 2000; **45**: 1293-1300
- 23 **Lemme EM**, Abrahao-Junior LJ, Manhaes Y, Shechter R, Carvalho BB, Alvariz A. Ineffective esophageal motility in gastroesophageal erosive reflux disease and in nonerosive reflux disease: are they different? *J Clin Gastroenterol* 2005; **39**: 224-227
- 24 **Simren M**, Silny J, Holloway R, Tack J, Janssens J, Sifrim D. Relevance of ineffective oesophageal motility during oesophageal acid clearance. *Gut* 2003; **52**: 784-790
- 25 **Grossi L**, Cappello G, Marzio L. Effect of an acute intraluminal administration of capsaicin on oesophageal motor pattern in GORD patients with ineffective oesophageal motility. *Neurogastroenterol Motil* 2006; **18**: 632-636
- 26 **Fass R**, Tougas G. Functional heartburn: the stimulus, the pain, and the brain. *Gut* 2002; **51**: 885-892
- 27 **Conchillo JM**, Nguyen NQ, Samsom M, Holloway RH, Smout AJ. Multichannel intraluminal impedance monitoring in the evaluation of patients with non-obstructive Dysphagia. *Am J Gastroenterol* 2005; **100**: 2624-2632

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# Inoperable esophageal cancer and outcome of palliative care

Sima Besharat, Ali Jabbari, Shahryar Semnani, Abbasali Keshtkar, Jeran Marjani

Sima Besharat, Ali Jabbari, Shahryar Semnani, Abbasali Keshtkar, Jeran Marjani, Golestan University of Medical Sciences, Golestan Research Center of Gastroenterology and Hepatology, Gorgan 49177-65181, Iran

**Author contributions:** Besharat S designed the research and wrote the paper; Jabbari A contributed new references and edited the paper; Semnani S gave the idea of the research and handled it scientifically; Keshtkar A analyzed data and Marjani J gathered data.

**Correspondence to:** Shahryar Semnani, MD, Gastroenterologist, Golestan University of Medical Sciences, Golestan Research center of Gastroenterology and Hepatology, 2nd Floor, Nabavi Polyclinic, 4th Azar, 5 Azar Boulevard, Gorgan 49177-65181, Golestan Province, Iran. [sh\\_semnani@yahoo.com](mailto:sh_semnani@yahoo.com)

Telephone: +98-171-2240835 Fax: +98-171-2269210

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## Abstract

**AIM:** To determine the outcome of esophageal cancer patients referred for palliative care, in Gorgan and Gonbad gastrointestinal clinics, northeast of Iran.

**METHODS:** This cross-sectional study was done on inoperable esophageal cancer cases referred to gastrointestinal clinics in Gorgan and Gonbad city (2005-2006). Demographic data were collected during the procedure and cases were followed up every one month. Improvement proportion was calculated with 95% confidence interval, to determine the rate of improvement. Survival analysis and Kaplan-Meier methods were used to estimate the duration of palliative care effectiveness.

**RESULTS:** We recruited 39 cases into the study. Squamous cell carcinoma was the most prevalent (92.3%). The middle third of the esophagus was involved predominantly (51.3%). Dilation was the most preferred method (89.7%) and stenting was done in 4 cases. Decreasing dysphagia score was not related to palliation method or pathology type of carcinoma. Age of the patients was significantly related to the improvement of dysphagia score. Mean survival time was 137.6 d and median was 103 d.

**CONCLUSION:** Results of this study showed a low survival rate after palliative care in esophageal cancer cases despite dysphagia scores' improvement after dilating or stenting.

## INTRODUCTION

Esophageal cancer patients have poor prognosis. Due to the lack of widespread screening methods, diagnosis is usually made at advanced stages; therefore, they have a short survival when diagnosed. The 5-year survival rate of patients with esophageal cancer is < 20%<sup>[1]</sup>.

This is more obvious especially in some regions like the northeast of Iran, where the prevalence rate of esophageal cancer is high.

Esophageal cancer five-year survival has slightly increased during past 20 years (5%-9%), but still remains low. Most patients present with locally advanced, unresectable or metastatic disease. At the time of diagnosis, 60% of the patients are only suitable for palliative therapy. Recent advances in therapeutic endoscopy have allowed improving dysphagia, and quality of life. Endoscopic techniques are chosen according to tumor characteristics, since the diagnosis is often made at an advanced stage, when radical treatment is unfeasible<sup>[1-3]</sup>.

Dysphagia, or the inability to swallow, is one of the most distressing and debilitating symptoms in patients with cancer-related oesophageal obstruction. Dysphagia leads to nutritional compromise, pain, and deterioration of quality of life<sup>[1,4-5]</sup>.

As the quality of life, and to some extent the quantity of life remaining to these patients depends to a large degree on their ability to swallow, the relief of dysphagia plays a vital role in the palliation of this disease<sup>[5]</sup>.

Endoscopic palliation aims to restore swallowing,

avoid re-intervention and reduce hospitalization<sup>[1,4-5]</sup>.

Palliation is an important goal of esophageal cancer therapy. Current management options for the palliation of dysphagia include: esophageal dilation, intraluminal stents, Nd:YAG laser therapy, photodynamic therapy, argon laser, systemic chemotherapy, external beam radiation therapy, brachytherapy, and combined chemoradiation therapy. The clinical situation, local expertise, and cost effectiveness play an important role in choosing the appropriate treatment modality<sup>[1]</sup>.

Treatment should ensure that the majority of these patients could avoid the consternation of total dysphagia, regardless of which stent is offered<sup>[5]</sup>.

Palliative treatment methods for esophageal and cardiac cancer include dilation, laser vaporization and other thermal methods, alcohol injection, and stent insertion. None of these procedures, however, has proved to be a simple, well-tolerated, and lasting method<sup>[5]</sup>. The aim of this study was to determining the rate of recovery after two methods of palliation in patients with inoperable esophageal carcinoma, in Golestan province, northeast of Iran.

## MATERIALS AND METHODS

This descriptive cross-sectional study was designed in two main and unique clinics of gastroenterology in the province (located in the central and eastern part of Golestan Province) and all inoperable esophageal cancer cases which referred from January 2005 to March 2007 were recruited. A basic checklist was completed for each case before the procedure and their demographic data were registered.

Dysphagia was graded as follows: 0 = able to eat normal diet/no dysphagia; 1 = able to swallow some solid foods; 2 = able to swallow only semi solid foods; 3 = able to swallow liquids only; 4 = unable to swallow anything/total dysphagia<sup>[5]</sup>.

Subjects were followed up every month, and the endpoint was considered as death or finishing the 6-mo period, due to the short survival of them. Improvement in dysphagia was evaluated 1 wk after stent placement and during monthly interviews.

Complications of palliative therapy were defined as major (aspiration, bleeding, stent misplacement or dislocation, perforation) or minor (reflux esophagitis, chest pain, pharyngeal discomfort). Tumor ingrowth or overgrowth was considered a treatment failure<sup>[6]</sup>.

The decrease in dysphagia severity for at least one degree was registered as dysphagia recovery.

After coding data and entering into the computer, improvement proportion was calculated with 95% confidence interval, to determine the rate of improvement. Survival analysis and Kaplan-Meier methods were used to estimate the duration of palliative care effectiveness.

## RESULTS

Thirty and nine cases fulfilled the inclusion criteria. Male to female ratio was 1.6 to 1. Mean age was  $67.5 \pm 13.7$  years. Among these cases, 89.7% were palliated with

**Table 1 Report of dysphagia after palliative care in patients suffering from inoperable esophageal cancer in Golestan province, northeast of Iran**

Dysphagia	Frequency	Percent
Recovery		
The first degree	18	46.2
The second degree	7	17.9
No recovery	4	10.3
Dysphagia aggravation	3	7.7
Not available	7	17.9
Total	39	100

**Table 2 Dysphagia relief after palliative care regarding the different variables in patients suffering from inoperable esophageal cancer in Golestan province, northeast of Iran**

Dysphagia relief	Variables	No recovery		Recovery	
		<i>n</i>	%	<i>n</i>	%
Type of palliative care	Stent	1	25	3	75
	Dilation	13	37.1	22	62.9
	Total	14	35.9	25	64.1
Age (yr)	< 65	3	21.4	11	78.6
	≥ 65	10	40	15	60
Pathology	SCC	13	36.1	23	63.9
	Adenocarcinoma	1	33.3	2	66.7

dilation method, and others ( $n = 3$ ) with stenting. Most of them (92.3%) were diagnosed as having SCC. The middle third of the esophagus was the most (51.3%) involved site.

At the beginning of the study, 22 cases (56.4%) had grade three dysphagia (dysphagia to water) and other 17 had complete or grade four dysphagia.

At the first follow up (one month after procedure), seven cases were not available (died). They passed away between 6-31 d (Table 1).

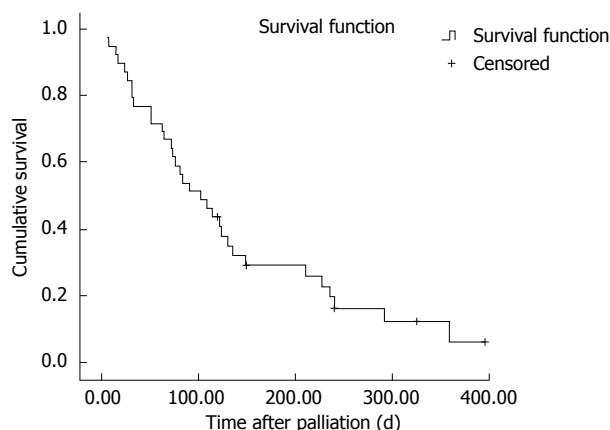
We considered these deaths as non-recovery of dysphagia, and then categorized the cases into two groups: 1, recovered and 2, not recovered (death, dysphagia aggravation or no change in dysphagia) and evaluated the relation between various variables with this condition (Table 2).

No significant relation was seen between relieving dysphagia, and method of palliative care or pathology type of the esophageal cancer ( $P = 0.96$ ). Age of the patients and the dysphagia recovery was not significantly related, too ( $P = 0.238$ ). Mean dysphagia score was significantly improved in the first follow up ( $3.37$  vs  $2.43$ ; 95% CI, 0.62-1.25;  $P < 0.0001$ ).

Among other 32 cases which were available at the first follow up, 25 were free of dysphagia. Survival analysis method was used to estimate the length of dysphagia relieving persistency after palliation. Aggravation of dysphagia at any time was considered as recurrence.

The mean time of persistent recovery was 172.1 d, and the median was 120 d. Among these 25 patients, only in ten cases no recurrence of dysphagia was reported up to the end of follow-up. At the end of 6-mo follow-up, only 6 (15.4%) patients were alive. The overall mean and median survival time was 137.6 d and 103 d after palliation, respectively.

In the recovered group, mean and median survival



**Figure 1** Survival function dysphagia recovery after palliative care by Kaplan-Meier method.

time were 177.1 d and 135 d after palliation, respectively; while it was 60.7 d and 31 d in the other group; respectively. Kaplan-Meier survival analysis and Log-rank used to evaluate the relation between dysphagia relief and the survival rate of the cases and significant differences were reported ( $\chi^2 = 13.21$ ,  $P < 0.0001$ ; Figure 1).

## DISCUSSION

Diagnostic and therapeutic management of esophageal cancer is a multidisciplinary challenge. Male to female ratio in our patients was 1.6 to 1. This proportion is reported in other similar studies<sup>[5]</sup>.

The most involved part of the esophagus was the middle third, and SCC was the most prominent type. While, in Western countries, the distal third is involved most<sup>[2,3]</sup>, maybe the higher incidence of adenocarcinoma, and its potentiality to appear in the distal part can explain this discrepancy. Among 39 recruited cases, 25 (64%) reported a relief in dysphagia. One grade decrease in dysphagia was seen in 46.2% and 2 grade in 17.9%.

In a study in France, a total of 120 patients treated in a single center by insertion of SEMS (Self-expanding Metal Stent), dysphagia scores decreased in 89.1% of patients, with median scores decreasing from 3.0 to 1.0 ( $P < 0.05$ )<sup>[7]</sup>. In the present study, mean dysphagia score in the first follow-up decreased significantly compared to pre-operation (from 3.37 to 2.43). Mean survival time after procedure was 177 d (5.9 mo) in recovered group, and 60.7 d (2 mo) in the other group, which was much lower than reported in other studies<sup>[8-10]</sup>.

In a study in the Netherlands (2006), data from 78 patients, rendered incurable at exploration, and who subsequently underwent palliative interventions, were analyzed retrospectively. Overall, intraluminal stenting was the palliative measure of dysphagia in 25 patients (32.3%). The median survival in the whole group was 8.9 (1-105) mo. Patients treated with chemotherapy had a higher median survival of 11.6 mo compared to that of the other palliatively-treated patients: 8.4 mo ( $P = 0.003$ ). They concluded that patients with incurable oesophageal carcinoma have a poor overall survival of less than 9 mo<sup>[8]</sup>.

In India (2006), thirty patients with inoperable esophageal carcinoma were treated with SEMS. Quality of life score improved significantly from 62-94 before stenting to 80-133 after the procedure. There was improvement in dysphagia grades. Pain was the most common complaint noted on follow up. There was no major morbidity or mortality related to the procedure<sup>[11]</sup>.

In the present study, no complaints were reported immediately after procedure and in the next follow-up, except for the seven cases that reported aggravation or no recovery of dysphagia. One important and disappointing result of this investigation was the high mortality rate of esophageal cancer in our area. Seven deaths occurred between procedures until the first follow-up (one-month later) and at the end of the study, only 6 cases were alive. Ross *et al* (USA, 2007) studied ninety-seven patients with malignant dysphagia who had SEMS placed from 2000 to 2003. Dysphagia scores improved in 86%. Early unexpected deaths occurred in 2 patients. Adenocarcinoma and female sex were factors associated with increased odds of a major complication. Median survival was 77 d<sup>[10]</sup>.

In the present study, dysphagia aggravation and re-dilation was implicated in 7.7%. This is a usual problem seen in all other investigations<sup>[9,11]</sup>. In a study in Norway (2006), 37 patients with unresectable esophageal and cardiac carcinoma treated with SEMS (January 1997-May 2004) were retrospectively analyzed. One patient died the day the stent was introduced. The median time to repeated hospital contact was 25 d, most often due to recurrence of dysphagia. Ten patients underwent repeated stent insertion. The median survival time after the first stent insertion was 88 d<sup>[9]</sup>.

In an Italian report (2007), in 60 cases with malignant dysphagia due to the various etiologies stent insertion was done. The mean dysphagia score of 2.8 improved to a mean score of 1.0 after stenting ( $P < 0.001$ ). Overall median survival time was 4.6 mo<sup>[4]</sup>. In Germany (2007), stent insertion was done in eighteen patients with esophageal carcinoma. Seventeen of 18 stents were placed technically successful in a single endoscopic procedure. Mean dysphagia score improved from 2.2 to 0.6. In 10 patients, a re-intervention was necessary mainly due to dislocation of the stent<sup>[12]</sup>.

Although placement of a stent is technically feasible, its application is hampered by frequent stent migration and insufficient prevention of gastroesophageal reflux. Further technical improvements of stents or alternative methods like brachytherapy are required for satisfactory palliation of malignant gastroesophageal stenosis<sup>[12,13]</sup>.

Comparing dilation and stenting in the present survey showed that dysphagia recovered in 63.6% after dilation and in 75% after stent insertion.

Although, dysphagia relief and median survival rate were lower in our study, maybe due to the delay in referring and the developed stages at the presentation; however, it seems that palliative care is effective in relieving dysphagia of inoperable esophageal carcinoma, and is suggested for increasing quality of life in the



remaining life-span of the patients.

Implantation of stents proved to be an effective and safe method in palliating severe dysphagia in patients with obstructing esophageal cancer<sup>[14-16]</sup>; but dilation seems more popular especially in our area; while stents are more expensive, and dilation is more preferred by patients and physicians.

Larger studies with higher sample size and facilities for screening in the lower dysphagia stages and evaluating other factors that impact on the survival rate of the patients are necessary.

Accurate and expanded results could not be achieved in the present report, due to the unavailability of some data and deaths occurred between the procedure and the first follow-up. Also, all included patients had grade 3 and 4 dysphagia, which can itself have an important impact on survival rate, because of prolonged inability of swallowing and the resulting malnutrition.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Esophageal cancer 5-year survival has slightly increased during past 20 years (5%-9%), but still remains low. Most patients present with locally advanced, unresectable or metastatic disease. At the time of diagnosis, 60% of the patients are only relevant for palliative therapy. As the quality of life, and to some extent the quantity of life remaining to these patients depends to a large degree on their ability to swallow, the relief of dysphagia plays a vital role in the palliation of this disease.

### Research frontiers

The clinical situation, local expertise, and cost effectiveness play an important role in choosing the appropriate treatment modality in these patients. Treatment should ensure that the majority of these patients could avoid the consternation of total dysphagia, regardless to which stent is offered.

### Innovations and breakthroughs

Although, dysphagia relief and median survival rate were lower in our study, maybe due to the delay in referring and developed stages at the presentation time; however, it seems that palliative care is effective in relieving dysphagia of inoperable esophageal carcinoma, and is suggested for increasing quality of life in the remaining life-span of the patients.

### Applications

Implantation of stents proved to be an effective and safe method in palliating severe dysphagia in patients with obstructing esophageal cancer; but dilation seems more popular especially in our area; while stents are more expensive and dilation is more preferred by patients and physicians.

### Peer review

Inoperable esophageal cancer cases face many challenges due to the difficulty in eating. When the tumor is considered not resectable, palliative care would be performed to provide a temporary canal for patients. Our area is placed on the esophageal cancer belt of the world, thus, investigators try to determine different aspects of this cancer in the region and help patients have a better life expectancy. Here, we assessed the outcome of esophageal cancer patients which underwent ballooning and stenting as a palliation for their dysphagia.

## REFERENCES

- 1 Javle M, Ailawadhi S, Yang GY, Nwogu CE, Schiff MD, Nava HR. Palliation of malignant dysphagia in esophageal cancer: a literature-based review. *J Support Oncol* 2006; **4**: 365-373, 379
- 2 Ferrante M, Feliziani M, Imperatori A, Ferraris L, Bernasconi G. Endoscopic palliation of esophageal cancer. *Rays* 2006; **31**: 3-7
- 3 Dahan L, Ries P, Laugier R, Seitz JF. [Palliative endoscopic treatments for esophageal cancers] *Gastroenterol Clin Biol* 2006; **30**: 253-261
- 4 Conigliaro R, Battaglia G, Repici A, De Pretis G, Ghezzi L, Bittinger M, Messmann H, Demarquay JF, Togni M, Bianchi S, Filiberti R, Conio M. Polyflex stents for malignant oesophageal and oesophagogastric stricture: a prospective, multicentric study. *Eur J Gastroenterol Hepatol* 2007; **19**: 195-203
- 5 Tate H. The Palliation of Dysphagia in Oesophageal Malignant Obstructions Using Endoprostheses: A Review of the Literature. Newcastle upon Tyne: Priory Lodge Education Ltd, 2007
- 6 Winkelbauer FW, Schofl R, Niederle B, Wildling R, Thurnher S, Lammer J. Palliative treatment of obstructing esophageal cancer with nitinol stents: value, safety, and long-term results. *AJR Am J Roentgenol* 1996; **166**: 79-84
- 7 Leclaire S, Di Fiore F, Antoniotti M, Ben Soussan E, Hellot MF, Grigioni S, Dechelotte P, Lerebours E, Michel P, Ducrotte P. Undernutrition is predictive of early mortality after palliative self-expanding metal stent insertion in patients with inoperable or recurrent esophageal cancer. *Gastrointest Endosc* 2006; **64**: 479-84
- 8 Pultrum BB, van Westreenen HL, Mulder NH, van Dullemen HM, Plukker JT. Outcome of palliative care regimens in patients with advanced oesophageal cancer detected during explorative surgery. *Anticancer Res* 2006; **26**: 2289-2293
- 9 Tangen M, Andresen SJ, Moum B, Hauge T. [Stent insertion as palliation of cancer in the esophagus and cardia] *Tidsskr Nor Lægeforen* 2006; **126**: 1607-1609
- 10 Ross WA, Alkassab F, Lynch PM, Ayers GD, Ajani J, Lee JH, Bismar M. Evolving role of self-expanding metal stents in the treatment of malignant dysphagia and fistulas. *Gastrointest Endosc* 2007; **65**: 70-76
- 11 Maraju NK, Anbalagan P, Kate V, Ananthakrishnan N. Improvement in dysphagia and quality of life with self-expanding metallic stents in malignant esophageal strictures. *Indian J Gastroenterol* 2006; **25**: 62-65
- 12 Schoppmeyer K, Golsong J, Schiefke I, Mossner J, Caca K. Antireflux stents for palliation of malignant esophagocardial stenosis. *Dis Esophagus* 2007; **20**: 89-93
- 13 Keller R, Flieger D, Fischbach W, Christl SU. Self-expanding metal stents for malignant esophagogastric obstruction: experience with a new design covered nitinol stent. *J Gastrointest Liver Dis* 2007; **16**: 239-243
- 14 Cwikiel W, Tranberg KG, Cwikiel M, Lillo-Gil R. Malignant dysphagia: palliation with esophageal stents--long-term results in 100 patients. *Radiology* 1998; **207**: 513-518
- 15 Carreira Villamor JM, Reyes Perez R, Gorris Gomez E, Pulido-Duque JM, Argiles Vives JM, Pardo Moreno MD, Maynar Moliner M. [Wallstent endoprostheses implanted by fluoroscopic guidance in the palliative treatment of malignant esophageal obstructions and esophago-tracheal fistulas] *Nutr Hosp* 1997; **12**: 141-146
- 16 Carreira JM, Gorris E, Reyes R, Argiles JM, Pulido JM, Pardo MD, Maynar M. [Treatment of dysphagia of malignant origin with the endoprosthesis of Strecker] *Med Clin (Barc)* 1998; **110**: 727-730

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# Honey prevents hepatic damage induced by obstruction of the common bile duct

B Imge Erguder, Sibel S Kilicoglu, Mehmet Namuslu, Bulent Kilicoglu, Erdinc Devrim, Kemal Kismet, Ilker Durak

B Imge Erguder, Mehmet Namuslu, Erdinc Devrim, Ilker Durak, Department of Biochemistry, Ankara University Faculty of Medicine, Ankara 06100, Turkey

Sibel S Kilicoglu, Department of Histology and Embriology, Ufuk University Faculty of Medicine, Ankara 06800, Turkey

Bulent Kilicoglu, Kemal Kismet, Ankara Research and Training Hospital, Clinics of General Surgery, Ankara 06100, Turkey

**Author contributions:** Erguder BI, Devrim E and Namuslu M performed biochemical analysis in this work, Kilicoglu SS performed histological examination, Kilicoglu B removed the tissues surgically, Kismet K analyzed data; and Erguder BI, Durak I, and Kilicoglu B wrote the paper.

**Correspondence to:** B Imge Erguder, Ankara Universities Tıp Fakultesi, Biyokimya Anabilim Dalı Dekanlık Binası, Sıhhiye 06100, Ankara 06100, Turkey. [imgeerguder@yahoo.com](mailto:imgeerguder@yahoo.com)

Telephone: +90-312-3103010 Fax: +90-312-3106370

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**CONCLUSION:** Honey was found to be beneficial in the prevention of hepatic damage due to obstruction of the common bile duct.

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**Key words:** Honey; Obstructive jaundice; Adenosine deaminase; Nitric oxide

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## Abstract

**AIM:** To examine the possible effects of honey supplementation on hepatic damage due to obstruction of the common bile duct in an experimental rat model.

**METHODS:** The study was performed with 30 male rats divided into three groups: a sham group, an obstructive jaundice group, and an obstructive jaundice plus honey group. At the end of the study period, the animals were sacrificed, and levels of nitric oxide (NO), and NO synthase (NOS) activities were measured in liver tissues, and levels of adenosine deaminase (ADA) and alanine transaminase (ALT) activities were measured in serum.

**RESULTS:** Blood ALT and ADA activities were significantly elevated in the jaundice group as compared to those of the sham group. In the obstructive jaundice plus honey group, blood ALT and ADA activities were significantly decreased as compared to those of the jaundice group. In erythrocytes and liver tissues, NO levels were found to be significantly higher in the obstructive jaundice plus honey group compared to those of the sham group. Additionally, NO levels were found to be significantly higher in liver tissues from the animals in the obstructive jaundice plus honey group than those of the jaundice group.

## INTRODUCTION

Honey is produced by honeybees. They obtain nectar from various flowers, and digest it in their bodies, enrich it with their salivary and enzymatic secretions, and put it in honeycombs, so that ripe honey is formed<sup>[1]</sup>. Since ancient times, honey has been known as both flavorful food and a traditional therapeutic material. It has rich flavonoid components, such as luteolin, quercetin, apigenin, fisetin, kaempferol, isorhamnetin, acacetin, tamarixetin, chrysin, and galangin, and therefore, exhibits antioxidant activity. Additionally, honey provides antibacterial, anti-inflammatory, immune-stimulant, anti-ulcer and wound/burn healing (regenerative) effects<sup>[2]</sup>.

Free radicals lead to oxidative damage in many molecules, such as lipids, proteins and nucleic acids. Many complications have been attributed to oxidative damage, including atherosclerosis, aging, and cancerous diseases. Antioxidant foods that are rich in flavonoids are protective agents against these ailments<sup>[3]</sup>.

Obstructive jaundice leads to oxidative injury and inflammation in hepatocytes<sup>[4,5]</sup>. Over production of hydroxyl radicals in blood and liver from rats with obstructive jaundice has been reported<sup>[6]</sup>.

Nitric oxide synthase (NOS) converts arginine to

citrulline and nitric oxide (NO). Nitric oxide leads to activation of guanylyl cyclase, formation of cyclic guanosine 3',5'-monophosphate (cGMP), stimulation of cGMP-protein kinases, and subsequent relaxation in smooth muscle. It has been reported that NOS gene knockout in mice causes an elevation in blood pressure and increased synthesis of cGMP prevents platelet aggregation<sup>[7]</sup>.

Adenosine deaminase (ADA; E.C. 3.5.4.4) is an enzyme that catalyses conversion of adenosine to inosine and ammonia<sup>[8]</sup>. ADA activity has been found to increase in cirrhotic patients, and increased ADA activity has been suggested as a nonspecific hepatic marker for disease with liver damage<sup>[9]</sup>.

Alanine transaminase (ALT; E.C. 2.6.1.1) is a transaminase enzyme that catalyzes the inter-conversion of the amino acid L-alanine to L-glutamate and vice versa. In liver diseases associated with hepatic necrosis, ALT levels characteristically are elevated<sup>[10]</sup>.

In this study, we investigated the effects of honey on the NO pathway and ADA enzyme activity in rats that had induced obstructive jaundice.

## MATERIALS AND METHODS

Thirty male Wistar albino type rats of 12 wk old ( $250 \pm 25$  g in weight) were housed individually in wire cages under constant temperature ( $21^\circ\text{C} \pm 2^\circ\text{C}$ ) with a 12 h light-dark cycle. Twelve hours before anesthesia animals were deprived of food, but had free access to water until 2 h before anesthesia. No enteral or parenteral antibiotics were administered at any time. The animals were divided randomly into 3 groups of 10 rats each: the sham group (group I), the obstructive jaundice group (group II) and the obstructive jaundice plus honey group (group III). The animals were anesthetized by intramuscular injection of 30 mg/kg ketamine hydrochloride (Ketalar; Parke-Davis, Istanbul, Turkey) and 5 mg/kg xylazine (Rompun, Bayer, Istanbul, Turkey). Midline laparotomy was performed under sterile conditions. In group I, the common bile duct (CBD) was freed from the surrounding soft tissue, and was manipulated without ligation and transection. In groups II and III, the CBDs of the rats were identified, double ligated with 5-0 silk, and divided between the ligatures. Group III was nourished with honey 10 mg/kg per day by using a nasogastric tube that was inserted daily and removed after honey supplementation (Balpamak LTD, Istanbul, Turkey). The animals were sacrificed on postoperative day 7 with high-dose diethyl ether inhalation. Subsequently, their liver tissues were removed and blood samples were obtained. The blood samples were put in tubes, and then centrifuged at 2000 g for 5 min. Upper clear supernatant (serum) was taken and used in the enzymatic analyses. The liver tissues were first homogenized in physiologic saline (1 g in 5 mL) and then were centrifuged at 4000 g for 20 min. Upper clear supernatants were removed to use in the analyses. Protein levels of the supernatants were determined using Lowry's method<sup>[11]</sup> were adjusted to equal concentrations

before analyses.

NO levels and NOS enzyme activities were measured in liver tissues, and ADA and ALT enzymes activities were measured in serum.

The level of NO was estimated by the method based on the diazotization of sulfanilic acid by NO at acid pH, and subsequent coupling to N-(1-naphthyl-ethylene diamine) (Griess reaction) as described previously<sup>[12]</sup>. Since nitrate anion does not give a diazotization reaction with sulfanilic acid, the samples were treated by cadmium (a reducing agent) to reduce nitrate anions into nitrite anions before the NO estimation<sup>[13]</sup>. The results were expressed as  $\mu\text{mol}/\text{mg}$  protein. The total NOS activity (IU/mL) method is based on the Griess reaction<sup>[12]</sup>. The results were expressed as IU/mg protein.

Adenosine deaminase activity was studied by the method of Guisti based on spectrophotometric detection of ammonia formation<sup>[8]</sup>. The results were expressed as IU/L.

Serum ALT levels were determined by using a spectrophotometric method<sup>[14]</sup>. The results were expressed as U/L.

The histopathological analyses were carried out in the Histology and Embryology Department of Ankara University School of Medicine. Histopathological examination was performed by using light microscopic analyses. The samples were obtained from the liver and fixed in 10% neutral buffered formalin solution for 2 d. Tissues were washed in flowing water, and were dehydrated with rising concentrations of ethanol (50%, 75%, 96% and 100%). After dehydration, specimens were put into xylene to obtain transparency and were then infiltrated with, and embedded in paraffin. Embedded tissues were cut into sections of 5  $\mu\text{m}$  thicknesses by Leica RM 2125 RT and were then stained with hematoxylin and eosin. Histopathologic examinations were performed and photographed by Nikon Eclipse E 600.

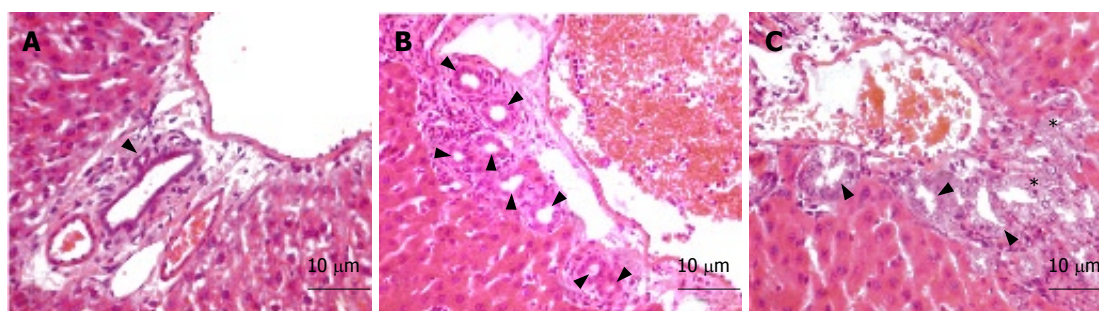
In the statistical evaluation of the results, one-way ANOVA, and post hoc LSD tests were used. *P* values of less than 0.05 were considered as significant.

## RESULTS

The results are given in Table 1. Serum ALT and ADA enzymes activities were significantly elevated in group II compared to those of the sham group. In group III, serum ALT and ADA enzymes activities were found to be significantly decreased compared to those of group II. In liver tissues, NO levels were found to be higher in group III than those of the sham group. Additionally, NO levels were found to be significantly higher in liver tissues of group III compared to those of group II. There were no significant differences between groups for NOS activities.

In group I, there were no morphological alterations in the portal tract and whole liver tissue (Figure 1A). Group II tissues displayed some histopathological changes in the portal tract, such as proliferation of the duct epithelial cells, and looping and reduplication of





**Figure 1** Portal areas of liver biopsies shown for group I (A), group II (B) and group III (C). Bile ducts (arrow heads) and the degenerating and regressing of bile ducts (\*) viewed with HE staining by light microscope.

**Table 1** The biochemical parameters for each group (mean  $\pm$  SD,  $n = 10$ )

Parameters	Group I	Group II	Group III
Serum			
ALT (U/L)	28.98 $\pm$ 7.24 <sup>a</sup>	59.49 $\pm$ 13.42 <sup>c</sup>	17.40 $\pm$ 5.71
ADA (IU/L)	6.13 $\pm$ 4.40 <sup>a</sup>	11.02 $\pm$ 3.14 <sup>c</sup>	6.05 $\pm$ 3.69
Liver			
NO ( $\mu$ mol/mg)	0.281 $\pm$ 0.080 <sup>a</sup>	0.265 $\pm$ 0.065 <sup>c</sup>	0.396 $\pm$ 0.085
NOS (IU/mg)	0.175 $\pm$ 0.031	0.183 $\pm$ 0.048	0.180 $\pm$ 0.032

ADA: Adenosine deaminase; ALT: Alanine transaminase; NO: Nitric oxide; NOS: NO synthase. Group I: Sham operated; Group II: Obstructive jaundice; Group III: Obstructive jaundice + honey. <sup>a</sup> $P < 0.05$  vs II; <sup>c</sup> $P < 0.05$  vs III.

the ducts and ductules. The surrounding hepatocytes were enlarged (Figure 1B). Histopathological evidence showing bile ductule proliferation was markedly reduced in group III. Regression of the bile duct epithelial cells, and phagocytosis of the debris from dying bile duct epithelial cells were observed. We also examined the conspicuous reduction in the size of enlarged hepatocytes (Figure 1C).

## DISCUSSION

Since ancient times, honey has been known to have antibacterial and antioxidant properties due to its phenolic compounds<sup>[3]</sup>. It has been emphasized that honey has a rapid wound healing property<sup>[15]</sup>. Gethin *et al* demonstrated that honey has repairing potential in leg ulceration when wounds are dressed with honey<sup>[16]</sup>. The antimicrobial property of honey on some microbial isolates has also been reported<sup>[17]</sup>. Moreover, the antibacterial effect of honey on ocular flora has been displayed<sup>[18]</sup>. Furthermore, the scolicidal efficacy of propolis, which is a resinous material obtained by honey bees from plants or flowers, has been shown in cystic hydatid disease<sup>[19]</sup>. Recently, it has been found that honey leads to increased levels of NO in biological fluids and to reduced liver enzymes, such as AST and ALT, in blood<sup>[20]</sup>.

In the obstructive jaundice group in our study, elevated serum ALT and ADA activities indicated liver damage. Additionally, reduced serum ALT and ADA activities in the obstructive jaundice plus honey group showed significant improvement in liver tissues.

Histopathological examination also showed damage in the obstructive jaundice group with improvement in the obstructive jaundice plus honey group.

Our results showed the protective potential of honey with liver damage. It is possible that NO levels increased in the liver tissue due to the rich NO content of the honey itself, which is supported by our finding of unchanged NOS activity in liver tissue. Increased NO levels in the obstructive jaundice plus honey group may contribute to the protective result possibly through the elimination of toxic free radicals by NO.

In conclusion, we suggest that honey supplementation may give beneficial results in the prevention of hepatic damage induced by obstruction of the common bile duct.

## COMMENTS

### Background

In liver diseases associated with hepatic necrosis, the enzyme level characteristically is elevated. In this study, it was aimed to investigate the effects of honey on NO pathway, and adenosine deaminase (ADA) enzyme activity in rats which have induced obstructive jaundice.

### Research frontiers

The authors suggest that honey supplementation may give beneficial results to prevent the hepatic damage induced by obstruction of common bile duct.

### Innovations and breakthroughs

This study tries to elucidate possible mechanism for honey supplementation in the hepatic damage induced by obstruction of common bile duct.

### Applications

Histopathological examination was performed by using light microscopic analyses.

### Peer review

This is a valuable study indicating hepatocellular damage in obstructive jaundice group and protective potential of honey in this process. It's a well-designed and important paper.

## REFERENCES

- 1 Naef R, Jaquier A, Velluz A, Bachofen B. From the linden flower to linden honey-volatile constituents of linden nectar, the extract of bee-stomach and ripe honey. *Chem Biodivers* 2004; **1**: 1870-1879
- 2 Fiorani M, Accorsi A, Blasa M, Diamantini G, Piatti E. Flavonoids from italian multifloral honeys reduce the extracellular ferricyanide in human red blood cells. *J Agric Food Chem* 2006; **54**: 8328-8334
- 3 Perez E, Rodriguez-Malaver AJ, Vit P. Antioxidant capacity of Venezuelan honey in wistar rat homogenates. *J Med Food*



- 2006; **9**: 510-516
- 4 **Celebi F**, Yilmaz I, Aksoy H, Gumus M, Taysi S, Oren D. Dehydroepiandrosterone prevents oxidative injury in obstructive jaundice in rats. *J Int Med Res* 2004; **32**: 400-405
- 5 **Takaoka M**, Kubota Y, Tsuji K, Yamamoto S, Ogura M, Yanagitani K, Shimatani M, Shibatani N, Inoue K. Human neutrophil functions in obstructive jaundice. *Hepatogastroenterology* 2001; **48**: 71-75
- 6 **Liu TZ**, Lee KT, Chern CL, Cheng JT, Stern A, Tsai LY. Free radical-triggered hepatic injury of experimental obstructive jaundice of rats involves overproduction of proinflammatory cytokines and enhanced activation of nuclear factor kappaB. *Ann Clin Lab Sci* 2001; **31**: 383-390
- 7 **Murray RK**. Muscle and the cytoskeleton. In: Murray RK, Granner DK, Mayes PA, Rodwell VW. Eds. *Harper's Biochemistry*. Stamford: Appleton & Lange, 2000: 729-730
- 8 **Guisti G**. Enzyme activities. In: Bergmayer UH, ed. *Methods of enzymatic analysis*. Weinheim Bergest: Verlag Chemie, 1974; 1087-1091
- 9 **Fernandez E**, Rodrigo L, Riestra S, Carcia S, Gutierrez F, Ocio G. Adenosine deaminase isoenzymes and neopterin in liver cirrhosis. *J Clin Gastroenterol* 2000; **30**: 181-186
- 10 **Moss DW**, Henderson AR. Clinical Enzymology. In: Burtis CA, Ashwood ER, eds. *Tietz textbook of Clinical Chemistry*. Philadelphia, Pennsylvania: W.B. Saunders Company, 1999: 652-654
- 11 **Lowry O**, Rosebrough N, Farr L, Randall R. Protein measurement with the Folin phenol reagent. *J Biol Chem* 1951; **193**: 265-275
- 12 **Durak I**, Kavutcu M, Kacmaz M, Avci A, Horasanli E, Dikmen B, Cimen MY, Ozturk HS. Effects of isoflurane on nitric oxide metabolism and oxidant status of guinea pig myocardium. *Acta Anaesthesiol Scand* 2001; **45**: 119-122
- 13 **Ridnour LA**, Sim JE, Hayward MA, Wink DA, Martin SM, Buettner GR, Spitz DR. A spectrophotometric method for the direct detection and quantitation of nitric oxide, nitrite, and nitrate in cell culture media. *Anal Biochem* 2000; **281**: 223-229
- 14 **Reitman S**, Frankel S. A colorimetric method for the determination of serum glutamic oxalacetic and glutamic pyruvic transaminases. *Am J Clin Pathol* 1957; **28**: 56-63
- 15 **Henriques A**, Jackson S, Cooper R, Burton N. Free radical production and quenching in honeys with wound healing potential. *J Antimicrob Chemother* 2006; **58**: 773-777
- 16 **Gethin G**, Cowman S. Case series of use of Manuka honey in leg ulceration. *Int Wound J* 2005; **2**: 10-15
- 17 **Al-Waili NS**, Akmal M, Al-Waili FS, Saloom KY, Ali A. The antimicrobial potential of honey from United Arab Emirates on some microbial isolates. *Med Sci Monit* 2005; **11**: BR433-BR438
- 18 **Albietz JM**, Lenton LM. Effect of antibacterial honey on the ocular flora in tear deficiency and meibomian gland disease. *Cornea* 2006; **25**: 1012-1019
- 19 **Kismet K**, Kilicoglu B, Koru O, Tanyuksel M, Oruc MT, Sorkun K, Salih B, Akkus MA. Evaluation on scolicidal efficacy of propolis. *Eur Surg Res* 2006; **38**: 476-481
- 20 **Al-Waili NS**, Saloom KY, Akmal M, Al-Waili F, Al-Waili TN, Al-Waili AN, Ali A. Honey ameliorates influence of hemorrhage and food restriction on renal and hepatic functions, and hematological and biochemical variables. *Int J Food Sci Nutr* 2006; **57**: 353-362

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## Construction and expression of eukaryotic plasmids containing lamivudine-resistant or wild-type strains of Hepatitis B Virus genotype C

Wei-Zhen Xu, Yong Fang, Di Li, Yan Wang, Qing-Long Shang, Gui-Qiu Li, Xu Teng, Hong-Xi Gu

Wei-Zhen Xu, Yong Fang, Di Li, Yan Wang, Qing-Long Shang, Gui-Qiu Li, Xu Teng, Hong-Xi Gu, Department of Microbiology, Harbin Medical University, Harbin 150081, Heilongjiang Province, China

**Author contributions:** Xu WZ and Li D contributed equally to this study; Xu WZ and Li D designed the research, performed the study and wrote the paper; Fang Y, Wang Y and Shang QL performed the study; Li GQ and Teng X analyzed the data; Gu HX was an instructor.

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**Correspondence to:** Hong-Xi Gu, Department of Microbiology, Harbin Medical University, No. 157, Baojian Road, Nangang District, Harbin 150081, Heilongjiang Province, China. [hxgu2432@163.com](mailto:hxgu2432@163.com)

Telephone: +86-451-86685122 Fax: +86-451-86685122

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### Abstract

**AIM:** To construct eukaryotic expression plasmids of full-length Hepatitis B Virus (HBV) genotype C genome, which contain lamivudine-resistant mutants (YIDD, YVDD) or wild-type strain (YMDD), and to observe the expression of HBV DNA and antigens [hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg)] of the recombinant plasmids in HepG2 cells.

**METHODS:** Three HBV full-length genomes were amplified from the plasmids pMD18T-HBV/YIDD, pMD18T-HBV/YVDD and pMD18T-HBV/YMDD, using PCR. Three recombinant plasmids were generated by inserting each of the PCR products into the eukaryotic expression vector pcDNA3.1 (+), between the *EcoRI* and *HindIII* sites. After being characterized by restriction endonuclease digestion, and DNA sequence analysis, the recombinant plasmids were transfected into HepG2 cells. At 48 and 72 h post-transfection, the levels of intracellular viral DNA replication were detected by real-time PCR, and the expression of HBsAg and HBeAg in the cell culture supernatant was determined by ELISA.

**RESULTS:** Restriction endonuclease digestion and DNA sequence analysis confirmed that the three

recombinant plasmids were correctly constructed. After transfecting the plasmids into HepG2 cells, high levels of intracellular viral DNA replication were observed, and HBsAg and HBeAg were secreted into the cell culture supernatant.

**CONCLUSION:** Eukaryotic expression plasmids pcDNA3.1 (+)-HBV/YIDD, pcDNA3.1 (+)-HBV/YVDD or pcDNA3.1 (+)-HBV/YMDD, which contained HBV genotype C full-length genome, were successfully constructed. After transfection into HepG2 cells, the recombinant plasmids efficiently expressed HBV DNA, HBsAg and HBeAg. Our results provide an experimental basis for the further study of HBV lamivudine-resistant mutants.

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**Key words:** Hepatitis B virus; Lamivudine-resistant mutant; Wild-type strain

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### INTRODUCTION

Hepatitis B Virus (HBV) infection has become one of the most serious health problems worldwide. In China, HBV prevalence is especially high. Presently, eight genotypes of HBV (A-H) have been identified, based on divergence over the entire genomic sequence of  $\geq 8\%$ <sup>[1-4]</sup>. Different HBV genotypes have specific geographical distributions<sup>[2,5]</sup>. According to previous studies, genotypes B and C are predominant in China<sup>[6,7]</sup>. In Heilongjiang Province in northern China, HBV genotype C is dominant<sup>[8,9]</sup>.

Lamivudine, a potent, non-toxic inhibitor of HBV replication in chronically infected patients, is currently one of the most effective anti-HBV drugs in the clinic.

Unfortunately, it has been found that long-term use of lamivudine leads to emergence of HBV YMDD mutants, which has been demonstrated to be associated with lamivudine resistance<sup>[10,11]</sup>. In YMDD variants, the methionine of the YMDD motif in HBV polymerase is substituted with either isoleucine, designated as YIDD, or valine, designated as YVDD. Much clinical data has indicated that patients who have developed HBV YMDD mutations show deterioration of their physical condition, and rebound of virus load in their serum<sup>[12-14]</sup>. In this study, we constructed the eukaryotic expression plasmids of HBV genotype C full-length genome, which contained wild-type, YVDD mutation or YIDD mutation, respectively. All these recombinant plasmids were shown to be able to express HBV DNA and antigens *in vitro*.

## MATERIALS AND METHODS

### Materials

Platinum Pfx DNA polymerase, T4 DNA ligase, and Lipofection 2000 reagent were purchased from Invitrogen (Carlsbad, CA, USA). Restriction endonucleases *Eco*RI and *Hind*III were purchased from New England Biolabs (Beijing, China). Dulbecco's modified Eagle's medium (DMEM) was purchased from GIBCO BRL (Gaithersburg, MD, USA). PCR primers were synthesized by Shanghai Sangon Biological Engineering, Technology and Services (Shanghai, China). DNA sequencing was performed by Invitrogen (Beijing, China). Enzyme immunoassay kit was purchased from Shanghai Kehua Biochemical Laboratory (Shanghai, China). Quantitative HBV PCR Fluorogenic Diagnostic Kit was purchased from PG Biotechnology (Nanjing, China). Axygen DNA Mini kit was purchased from Axygen Biosciences (Union City, CA, USA). The recombinant plasmids pMD18T-HBV were constructed in our laboratory. The HBV genotype C full-length genome of wild-type strain or YIDD, YVDD mutants were obtained from serum of chronic HBV patients, and inserted into the vector pMD18T. The expression vector pcDNA3.1 (+), HepG2 cells and *Escherichia coli* DH5 $\alpha$  were maintained in our laboratory.

### Construction of the recombinant plasmids

The HBV full-length genome of wild-type HBV DNA, YVDD or YIDD mutants was amplified from the plasmids pMD18T-HBV, by PCR using a primer set that consisted of a sense primer: 5'TACCATGGCCCTTTTTCACCTCTGCCTAATC-3', and an antisense primer: 5'CGAGCTCTTCAAAAAGTTGCATGGTGCTGG-3'. Amplification was performed for 30 cycles using the Platinum Pfx DNA Polymerase. The PCR hot-start procedure was as follows: 95°C for 6 min, 94°C for 40 s, 68°C for 3 min, plus 1 min after each 10 cycles, and 68°C for 10 min. The *Hind*III/*Eco*RI-digested PCR products were ligated into *Hind*III/*Eco*RI-digested pcDNA3.1 (+) vector using T4 DNA ligase. The recombinant plasmids were then transformed into *Escherichia coli* (*E. coli*) DH5 $\alpha$  and confirmed by restriction endonuclease

digestion and DNA sequence analysis. The sequences were aligned using the Gene Runner version 3.05 (Hastings Software Inc., Hastings, NY, USA).

### Cell culture and transfection

HepG2 cells were cultured in DMEM, supplemented with 10% fetal calf serum, penicillin (100 U/mL) and streptomycin (100 g/mL) at 37°C in a humidified incubator with 5% CO<sub>2</sub>. HepG2 cells in the exponential phase of growth were strictly counted and seeded onto 24-well culture plates with  $1.0 \times 10^5$  cells/well. After 24 h, cells at 80%-90% confluence were transfected with the recombinant plasmids using Lipofection 2000 reagent, following the manufacturer's guidelines. The transfected cells and supernatants were then harvested after 48 or 72 h. Vector pcDNA3.1 (+) was used as a mock transfection control.

### Assays of hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg)

At 48 or 72 h post-transfection, the culture supernatant was collected, centrifuged at 3000 r/min for 5 min to remove cellular debris, and transferred to a clean tube for further analysis. The expression levels of HBsAg and HBeAg were separately assayed using an enzyme immunoassay kit. According to the instructions, a ratio of sample/negative (S/N)  $\geq 2.1$  was considered as a positive response to HBsAg or HBeAg antigen.

### Real-time fluorimetry PCR analysis of HBV DNA

Real-time fluorimetry PCR using TaqMan probe was performed to quantify HBV DNA at 48 or 72 h post-transfection. HBV DNA was extracted from the intracellular core particles using Axygen DNA Mini kit, and then examined by Quantitative HBV PCR Fluorogenic Diagnostic kit. According to the instructions, an HBV DNA level  $\geq 5.0 \times 10^2$  copies/mL was considered as a positive response.

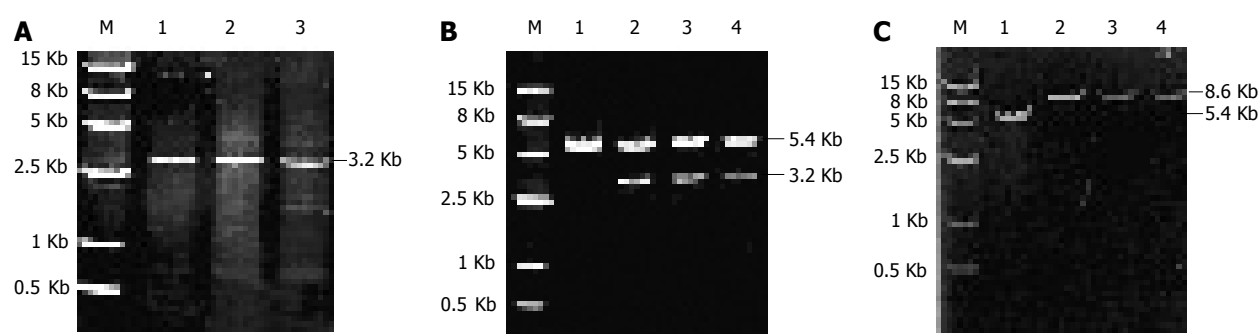
### Statistical analysis

All experiments were performed at least three times. All data were indicated as mean  $\pm$  SD. Data analysis was performed by SPSS 10.0 software (Spss Inc., Chicago, IL, USA).

## RESULTS

### Construction and characterization of recombinant plasmids pcDNA3.1 (+)-HBV/C-YMDD, YIDD or YVDD

As shown in Figure 1A, the PCR products had the expectant molecular weight (3.2 kb). The target genes were cloned to the expression vector pcDNA3.1 (+), and transformed into *E. coli* DH5 $\alpha$ , which generated the reconstructed plasmids pcDNA3.1 (+)/HBV/C-YMDD, YIDD or YVDD. After amplification by *E. coli*, the recombinant plasmids were extracted from the positive clones, and then characterized by digestion with restriction enzymes *Hind*III/*Eco*RI (Figure 1B, lanes 2-4) and *Eco*RI (Figure 1C, lanes 2-4). Vector pcDNA3.1 (+),



**Figure 1** A: Electrophoresis of PCR results of HBV genotype C full-length genomes. M: Marker; lanes 1-3: PCR products of YMDD, YIDD and YVDD, respectively; B: Electrophoresis of digestion with *EcoRI* / *HindIII*. M: marker; lane 1: pcDNA3.1 (+)/*EcoRI* ; lane 2: pcDNA3.1 (+)/HBV-YMDD/*EcoRI* / *HindIII* ; lane 3: pcDNA3.1 (+)/HBV-YIDD/*EcoRI* / *HindIII* ; lane 4: pcDNA3.1 (+)/HBV-YVDD/*EcoRI* / *HindIII* ; C: Electrophoresis of digestion with *EcoRI* . M: marker; lane 1: pcDNA3.1 (+)/*EcoRI* ; lane 2: pcDNA3.1 (+)/HBV-YMDD/*EcoRI* ; lane 3: pcDNA3.1 (+)/HBV-YIDD/*EcoRI* ; lane 4: pcDNA3.1 (+)/HBV-YVDD/*EcoRI* .

used as a negative control, was also digested with *EcoRI*, which yielded a product of approximate 5.4 kb in size (Figure 1B and C, lane 1). The digested products of the recombinant plasmids were visualized on 7 g/L agarose gel (Figure 1B), which demonstrated that recombinant plasmids were digested to 5.4 and 3.2 kb DNA fragments, which corresponded to the lined vector pcDNA3.1 (+) (5.4 kb) and the target gene HBV full-length genome (3.2 kb), respectively. As shown in Figure 1C, the fragment digested from the recombinant plasmids by *EcoRI* was approximate 8.6 kb in size, as expected.

#### DNA sequence analysis of recombinant plasmids

DNA sequence analysis of positive clones confirmed the result. The inserted HBV full-length genome had the correct reading frame and length. Compared with the sequence of the recombinant plasmids that contained wild-type strain (Figure 2A), it was clearly shown that, in the HBV YIDD mutant (Figure 2B), the 741th base G mutated to T, and in the HBV YVDD mutant (Figure 2C), the 739th base A mutated to G. These mutations resulted in replacement of the methionine residue (amino acid 204) by isoleucine (rtM204 I), or valine (rtM204 V), respectively.

#### Extracellular expression of HBsAg and HBeAg

At 48 or 72 h post-transfection, culture supernatants were collected. The expression levels of HBV HBsAg and HBeAg were then detected by ELISA. According to the instructions, an S/N ratio  $\geq 2.1$  was considered as positive HBeAg response. As shown in Table 1, our results indicated that each of the recombinant plasmids could express the antigens, HBsAg and HBeAg in HepG2 cells. The blank control group had a negative HBsAg and HBeAg response.

#### Intracellular expression of HBV DNA

At 48 or 72 h post-transfection, HepG2 cells were harvested and real-time fluorimetry PCR was then performed. As shown in Table 2, the three transfection groups could be considered as positive (all  $\geq 5.0 \times 10^2$  copies/mL), which indicated that HBV DNA was

expressed efficiently. The blank control group was negative.

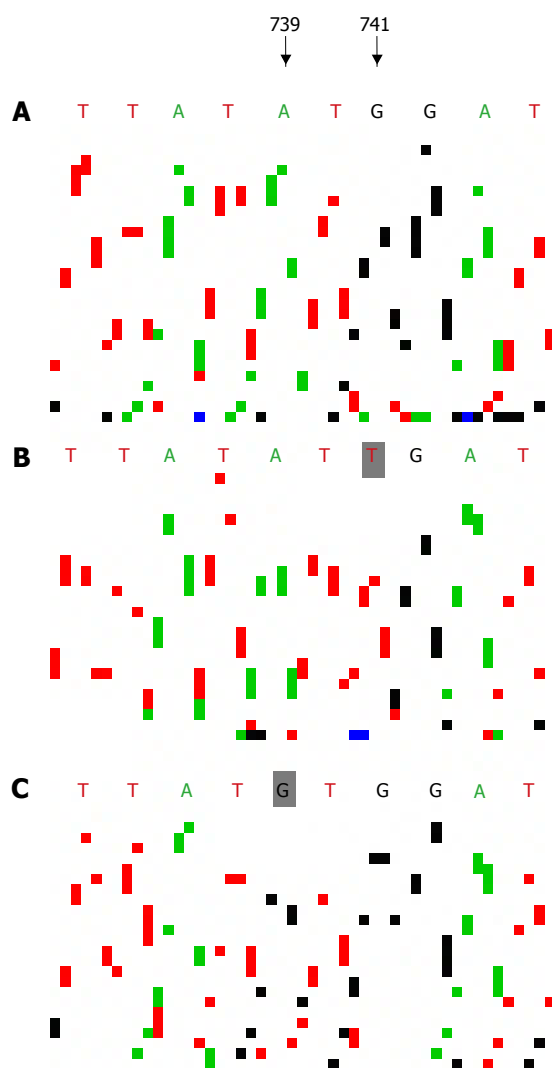
## DISCUSSION

Lamivudine, a potent inhibitor of HBV replication has been the main therapeutic option for treatment of chronic hepatitis B. It functions by interfering with HBV reverse transcriptase activity, and leads to a marked decrease in serum HBV DNA levels, a significant increase in the rate of HBeAg seroconversion, as well as improvement in serum alanine aminotransferase (ALT) levels<sup>[15]</sup> and liver histopathological parameters<sup>[16]</sup>. Several data have revealed that lamivudine can efficiently promote the treatment of hepatitis B in the short term. However, the long-term effectiveness of lamivudine is hampered by the development of viral resistance<sup>[15,17]</sup>. Lamivudine resistance is associated with mutations in the highly conserved YMDD motif of the reverse transcriptase, in which, methionine 204 is replaced by either isoleucine (rtM204 I, YIDD variant) or valine (rtM204 V, YVDD variant).

It has been reported that the rate of HBV YMDD mutation increases with the duration of lamivudine therapy, with an increase from 15% in one year to 38% and 53% after two and three years of treatment, respectively<sup>[18]</sup>. Our previous research has also indicated that in northern China, the YMDD mutation rate is approximate 56.3% after four years of lamivudine treatment<sup>[8]</sup>. YMDD mutations not only result in a reduction in the susceptibility to lamivudine, but also cause virological and biochemical breakthrough, which are represented as rebound of HBV DNA and ALT levels<sup>[19,20]</sup>. Moreover, acute exacerbation of hepatitis and hepatic failure may occur after the emergence of YMDD mutants. Therefore, the antiviral treatment of YMDD mutants has become a crucial issue in the clinic.

HBV genotypes have distinct geographical distributions and are potential factors that affect virus replication, virus variation, clinical course, and therapy of HBV infection. In northern China, genotype C is predominant, and accounts for 77%-88% of cases of chronic hepatitis B<sup>[6,8]</sup>. Sugiyama reported that the





**Figure 2** Sequence analysis of wild-type strain and YMDD mutants (YIDD, YVDD). **A:** Wild-type strain: 739th base is A; 741th base is G. **B:** YIDD mutant: 741th base G mutated to T. **C:** YVDD mutant: 739th base A mutated to G.

replication capacity of HBV in transfected Huh7 cells varied among genotype A and B, as well as C and D, with genotype C having the highest replication capacity<sup>[21]</sup>. HBV genotype C is associated with more severe histological liver damage and low-grade responses to interferon therapy<sup>[22]</sup>. Moreover, patients with genotype C show poor responses to embolization therapy and may die from hepatic failure because of rapid hepatocellular carcinoma (HCC) progression<sup>[23]</sup>. Another study has reported that HBV genotype C has more rapid selection of lamivudine resistance than genotype B<sup>[17]</sup>. Therefore, further studies of HBV YMDD mutants with genotype C are of great significance.

To date, many *in vitro* studies on lamivudine resistance have been reported<sup>[24-27]</sup>. In most of these, recombinant plasmids containing HBV full-length or fragment genome were constructed first, and then expressed in liver-derived cell lines. For example, Gunther *et al* have reported an original and efficient method of amplifying full-length HBV genomes by PCR<sup>[24]</sup>. Chen *et al* have described a

**Table 1** HBsAg and HBeAg in transfected HepG2 cells determined by ELISA

Clone	HBsAg (Sample/Negative)		HBeAg (Sample/Negative)	
	48 h	72 h	48 h	72 h
pcDNA3.1 (+)	0.33 ± 0.028	0.37 ± 0.094	0.39 ± 0.046	0.38 ± 0.050
pcDNA3.1 (+)-HBV/C YMDD	3.14 ± 0.069	3.47 ± 0.413	8.72 ± 0.059	8.77 ± 0.256
pcDNA3.1 (+)-HBV/C YIDD	6.77 ± 0.099	8.26 ± 0.334	2.06 ± 0.318	2.18 ± 0.028
pcDNA3.1 (+)-HBV/C YVDD	10.30 ± 0.065	10.37 ± 0.205	5.03 ± 0.132	5.30 ± 0.117

The ratio of sample/negative (S/N) ≥ 2.1 was considered as positive HBsAg and HBeAg response.

**Table 2** Real-time PCR detection of HBV DNA in transfected HepG2 cells (× 10<sup>8</sup> copies/mL)

Clone	48 h	72 h
pcDNA3.1 (+) <sup>1</sup>	-	-
pcDNA3.1 (+)-HBV/C YMDD	3.57 ± 0.084	3.80 ± 0.078
pcDNA3.1 (+)-HBV/C YIDD	6.85 ± 0.143	6.90 ± 0.038
pcDNA3.1 (+)-HBV/C YVDD	17.64 ± 0.240	18.55 ± 0.127

The HBV DNA levels ≥ 5 × 10<sup>2</sup> copies/mL was considered as positive HBV DNA response. <sup>1</sup>The results were negative.

method of constructing baculovirus recombinants that contain multiple HBV lamivudine-resistant mutations, introduced by successive rounds of site-directed mutagenesis in laboratory strains<sup>[25]</sup>. However, in all these studies, either one type of HBV YMDD mutant or wild-type strains was included in the plasmids without specification of HBV genotype. Therefore, to date, serial plasmids that contain a specific HBV genotype, such as genotype C, and lamivudine-resistant sequences, which allow systematic studies on the combined effects of HBV genotype together with lamivudine-resistant mutations, have not been reported.

In this study, we successfully constructed a series of eukaryotic expression plasmids that contained genotype C HBV strain with either wild-type, YVDD or YIDD mutation, namely the plasmids pcDNA3.1 (+)-HBV/C-YMDD, pcDNA3.1 (+)-HBV/C-YVDD and pcDNA3.1 s(+)-HBV/C-YIDD, respectively. In order to achieve high-level expression *in vitro*, the Kozak sequence, ACCATGGCC-which has been found to contribute to the fidelity and efficiency of initiation and expression<sup>[28]</sup>-was coupled to the 5' end of the sense primer. Moreover, to further assure high fidelity, the PCR analyses were performed following a hot-start protocol and using high-fidelity enzymes. After transfecting the constructed plasmids into HepG2 cells, we analyzed the expression levels of HBsAg and HBeAg by ELISA, and the replication level of HBV DNA by real-time PCR. It was found that both HBV DNA and the antigens were expressed in the transfected cells, but not in the negative control cells transfected with pcDNA3.1 (+). As shown in Table 1, all the recombinant plasmids could express HBsAg in HepG2 cells. At 48 and 72 h, the expression levels of HBeAg were 8.723 ± 0.0585 and 8.77 ± 0.256,

respectively, in YMDD strains and  $5.03 \pm 0.132$  and  $5.3 \pm 0.117$  in YVDD mutants. However, HBeAg expression levels in YIDD mutants were only  $2.06 \pm 0.318$  and  $2.18 \pm 0.028$  at 48 and 72 h, respectively. This difference was probably caused by the emergence of BCP mutations (A1762T/G1764A) in YIDD mutants, while this mutation was not present in YVDD mutants. Our observation is consistent with previous reports that BCP mutation can result in a decrease in HBeAg levels<sup>[29]</sup>. In addition, HBV DNA expression levels of each of the recombination plasmids were  $\geq 10^8$  copies/mL in HepG2 cells (Table 2), which indicates that the three recombinant plasmids can be expressed efficiently. Successful construction of the three eukaryotic plasmids pcDNA3.1 (+)-HBV/C-YMDD, pcDNA3.1 (+)-HBV/C-YVDD and pcDNA3.1 (+)-HBV/C-YIDD, provides an experimental basis for the establishment of stable expression system of HBV genotype C lamivudine-resistant mutants. The results may contribute to future *in vitro* antiviral studies of HBV genotype C lamivudine-resistant mutants.

## ACKNOWLEDGMENT

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## COMMENTS

### Background

HBV infection remains a major public health problem worldwide. Lamivudine is currently one of the most effective anti-hepatitis B virus (HBV) drugs in use clinically. However, the long-term use of lamivudine leads to the emergence of lamivudine-resistant mutants (YMDD mutants). It was reported that the rate of YMDD mutations was up to 70% after three years of treatment. The development of YMDD mutants has hampered anti-HBV therapy.

### Research frontiers

*In vivo* and *in vitro* studies on the HBV drug-resistance mechanism have been of great interest. *In vivo* studies have mainly focused on the rate, types and detection method of YMDD mutation. However, there is still little known about the effects of YMDD mutations *in vitro*.

### Innovations and breakthroughs

Appropriate and effective eukaryotic expression plasmids that are able to efficiently express HBV DNA and antigens are necessary for further *in vitro* investigations. However, to date, serial plasmids that contain a specific HBV genotype, such as genotype C, and a certain lamivudine-resistance mutation, which allow systematic studies of the combined effects of HBV genotype, together with lamivudine-resistance mutations, have not been reported. In this study, authors successfully constructed eukaryotic expression plasmids pcDNA3.1 (+)-HBV/C-YMDD, pcDNA3.1 (+)-HBV/C-YVDD and pcDNA3.1 (+)-HBV/C-YIDD, which contained genotype C HBV strain with either wild-type, YVDD or YIDD mutations, respectively, and had the ability to express HBV DNA and antigens *in vitro* with a high capacity.

### Applications

The successful construction of three eukaryotic plasmids, pcDNA3.1 (+)-HBV/C-YMDD, pcDNA3.1 (+)-HBV/C-YVDD and pcDNA3.1 (+)-HBV/C-YIDD, provides an experimental basis for the establishment of a stable expression system of HBV genotype C lamivudine-resistant mutants. The results may contribute to further *in vitro* antiviral studies of HBV genotype C lamivudine-resistant mutants. This could include establishing a stable expression system for HBV genotype C lamivudine-resistant mutants for studying the mechanism of HBV lamivudine resistance.

### Terminology

HBV genotype C is predominant in China, and is associated with more severe

histological liver damage, lower response to anti-HBV treatment, and more rapid development of lamivudine resistance.

### Peer review

The paper describes a technique for constructing eukaryotic expression plasmids of HBV genotype C with lamivudine-resistant mutants. This is an interesting topic and the manuscript is well written.

## REFERENCES

- 1 Miyakawa Y, Mizokami M. Classifying hepatitis B virus genotypes. *Intervirology* 2003; **46**: 329-338
- 2 Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF, Rossau R. A new genotype of hepatitis B virus: complete genome and phylogenetic relatedness. *J Gen Virol* 2000; **81**: 67-74
- 3 Kao JH, Chen PJ, Lai MY, Chen DS. Genotypes and clinical phenotypes of hepatitis B virus in patients with chronic hepatitis B virus infection. *J Clin Microbiol* 2002; **40**: 1207-1209
- 4 Arauz-Ruiz P, Norder H, Robertson BH, Magnus LO. Genotype H: a new Amerindian genotype of hepatitis B virus revealed in Central America. *J Gen Virol* 2002; **83**: 2059-2073
- 5 Jazayeri M, Basuni AA, Sran N, Gish R, Cooksley G, Locarnini S, Carman WF. HBV core sequence: definition of genotype-specific variability and correlation with geographical origin. *J Viral Hepat* 2004; **11**: 488-501
- 6 Gu HX, Xu ZL, Liu JY, Zhong ZH, Wang HQ, Zhang SY, Li D, Zhang HH, Abe K. Epidemiology of HBV genotypes by nested PCR with multi-paired primers. *Shijie Huaren Xiaohua Zazhi* 2004; **12**: 1073-1076
- 7 Ding X, Mizokami M, Yao G, Xu B, Orito E, Ueda R, Nakanishi M. Hepatitis B virus genotype distribution among chronic hepatitis B virus carriers in Shanghai, China. *Intervirology* 2001; **44**: 43-47
- 8 Li D, Gu HX, Zhang SY, Zhong ZH, Zhuang M, Hattori T. YMDD mutations and genotypes of hepatitis B virus in northern China. *Jpn J Infect Dis* 2006; **59**: 42-45
- 9 Ding X, Gu H, Zhong ZH, Zilong X, Tran HT, Iwaki Y, Li TC, Sata T, Abe K. Molecular epidemiology of hepatitis viruses and genotypic distribution of hepatitis B and C viruses in Harbin, China. *Jpn J Infect Dis* 2003; **56**: 19-22
- 10 Allen MI, Deslauriers M, Andrews CW, Tipples GA, Walters KA, Tyrrell DL, Brown N, Condreay LD. Identification and characterization of mutations in hepatitis B virus resistant to lamivudine. Lamivudine Clinical Investigation Group. *Hepatology* 1998; **27**: 1670-1677
- 11 Stuyver L, Van Geyt C, De Gendt S, Van Reybroeck G, Zoulim F, Leroux-Roels G, Rossau R. Line probe assay for monitoring drug resistance in hepatitis B virus-infected patients during antiviral therapy. *J Clin Microbiol* 2000; **38**: 702-707
- 12 Sun J, Wang Z, Ma S, Zeng G, Zhou Z, Luo K, Hou J. Clinical and virological characteristics of lamivudine resistance in chronic hepatitis B patients: a single center experience. *J Med Virol* 2005; **75**: 391-398
- 13 Pallier C, Castera L, Soulier A, Hezode C, Nordmann P, Dhumeaux D, Pawlotsky JM. Dynamics of hepatitis B virus resistance to lamivudine. *J Virol* 2006; **80**: 643-653
- 14 Suzuki Y, Yotsuyanagi H, Okuse C, Nagase Y, Takahashi H, Moriya K, Suzuki M, Koike K, Iino S, Itoh F. Fatal liver failure caused by reactivation of lamivudine-resistant hepatitis B virus: a case report. *World J Gastroenterol* 2007; **13**: 964-969
- 15 Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; **339**: 61-68
- 16 Dienstag JL, Goldin RD, Heathcote EJ, Hann HW, Woessner M, Stephenson SL, Gardner S, Gray DF, Schiff ER. Histological outcome during long-term lamivudine therapy.

- Gastroenterology* 2003; **124**: 105-117
- 17 **Pan XP**, Li LJ, Du WB, Li MW, Cao HC, Sheng JF. Differences of YMDD mutational patterns, precore/core promoter mutations, serum HBV DNA levels in lamivudine-resistant hepatitis B genotypes B and C. *J Viral Hepat* 2007; **14**: 767-774
- 18 **Leung NW**, Lai CL, Chang TT, Guan R, Lee CM, Ng KY, Lim SG, Wu PC, Dent JC, Edmundson S, Condeelis LD, Chien RN. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. *Hepatology* 2001; **33**: 1527-1532
- 19 **Yuen MF**, Sablon E, Hui CK, Yuan HJ, Decraemer H, Lai CL. Factors associated with hepatitis B virus DNA breakthrough in patients receiving prolonged lamivudine therapy. *Hepatology* 2001; **34**: 785-791
- 20 **Wang JH**, Lu SN, Lee CM, Lee JF, Chou YP. Fatal hepatic failure after emergence of the hepatitis B virus mutant during lamivudine therapy in a patient with liver cirrhosis. *Scand J Gastroenterol* 2002; **37**: 366-369
- 21 **Sugiyama M**, Tanaka Y, Kato T, Orito E, Ito K, Acharya SK, Gish RG, Kramvis A, Shimada T, Izumi N, Kaito M, Miyakawa Y, Mizokami M. Influence of hepatitis B virus genotypes on the intra-and extracellular expression of viral DNA and antigens. *Hepatology* 2006; **44**: 915-924
- 22 **Wai CT**, Chu CJ, Hussain M, Lok AS. HBV genotype B is associated with better response to interferon therapy in HBeAg (+) chronic hepatitis than genotype C. *Hepatology* 2002; **36**: 1425-1430
- 23 **Tsubota A**, Arase Y, Ren F, Tanaka H, Ikeda K, Kumada H. Genotype may correlate with liver carcinogenesis and tumor characteristics in cirrhotic patients infected with hepatitis B virus subtype adw. *J Med Virol* 2001; **65**: 257-265
- 24 **Gunther S**, Li BC, Miska S, Kruger DH, Meisel H, Will H. A novel method for efficient amplification of whole hepatitis B virus genomes permits rapid functional analysis and reveals deletion mutants in immunosuppressed patients. *J Virol* 1995; **69**: 5437-5444
- 25 **Chen RY**, Edwards R, Shaw T, Colledge D, Delaney WE 4th, Isom H, Bowden S, Desmond P, Locarnini SA. Effect of the G1896A precore mutation on drug sensitivity and replication yield of lamivudine-resistant HBV *in vitro*. *Hepatology* 2003; **37**: 27-35
- 26 **Sun D**, Nassal M. Stable HepG2- and Huh7-based human hepatoma cell lines for efficient regulated expression of infectious hepatitis B virus. *J Hepatol* 2006; **45**: 636-645
- 27 **Brunelle MN**, Jacquard AC, Pichoud C, Durantel D, Carrouee-Durantel S, Villeneuve JP, Trepo C, Zoulim F. Susceptibility to antivirals of a human HBV strain with mutations conferring resistance to both lamivudine and adefovir. *Hepatology* 2005; **41**: 1391-1398
- 28 **Kozak M**. An analysis of vertebrate mRNA sequences: intimations of translational control. *J Cell Biol* 1991; **115**: 887-903
- 29 **Parekh S**, Zoulim F, Ahn SH, Tsai A, Li J, Kawai S, Khan N, Trepo C, Wands J, Tong S. Genome replication, virion secretion, and e antigen expression of naturally occurring hepatitis B virus core promoter mutants. *J Virol* 2003; **77**: 6601-6612

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## Reduced expression of P120 catenin in cholangiocarcinoma correlated with tumor clinicopathologic parameters

Bo Zhai, He-Xin Yan, Shu-Qin Liu, Lei Chen, Meng-Chao Wu, Hong-Yang Wang

Bo Zhai, Department of Ultrasonic Intervention, Eastern Hepatobiliary Surgery Hospital, Second Military Medical University, Shanghai 200438, China

He-Xin Yan, Shu-Qin Liu, Lei Chen, Meng-Chao Wu, Hong-Yang Wang, International Cooperation Laboratory on Signal Transduction, Eastern Hepatobiliary Surgery Institute, Second Military Medical University, Shanghai 200438, China

Author contributions: Zhai B wrote the paper and organized the figures and patient data, Yan HX and Liu SQ did the immunohistochemical staining assays; Chen L carried out the statistical analysis; Wu MC and Wang HY helped write, organize, and correct the paper; Wang HY supervised the writing and organization process.

Correspondence to: Hong-Yang Wang, International Cooperation Laboratory on Signal Transduction, Eastern Hepatobiliary Surgery Institute, Second Military Medical University, Shanghai 200438, China. [hywangk@online.sh.cn](mailto:hywangk@online.sh.cn)  
Telephone: +86-21-25070846 Fax: +86-21-65566851

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for predicting tumor invasion, metastasis and patients' survival, but only P120 is an independent prognostic factor for ICC.

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**Key words:** P120; Intrahepatic cholangiocarcinoma; Clinicopathologic feature; Invasion and metastasis; Survival

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### Abstract

**AIM:** To investigate the relationship between the expression of P120 and the clinicopathologic parameters in intrahepatic cholangiocarcinoma (ICC).

**METHODS:** An immunohistochemical study of E-cadherin and P120 catenin was performed on 42 specimens of ICC with a Dako Envision kit.

**RESULTS:** The expression of E-cadherin and P120 was reduced in 27 cases (64.3%) and 31 cases (73.8%), respectively. Both E-cadherin and P120 expressions were significantly correlated with the tumor histological grade ( $\chi^2 = 9.333, P = 0.009$  and  $\chi^2 = 11.71, P = 0.003$ ), TNM stage ( $\chi^2 = 8.627, P = 0.035$  and  $\chi^2 = 13.123, P = 0.004$ ), intrahepatic metastasis ( $\chi^2 = 7.292, P = 0.007$  and  $\chi^2 = 4.657, P = 0.041$ , respectively) and patients' survival ( $\chi^2 = 6.351, P = 0.002$  and  $\chi^2 = 4.023, P = 0.000$ , respectively). In addition, the expression of P120 was in concordance with that of E-cadherin ( $\chi^2 = 13.797, P = 0.000$ ), indicating that the expression of P120 may be dependent on that of E-cadherin. Finally, only P120 expression was found to be an independent prognostic factor in Cox regression model ( $r = 0.088, P = 0.049$ ).

**CONCLUSION:** Down-regulated expression of E-cadherin and P120 occurs frequently in ICC and contributes to the progression and development of tumor. Both of them may be valuable biologic markers

### INTRODUCTION

P120-catenin is a member of the Armadillo (ARM)/ $\beta$ -catenin gene family and is essential for mesenchymal cadherin-mediated regulation of cell motility and invasiveness<sup>[1,2]</sup>. Cadherin, one of the transmembrane cell-cell adhesion receptors involved in development, and morphogenesis of ICC<sup>[3]</sup>, is necessary and sufficient for P120 targeting cell-cell junctions.

A main function of P120 is to stabilize cadherins at the cell membrane by regulating cadherin turnover and degradation. In this way, P120 level acts as a set point mechanism underlying cell-cell adhesive interactions. P120 may function as a "cap" to bind to the cadherin cytoplasmic tail and prevent cadherin interactions with endocytic membrane trafficking machinery. Alternatively, P120 may stabilize cell junctions or regulate membrane trafficking machinery through interactions with small GTPases, such as Rho A, Rac and Cdc42. Through these mechanisms, P120 exerts its influence over a wide range of biological processes that are dependent upon tight regulation of cell surface cadherin levels<sup>[4]</sup>.

Intrahepatic cholangiocarcinoma (ICC) is the second most common tumor of primary liver cancers in adults worldwide, accounting for about 15% of liver cancers, and its incidence has increased in recent years<sup>[5]</sup>. Despite



improved diagnostic and operative techniques, the prognosis of ICC remains poor. In addition, molecular events involving the development of ICC are not well understood. Some studies examined the expression of E-cadherin/catenin complex in ICC, but the conclusion is still controversial. Moreover, to our knowledge, no study has demonstrated the expression characteristics of P120 and the relationship between the expression of P120, and the clinicopathologic parameters in ICC. Therefore, in the present study, we used immunohistochemical staining for the E-cadherin/P120 complex in primary ICC to correlate its expression with its clinicopathologic features.

## MATERIALS AND METHODS

### *Selection of patients and definition of clinicopathologic parameters*

In this study, we selected 42 specimens of intrahepatic cholangiocarcinoma collected and diagnosed at the Eastern Hepatobiliary Surgery Hospital, the Second Military Medical University from October 1997 to March 2004. The patients were consisted of 32 men and 10 women. Their age ranged from 27 to 73 years, with an average age of 51 years. Cancer tissue and non-tumorous liver tissue were obtained from each patient for pathological examination. The detailed pathologic data were obtained from the Department of Pathology of Eastern Hepatobiliary Surgery Hospital. Background liver showed cirrhosis in 19 (45.2%) patients, and chronic hepatitis in 15 (35.7%) patients.

Clinicopathologic parameters included histological grade, pTNM stage, tumor size, capsular and vascular invasion, satellite nodules, intrahepatic metastasis, lymph node status and patients' survival. Histological grade of ICC was sub-classified into well, moderately and poorly differentiated ICC based on the criteria for a liver cancer study in Japan<sup>[6]</sup>. Tumor staging was performed according to the pTNM staging system of the International Union against Cancer UICC<sup>[7]</sup>.

### *Immunohistochemistry*

Formalin-fixed and paraffin-embedded tissues were cut to 5- $\mu$ m thick sections. Immunohistochemical staining for E-cadherin and P120 was performed with a Dako EnVision™ kit (Dakocytomation Company, Denmark). The sections were dewaxed, incubated with methanol containing 30% H<sub>2</sub>O<sub>2</sub> for 20 min to block endogenous peroxidase activity, immersed in 0.01 mol/L citrate buffer (pH 6.0), heated at 100°C in a microwave oven for 20 min, washed three times with distilled water and blocked with 1% BSA for 30 min. The sections were then incubated overnight at 4°C with rabbit polyclonal IgG of E-cadherin (H-297, SC-7870, Santa Clauze Corporation, USA) and rabbit polyclonal IgG of P120 (H-90, SC-13957, Santa Clauze Corporation, USA) at a 1:200 dilution. A subsequent reaction was carried out using second antibodies (Dakocytomation Company, Denmark) at 37°C for 30 min. Then, the sections were washed three times with

phosphate-buffered saline (PBS) and subsequently the color was displayed with DAB (Dakocytomation Company, Denmark) for about 5 min. Nuclei were lightly counterstained with hematoxylin. No staining was obtained when immune serum or PBS was used instead of primary antibodies, thus confirming the specificity of each primary antibody.

### *Evaluation of immunostaining*

A scoring system was used to semiquantitatively evaluate the immunoexpression of E-cadherin and P120 in ICC as described previously<sup>[8]</sup>. The expression of E-cadherin and P120 in nontumorous tissue was used as an internal control. Briefly, immune activities of E-cadherin and P120 were assessed by the extent (broadness) and intensity (color strength). Depending on the percentage of positive cells, the extent was scored as follows: 0 = no positive cells or less than 5%, +1 = 5%-25% positive cells, +2 = 26%-50% positive cells, +3 = 51%-75% positive cells, and +4 = 76%-100% positive cells. The intensity was also scored as follows: 0 = no immunoreaction, +1 = mild immunoreaction, +2 = moderate immunoreaction, +3 = marked immunoreaction. E-cadherin or P120 expression was defined as positive when the composite score was 6 or 7, and as "absent or loss" when the total score was 0.

### *Statistical analysis*

Results from immunohistochemistry were analyzed by  $\chi^2$  or Fisher's exact test.  $P < 0.05$  was considered statistically significant. Survival analysis was performed using the log-rank test ( $P < 0.05$ ). Survival curves were plotted according to the method of Kaplan and Meier. The prognosis value of E-cadherin and P120 for ICC was evaluated with univariate (log-rank test) and multivariate analysis (Cox regression model). SPSS 10.1 software package for Windows (SPSS, Inc., Chicago, IL) was used.

## RESULTS

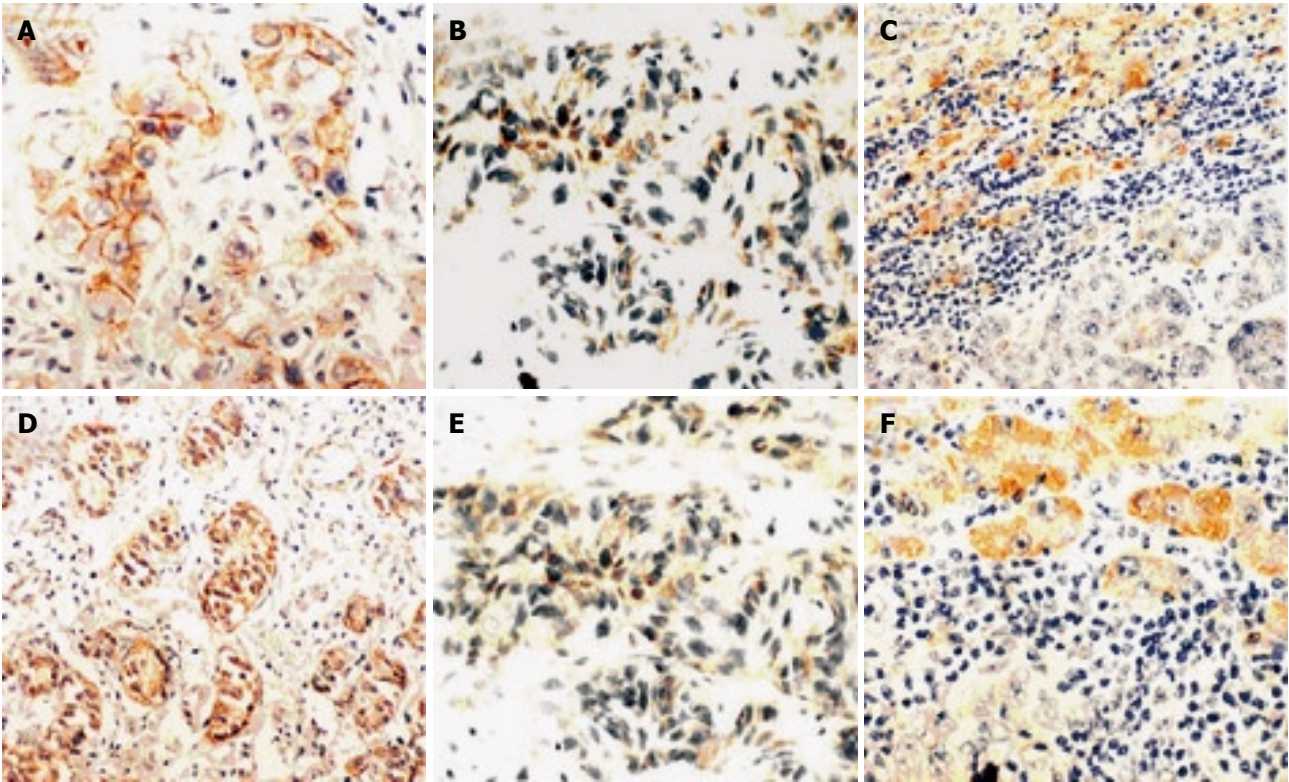
### *Observation under microscope*

In nontumorous liver tissue, both E-cadherin and P120 were expressed strongly on cell membranes, but the staining intensity was gradually decreased. In addition, these molecules were normally expressed on cell membranes of bile ducts, proliferating ductules and intra-hepatic vessels. No expression was found in other types of cells in the liver.

In ICC, the expression of E-cadherin and P120 catenin was reduced in 27 (64.3%) and 31 cases (73.8%), being absent in 8 and 10 cases, respectively. In addition, P120 was expressed in 17 cases (40.5%) (Figure 1).

### *Relationship between expression of E-cadherin/P120 and histological features of ICC*

As shown in Table 1, the membranous expression of E-cadherin and P120 was significantly correlated with the tumor grade ( $P = 0.009$  and  $P = 0.003$ , respectively).



**Figure 1** Immunoreactivity of E-cadherin and P120 in intrahepatic cholangiocarcinomas. "Preserved type"(+) (A, D), "reduced type"(-) (B, E), and "complete absent" (C, F) of E-cadherin and P120 induced type"(-) and staining, respectively ( $\times 200$ ).

Table 1 Relationship between expressions of E-cadherin/P120 catenin and histological features of ICC <i>n</i> (%)							
	<i>n</i>	E-cadherin			P120 catenin		
		+	-	<i>P</i> value	+	-	<i>P</i> value
Differentiation grade							
Well	3	3 (100)	0 (0)	0.009	3 (100)	0 (0)	0.003
Mediate	14	7 (50.0)	7 (50.0)		5 (35.7)	9 (64.3)	
Poor	25	5 (20.0)	20 (80.0)		3 (12.0)	22 (88.0)	
pTNM							
I	2	2 (100)	0 (0)	0.035	2 (100)	0 (0)	0.004
II	9	5 (55.6)	4 (44.4)		5 (55.6)	4 (44.4)	
III	25	8 (32.0)	17 (68.0)		4 (16.0)	21 (84.0)	
IV	6	0 (0)	6 (100)		0 (0)	6 (100)	

The expression of E-cadherin and P120 tended to be reduced in poorly-differentiated tumors compared with well- and moderately-differentiated tumors. In addition, the expression of E-cadherin and P120 was inversely associated with the pTNM stage of tumors ( $P = 0.035$  and  $P = 0.004$ , respectively).

**Relationship between expression of E-cadherin and P120 and clinical parameters of ICCs**

As shown in Table 2, the expression of E-cadherin or P120 was significantly associated with intra-hepatic metastasis of ICC ( $P = 0.007$  and  $P = 0.041$ , respectively). No statistically significant difference was observed between the expression level of E-cadherin or P120 and tumor size, capsular and vascular invasion, lymph node permission and satellite nodules.

**Relationship between expressions of E-cadherin and P120 in ICC**

As shown in Table 3, positive and negative expression of E-cadherin and P120 was found in 9 and 25 cases, respectively. However, negative expression of P120 was observed in 7 cases. There was a significant concordance between the expressions of E-cadherin and P120 ( $P = 0.000$ ).

**Relationship between expression of E-cadherin/P120 and survival of ICC patients**

The patients were followed up for 4-67 months. The overall survival rate of patients according to the expression of E-cadherin and P120 in tumor is shown in Figure 2. Analysis of the survival of all patients showed that abnormal expression of E-cadherin and P120 was

**Table 2** Relationship between expressions of E-cadherin/P120 catenin and clinical parameters of ICC  
*n* (%)

	<i>n</i>	E-cadherin			P120 catenin		
		+	-	<i>P</i> value	+	-	<i>P</i> value
Size							
< 5 cm	17	7 (41.2)	10 (58.8)	0.826	6 (35.3)	11 (64.7)	0.584
5-10 cm	16	5 (31.3)	11 (68.7)		3 (18.8)	12 (81.2)	
> 10 cm	9	3 (33.3)	6 (66.7)		2 (22.2)	7 (77.8)	
Capsular invasion							
+	6	4 (66.7)	2 (33.3)	0.164	3 (50)	3 (50)	0.391
-	36	11 (30.6)	25 (69.4)		8 (22.2)	28 (77.8)	
Satellite nodules							
+	11	4 (36.4)	7 (65.6)	1	3 (27.3)	8 (72.7)	0.314
-	31	11 (35.5)	20 (64.5)		8 (25.8)	23 (74.2)	
Vascular invasion							
+	13	2 (15.4)	11 (84.6)	0.089	1 (7.7)	12 (92.3)	0.127
-	29	13 (44.8)	16 (55.2)		10 (34.5)	19 (65.5)	
L.N.P							
+	7	1 (14.3)	6 (85.7)	0.39	0 (0)	7 (100)	0.161
-	35	14 (40)	21 (60)		11 (31.4)	24 (68.6)	
I.M.							
+	10	0 (0)	10 (100)	0.007	0 (0)	10 (100)	0.041
-	32	15 (46.9)	17 (53.1)		11 (34.4)	21 (65.6)	

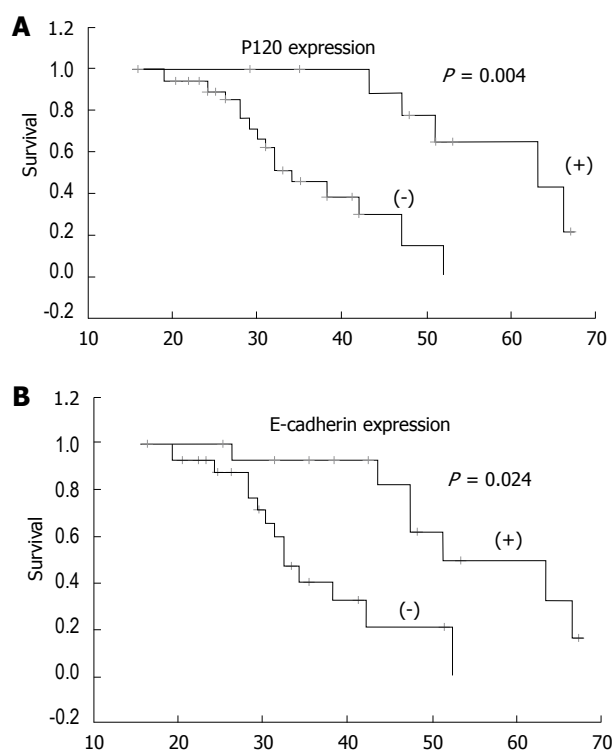
**Table 3** Relationship between expression of E-cadherin and P120 catenin in ICC

E-cadherin	P120		<i>P</i> value
	+	-	
+	9	6	0.000
-	2	25	

**Table 4** Cox multivariate analysis for survival of 37 patients

	Sig	RR	95% CI	
			Lower	Upper
E-cadherin expression	0.724	1.525	0.147	15.827
P120 expression	0.049	0.088	0.008	0.991
Differentiation	0.194	0.407	0.105	1.583
pTNM stage	0.073	2.898	0.904	9.288
Tumor size	0.037	0.387	0.159	0.944
Capsular invasion	0.052	17.046	0.981	6.166
Satellite nodules	0.709	1.597	0.137	18.578
Vascular invasion	0.948	0.961	0.284	3.247
Lymph node invasion	0.087	4.72	0.8	27.855
Intrahepatic metastasis	0.786	1.352	0.154	11.899

Sig: Significance; RR: Relative risk; CI: Confidence interval.

**Figure 2** Kaplan-Meier survival curves. A: Expression of P120 induced type(-) and B: Expression of E-cadherin.

significantly correlated with the poor survival of patients ( $P = 0.024$  and  $P = 0.004$ , respectively). However,

when the expression of E-cadherin or P120 and the clinicopathological parameters were analyzed by the Cox regression model, abnormal expression of P120 was found to be an independent prognostic factor for ICC patients ( $P = 0.049$ ) (Table 4).

## DISCUSSION

Usually, ICC is an adenocarcinoma and may arise from the large intra-hepatic bile ducts near the hepatic hilus or from the bile ducts at the border of hepatic parenchyma. It was reported that altered expression of E-cadherin/catenins complex in ICC occurs frequently and is significantly correlated with tumor histological features and/or vascular invasion and metastasis<sup>[9-14]</sup>.

It was recently reported that P120 plays a role in the occurrence of various cancers, and that P120 may behave either as a tumor suppressor or as a metastasis promoter, depending on the loss of E-cadherin and P120. If E-cadherin is lost first, P120 may directly and actively promote metastasis. If P120 is lost first, E-cadherin levels would fall significantly, which is likely to be parallel to



the reduced levels of  $\alpha$ - and  $\beta$ -catenins<sup>[15]</sup>. P120 down-regulation results in a striking dose-dependant loss of endogenous cadherins, indicating that P120 is essential for cadherin stability. Moreover, P120 down-regulation occurs frequently in almost all carcinomas<sup>[16]</sup>. P120 loss is often associated with the stage and poor prognosis of tumors, suggesting that its loss may be associated with biological aggressiveness and progression of tumors. Nevertheless, to our knowledge, no report is available on the expression of P120 in human intrahepatic cholangiocarcinoma.

The present study showed that reduced or absent expression of E-cadherin and P120 was associated with the histological grade of tumors, which is consistent with reported data<sup>[17-21]</sup>. In well-differentiated tumors, there were obvious and strong staining along the cell-cell boundaries, whereas in poorly-differentiated tumors, the immunohistostaining was focally and heterogeneously distributed, with patchy or spotty features along the cell-cell boundaries, indicating that the staining of E-cadherin and P120 is related with the differentiation of ICC, namely both E-cadherin and P120 may be regarded as differentiation markers of tumor. In addition, the staining intensity of the E-cadherin and P120 complex was gradually decreased, suggesting that P120 may play a critical role in ICC progression.

Microscopy revealed that E-cadherin was located on the membrane either in non-tumor tissues or in tumor cells, whereas P120 was expressed on the membrane or in cytoplasm of tumor cells. However, it was reported that P120 is also in nuclei<sup>[22]</sup>, suggesting that P120 plays an important role in cell signal transduction. P120 has an intrinsic nucleocytoplasmic shuttling activity that is modulated, in part, by extrinsic factors such as cadherin binding and interactions with the microtubule network<sup>[22]</sup>. Julia and his colleagues reported<sup>[23]</sup> that P120 displays up-regulation and nuclear expression in pancreatic cancer. No expression of P120 in nuclei of cancer cells, however, was observed in our study, suggesting that it is necessary to further investigate the mechanism underlying P120 expression in nuclei of cancer cells.

In this study, we observed the relationship between reduced expression of E-cadherin and P120 and several clinicopathologic parameters of ICC. The expression of P120 and E-cadherin was significantly associated with tumor pTNM stage and intrahepatic metastasis (IM), but not with tumor stage and size, capsular and vascular invasion, and lymph node invasion. Osada and his colleagues<sup>[24]</sup> revealed that E-cadherin is involved in intra-hepatic metastasis of hepatocellular carcinoma. Asayama *et al.*<sup>[13]</sup> detected the expression of E-cadherin in hepatocellular carcinoma and cholangiocarcinoma, and found that reduced expression of E-cadherin is significantly correlated with the grade and IM of ICC. Therefore, E-cadherin and P120 may be important mediators in tumor progression, and can be considered as invasion and metastasis markers of ICC.

Several studies on other cancers have evaluated the relationship between the expression of E-cadherin/P120 and the survival of patients, but the results remain debatable<sup>[25-29]</sup>. In the present study, reduced expression of

both E-cadherin and P120 was significantly related with the survival of patients. However, when the expression of E-cadherin/P120 and the clinicopathological parameters of ICC were analyzed by the Cox regression model, only the abnormal expression of P120 was found to be an independent prognostic factor for ICC, suggesting that P120 can be considered a valuable biological marker for predicting the prognosis of ICC patients.

In summary, abnormal expression of E-cadherin and P120 catenin occurs frequently in intrahepatic cholangiocarcinoma. Reduced expression of P120 catenin and E-cadherin is correlated with tumor differentiation, pTNM stage, intrahepatic metastasis and survival of patients. Both P120 catenin and E-cadherin may play an important role in the development and progression of human intrahepatic cholangiocarcinoma.

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## COMMENTS

### Background

P120-catenin is a member of the E-cadherin/catenin complex family and may be associated with biological aggressiveness and progression of tumors. However, no report is available on the expression of P120 catenin in human intra-hepatic cholangiocarcinoma.

### Research frontiers

P120 down-regulation occurs frequently in almost all carcinomas. P120 loss is often associated with the stage and poor prognosis of tumors.

### Innovations and breakthroughs

Our results suggest that down-regulated expression of E-cadherin and P120 catenin occurred frequently in intrahepatic cholangiocarcinoma (ICC) and contributed to the progression and development of tumors. Both E-cadherin and P120 catenin may be valuable biologic markers for predicting tumor invasion, metastasis and survival of patients, but only P120 catenin is an independent prognostic factor for ICC.

### Applications

Because down-regulated expression of P120 contributes to the progression and development of ICC, P120 can be used as a valuable biologic marker for predicting the invasion and metastasis of ICC, and the survival of patients.

### Peer review

This is an interesting report on E-cadherin and P120 catenin in human intra-hepatic cholangiocarcinoma. The study was performed on 42 specimens of ICC with a Dako Envision kit, indicating that. Both E-cadherin and P120 catenin may be valuable biological markers for predicting tumor invasion, metastasis and survival of patients. However, its clinical application should be further studied.

## REFERENCES

- 1 Reynolds AB, Herbert L, Cleveland JL, Berg ST, Gaut JR. p120, a novel substrate of protein tyrosine kinase receptors and of p60v-src, is related to cadherin-binding factors beta-catenin, plakoglobin and armadillo. *Oncogene* 1992; 7: 2439-2445
- 2 Yanagisawa M, Anastasiadis PZ. p120 catenin is essential for mesenchymal cadherin-mediated regulation of cell motility and invasiveness. *J Cell Biol* 2006; 174: 1087-1096
- 3 Nollet F, Berx G, van Roy F. The role of the E-cadherin/catenin adhesion complex in the development and



- progression of cancer. *Mol Cell Biol Res Commun* 1999; **2**: 77-85
- 4 **Xiao K**, Oas RG, Chiasson CM, Kowalczyk AP. Role of p120-catenin in cadherin trafficking. *Biochim Biophys Acta* 2007; **1773**: 8-16
  - 5 **Patel T**. Increasing incidence and mortality of primary intrahepatic cholangiocarcinoma in the United States. *Hepatology* 2001; **33**: 1353-1357
  - 6 **The general rules for the clinical and pathological study of primary liver cancer**. Liver Cancer Study Group of Japan. *Jpn J Surg* 1989; **19**: 98-129
  - 7 **Hermanek P**, Hutter RVP, Sobin LH. TNM Atlas, UICC. 4th ed. Berlin: Springer 1997: 115-123
  - 8 **Gamallo C**, Palacios J, Suarez A, Pizarro A, Navarro P, Quintanilla M, Cano A. Correlation of E-cadherin expression with differentiation grade and histological type in breast carcinoma. *Am J Pathol* 1993; **142**: 987-993
  - 9 **Ashida K**, Terada T, Kitamura Y, Kaibara N. Expression of E-cadherin, alpha-catenin, beta-catenin, and CD44 (standard and variant isoforms) in human cholangiocarcinoma: an immunohistochemical study. *Hepatology* 1998; **27**: 974-982
  - 10 **Sato K**, Murai H, Ueda Y, Katsuda S. Intrahepatic sarcomatoid cholangiocarcinoma of round cell variant: a case report and immunohistochemical studies. *Virchows Arch* 2006; **449**: 585-590
  - 11 **Settakorn J**, Kaewpila N, Burns GF, Leong AS. FAT, E-cadherin, beta catenin, HER 2/neu, Ki67 immun-expression, and histological grade in intrahepatic cholangiocarcinoma. *J Clin Pathol* 2005; **58**: 1249-1254
  - 12 **Tokumoto N**, Ikeda S, Ishizaki Y, Kurihara T, Ozaki S, Iseki M, Shimizu Y, Itamoto T, Arihiro K, Okajima M, Asahara T. Immunohistochemical and mutational analyses of Wnt signaling components and target genes in intrahepatic cholangiocarcinomas. *Int J Oncol* 2005; **27**: 973-980
  - 13 **Asayama Y**, Taguchi Ki K, Aishima Si S, Nishi H, Masuda K, Tsuneyoshi M. The mode of tumour progression in combined hepatocellular carcinoma and cholangiocarcinoma: an immunohistochemical analysis of E-cadherin, alpha-catenin and beta-catenin. *Liver* 2002; **22**: 43-50
  - 14 **Sugimachi K**, Taguchi K, Aishima S, Tanaka S, Shimada M, Kajiyama K, Sugimachi K, Tsuneyoshi M. Altered expression of beta-catenin without genetic mutation in intrahepatic cholangiocarcinoma. *Mod Pathol* 2001; **14**: 900-905
  - 15 **Thoreson MA**, Reynolds AB. Altered expression of the catenin p120 in human cancer: implications for tumor progression. *Differentiation* 2002; **70**: 583-589
  - 16 **Reynolds AB**, Carnahan RH. Regulation of cadherin stability and turnover by p120ctn: implications in disease and cancer. *Semin Cell Dev Biol* 2004; **15**: 657-663
  - 17 **Bremnes RM**, Veve R, Gabrielson E, Hirsch FR, Baron A, Bemis L, Gemmill RM, Drabkin HA, Franklin WA. High-throughput tissue microarray analysis used to evaluate biology and prognostic significance of the E-cadherin pathway in non-small-cell lung cancer. *J Clin Oncol* 2002; **20**: 2417-2428
  - 18 **Sarrio D**, Perez-Mies B, Hardisson D, Moreno-Bueno G, Suarez A, Cano A, Martin-Perez J, Gamallo C, Palacios J. Cytoplasmic localization of p120ctn and E-cadherin loss characterize lobular breast carcinoma from preinvasive to metastatic lesions. *Oncogene* 2004; **23**: 3272-3283
  - 19 **Ishizaki Y**, Omori Y, Momiyama M, Nishikawa Y, Tokairin T, Manabe M, Enomoto K. Reduced expression and aberrant localization of p120catenin in human squamous cell carcinoma of the skin. *J Dermatol Sci* 2004; **34**: 99-108
  - 20 **Qian ZR**, Sano T, Yoshimoto K, Asa SL, Yamada S, Mizusawa N, Kudo E. Tumor-specific downregulation and methylation of the CDH13 (H-cadherin) and CDH1 (E-cadherin) genes correlate with aggressiveness of human pituitary adenomas. *Mod Pathol* 2007; **20**: 1269-1277
  - 21 **Bremnes RM**, Veve R, Gabrielson E, Hirsch FR, Baron A, Bemis L, Gemmill RM, Drabkin HA, Franklin WA. High-throughput tissue microarray analysis used to evaluate biology and prognostic significance of the E-cadherin pathway in non-small-cell lung cancer. *J Clin Oncol* 2002; **20**: 2417-2428
  - 22 **Roczniak-Ferguson A**, Reynolds AB. Regulation of p120-catenin nucleocytoplasmic shuttling activity. *J Cell Sci* 2003; **116**: 4201-4212
  - 23 **Mayerle J**, Friess H, Buchler MW, Schneckeburger J, Weiss FU, Zimmer KP, Domschke W, Lerch MM. Up-regulation, nuclear import, and tumor growth stimulation of the adhesion protein p120 in pancreatic cancer. *Gastroenterology* 2003; **124**: 949-960
  - 24 **Osada T**, Sakamoto M, Ino Y, Iwamatsu A, Matsuno Y, Muto T, Hirohashi S. E-cadherin is involved in the intrahepatic metastasis of hepatocellular carcinoma. *Hepatology* 1996; **24**: 1460-1467
  - 25 **Bellovin DI**, Bates RC, Muzikansky A, Rimm DL, Mercurio AM. Altered localization of p120 catenin during epithelial to mesenchymal transition of colon carcinoma is prognostic for aggressive disease. *Cancer Res* 2005; **65**: 10938-10945
  - 26 **Wang EH**, Liu Y, Xu HT, Dai SD, Liu N, Xie CY, Yuan XM. Abnormal expression and clinicopathologic significance of p120-catenin in lung cancer. *Histol Histopathol* 2006; **21**: 841-847
  - 27 **Wijnhoven BP**, Pignatelli M, Dinjens WN, Tilanus HW. Reduced p120ctn expression correlates with poor survival in patients with adenocarcinoma of the gastroesophageal junction. *J Surg Oncol* 2005; **92**: 116-123
  - 28 **Bantis A**, Giannopoulos A, Gonidi M, Liossi A, Aggelonidou E, Petrakakou E, Athanassiades P, Athanassiadou P. Expression of p120, Ki-67 and PCNA as proliferation biomarkers in imprint smears of prostate carcinoma and their prognostic value. *Cytopathology* 2004; **15**: 25-31
  - 29 **Nakopoulou L**, Gakiopoulou-Givalou H, Karayiannakis AJ, Giannopoulou I, Keramopoulos A, Davaris P, Pignatelli M. Abnormal alpha-catenin expression in invasive breast cancer correlates with poor patient survival. *Histopathology* 2002; **40**: 536-546

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## Effects of different *Helicobacter pylori* culture filtrates on growth of gastric epithelial cells

Yan-Guo Yan, Gang Zhao, Jin-Ping Ma, Shi-Rong Cai, Wen-Hua Zhan

Yan-Guo Yan, Department of General Surgery, The Affiliated Hospital of Wuhan University of Science & Technology, Wuhan 430064, Hubei Province, China

Gang Zhao, The Second Department of General Surgery, Guangdong Province Hospital, Guangzhou 510080, Guangdong Province, China

Jin-Ping Ma, Shi-Rong Cai, Wen-Hua Zhan, Department of Gastrointestinal and Pancreatic Surgery, The First Affiliated Hospital, Research Center for Gastric Cancer, Sun Yat-sen University, Guangzhou 510080, Guangdong Province, China

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**Author contributions:** Yan YG, Zhao G, performed the research and wrote the paper and contributed equally to this work; Zhan WH and Ma JP designed the research; Cai SR recorded the data.

**Correspondence to:** Yan-Guo Yan, Department of General Surgery, The Affiliated Hospital of Wuhan University of Science & Technology, Wuhan 430064, Hubei Province, China. [yan969400@yahoo.com.cn](mailto:yan969400@yahoo.com.cn)

Telephone: +86-27-51164927 Fax: +86-27-51163527

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### Abstract

**AIM:** To study the effects of different *Helicobacter pylori* (*H. pylori*) culture filtrates on growth of gastric epithelial cells.

**METHODS:** Broth culture filtrates of *H. pylori* were prepared. Gastric epithelial cells were treated with the filtrates, and cell growth was determined by growth curve and flow cytometry. DNA damage of gastric epithelial cells was measured by single-cell microgel electrophoresis.

**RESULTS:** Gastric epithelial cells proliferated actively when treated by *CagA*-gene-positive broth culture filtrates, and colony formation reached 40%. The number of cells in S phase increased compared to controls. Comet assay showed 41.2% comet cells in GES-1 cells treated with *CagA*-positive filtrates ( $P < 0.05$ ).

**CONCLUSION:** *CagA*-positive filtrates enhance the changes in morphology and growth characteristics of human gastric epithelial tumor cells. DNA damage maybe one of the mechanisms involved in the growth changes.

*Helicobacter pylori*; Single cell microgel electrophoresis

**Peer reviewer:** Yvan Vandenplas, Professor, Department of Pediatrics, AZ-VUB, Laarbeeklaan 101, Brussels 1090, Belgium

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### INTRODUCTION

Infection with the gastric bacterium *Helicobacter pylori* (*H. pylori*) the causative agent of chronic gastritis is associated with an increased risk of developing gastric cancer<sup>[1-6]</sup>. The international agency for research on cancer has classified *H. pylori* as a type I carcinogen<sup>[7-10]</sup>. A number of studies have pointed to a link between carriage of *CagA*+ strains and an increased risk of gastric cancer<sup>[11-13]</sup>. The mechanism by which *H. pylori* promotes the development of gastric cancer is presently unclear. Studies have shown that *CagA* can bind to other signaling molecules, such as SHP2, GRB2, c-MET and phospholipase C to promote gastric carcinogenesis<sup>[14-19]</sup>, which depends on a type IV secretion system to translocate *CagA* into host cells.

The aim of this study was to investigate the effects of *H. pylori* culture filtrates that contained *CagA* protein on the growth of gastric epithelial cells. *CagA*-gene-positive and negative *H. pylori* culture filtrates were prepared to treat gastric epithelial cells, to observe DNA damage and growth changes.

### MATERIALS AND METHODS

#### Bacterial strains

*H. pylori* strains NCTC11639 (*CagA*-positive, *VacA*-negative) and G50 (*CagA*-negative, *VacA*-negative) were provided by the Chinese Center for Disease Control and Prevention.

#### Production of culture filtrates

*H. pylori* was grown in Brucella broth, supplemented with 5% fetal calf serum (Gibco, Grand Island, NY,

USA), for 24–36 h at 37°C in a thermostatic shaker under microaerophilic conditions. As described by Sommi *et al*<sup>[20]</sup>, when bacterial suspensions reached  $A_{450}$  1.2 (corresponding to a bacterial concentration of  $5 \times 10^8$  CFU/mL), to obtain the *H. pylori* culture filtrate, we then removed bacteria by centrifugation (12 000 *g* for 10 min) and sterilized the supernatants by passage through a 0.22  $\mu$ m cellulose acetate filters (Nalge, Rochester, NY, USA). Uninoculated broth filtrate served as a blank control. To remove ammonia, we dialyzed control and culture filtrates against Hanks' balanced salt solution for 36 h in dialysis tubing, with a 12 kDa molecular mass cutoff (Sigma, St. Louis, MO, USA). The presence of CagA in the culture filtrates was tested by means of SDS-PAGE, followed by Western blotting<sup>[21,22]</sup>. Total protein content was determined by measuring  $A_{280}$ . The conditioned broth was stored at -70°C until use. There were three groups: CagA+, CagA- and blank control.

### Western blotting

Culture filtrates were subjected to 12% SDS-PAGE and transferred to polyvinylidene membranes using standard procedures, and incubated with primary monoclonal anti-CagA antibodies (1:5000) (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Horseradish peroxidase-conjugated secondary antibodies (Boster, China) were used and the immunoreactive proteins were visualized by an ECL detection system (Amersham Biosciences, Uppsala, Sweden).

### Human gastric epithelial cells

GES-1 cells (Beijing Institute for Cancer Research Collection) were maintained in DMEM high-glucose medium (Gibco) containing 10% FCS (Sigma, Poole, UK) and different *H. pylori* culture filtrates (10% v/v), in 24-well plates. Cell cultures were maintained at 37°C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>, continuously for 1 mo. The culture medium was changed every 3 d.

### MTT assay for cell proliferation

The effect of culture filtrate on endothelial cell proliferation was determined by MTT assay (Sigma). Briefly, GES-1 cells were plated in 24-well plates and cultured overnight in growth medium for 1 mo.  $2.5 \times 10^4$  cells were inoculated in three groups: CagA+, CagA- and blank control. The viable cells were quantified by MTT assay at 1–7 d, following the manufacturer's instructions.

### Flat clone formation test

The gastric epithelial cells in logarithmic phase were digested to produce single-cell suspension by 0.25% trypsin. Five hundred cells were dispersed in a culture dish (diameter 60 mm); culture medium was added, and the cells were cultured for 2 wk. When clones were visible, the supernatant was removed, and fixed in methanol for 15 min. The samples were stained with 0.4% crystal violet for 20 min, rinsed in water, air dried,

and the number of clones that contained above 50 cells was calculated, according to the formula: cloning rate = average clone number/500  $\times$  100%.

### Cell cycle and apoptosis rate analysis by flow cytometry

For analysis of DNA content, cells treated with different *H. pylori* culture broths were harvested after 72 h and fixed with 70% cold ethanol for 4 h. The apoptosis rate was detected by flow cytometry (FACSalibur, BD Biosciences). Briefly,  $1 \times 10^6$  cells/mL were suspended in 0.2% triton X-100/PBS solution containing 1 g/L ribonuclease A. After incubation for 20 min, DNA was stained with 50 mg/L propidium iodide. Flow cytometry was performed to determine the apoptosis rate.

### Single-cell microgel electrophoresis (comet assay)

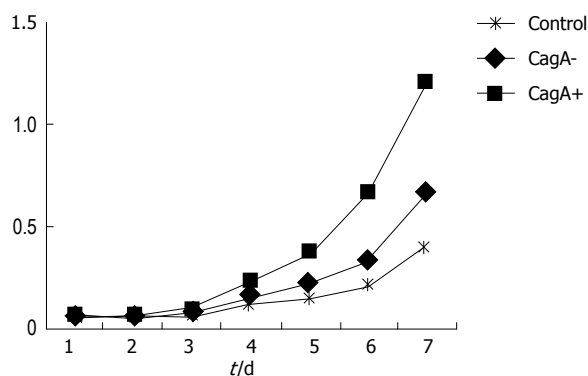
The alkaline comet assay in single-cell suspensions was performed according to the method of Singh *et al*<sup>[23]</sup>, with some modifications<sup>[24]</sup>. Briefly, 15  $\mu$ L of the single cell suspension ( $2 \times 10^4$  cells) was embedded in 0.5% low-melting-point agarose (Sigma) and spread on agarose-precoated microscope slides. Slides were immersed overnight at 4°C in freshly prepared cold lysis solution [2.5 mol/L NaCl, 100 mmol/L EDTA, 10 mmol/L Tris, 1% sodium salt N-lauryl sarcosine (pH 10), with 1% triton X-100, and 10% DMSO added fresh; all supplied by Sigma]. Subsequently, the cells were exposed to alkaline buffer (1 mmol/L EDTA and 300 mmol/L NaOH, pH 13.4) at 4°C for 40 min, to allow DNA unwinding, and expression of alkali-labile sites. In the same solution, electrophoresis was conducted at 4°C for 20 min, at 25 V and 300 mA. After electrophoresis, the slides were neutralized (0.4 mol/L Tris, pH 7.5), stained with 40  $\mu$ L ethidium bromide (EtBr) (20  $\mu$ g/mL), and analyzed with a fluorescence microscope (Axioplan II; Zeiss, Oberkochen, Germany), under green light at 400 nm, using an image analysis system (Comet Assay II; Perceptive Instruments, Suffolk, UK). Two hundred randomly selected cells (100 from each of two replicate slides) were evaluated from each sample, and the mean of the tail moment was determined. Tail moment according to Comet Assay II is defined as "the product of DNA in the tail, and the mean distance of migration in the tail. It is calculated by multiplying tail intensity/sum of comet intensity by tail center of gravity minus peak position." Using this method, the extent of DNA migration is related to the level of DNA damage in each cell, which creates the comet tail images.

### Gastric epithelial cell morphology

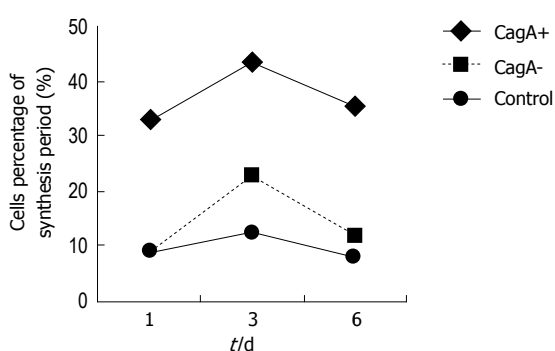
Gastric epithelial cells treated continuously for 1 mo were collected and digested by 0.25% trypsin to yield a cell suspension, centrifuged at 1000 *g* for 10 min. The supernatant was removed, and the precipitate was fixed with 5% glutaraldehyde. Ultrathin sections were cut and observed under an electron microscope (H-600, Hitachi, Japan).

### Statistical analysis

All data were expressed as mean  $\pm$  SE. SPSS 10.0



**Figure 1** Cell proliferation determined by MTT assay.  $P < 0.05$ , CagA+ vs control and CagA+ vs CagA-.



**Figure 2** S-phase cells.  $P < 0.05$ , CagA+ vs CagA-.

software was used for one-way analysis of variance and  $q$  test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Western blotting of CagA and cell proliferation determined by MTT assay

CagA expression was detected in the CagA+, CagA- and control groups. GES-1 cells proliferated actively when treated by CagA and *H pylori* culture filtrates. There were significant differences between the two groups (Figure 1).

### Flat clone formation test

After treatment with *H pylori* culture filtrates, the flat clone formation test in the CagA+ group showed that the GES-1 clone number was larger than that of the CagA-, and control groups (200 vs 88.12,  $P = 0.005$ ). The clone formation rates were 40%, 17.6% and 2.4%, respectively. Statistical analysis revealed a significant difference between the CagA+ and CagA- groups.

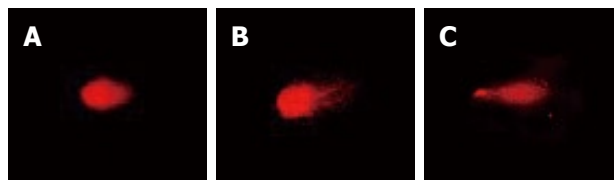
### Effects of culture filtrates on GES-1 cell cycle and apoptosis rate

Flow cytometry showed that the number of S-phase cells increased more in the CagA+ group than in the control group at 1, 3 and 6 d (33.03% vs 9.16%, 53.46% vs 6.83%, and 35.55% vs 8.10%, respectively), which was higher than in the CagA-group ( $P = 0.034$ ). After GES-1 cells had been treated with different *H pylori* culture filtrates

**Table 1** Determination of DNA damage by single-cell microgel electrophoresis

	CagA+	CagA-	Control
Comet cell rate (%)	41.2 <sup>a</sup>	12.5	5
Comet tail length	78.6 ± 5.0 <sup>a</sup>	14.2 ± 6.3	6.8 ± 2.1

<sup>a</sup> $P < 0.05$  vs control and CagA-.



**Figure 3** DNA damage measured by single-cell microgel electrophoresis. A: Comet tails in control group; B: Comet tails in CagA- group; C: Comet tails in CagA+ group.

for 1 mo, the detection of apoptotic cells was carried out by flow cytometry, and repeated eight times. The mean percentage of hypodiploid cells in the CagA+, CagA- and control cells was 5.6%, 4.4% and 3.7%, respectively. Statistical analysis revealed no significant difference among the three groups (Figure 2).

### Determination of DNA damage by single-cell microgel electrophoresis

Single-cell microgel electrophoresis showed in the control group that the ratio of comet cells was 5%, less than that in the CagA+ and CagA- groups. There were more comet cells after treatment with CagA+ broth than in the CagA- group, (41.2% vs 12.5%,  $P = 0.024$ , Table 1). The average comet tail length in the CagA+ group was  $78.6 \pm 5.0$ , which was larger than that in the CagA-group ( $14.2 \pm 6.3$ ,  $P = 0.036$ , Figure 3).

### Cell morphology

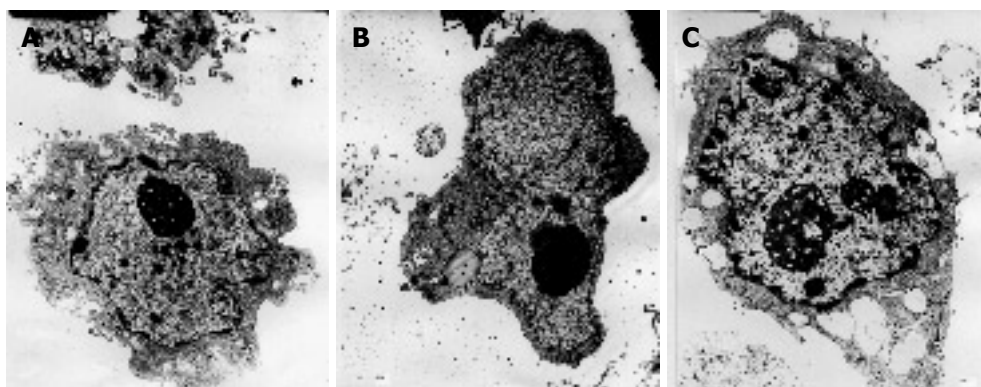
Electron microscopy revealed dyskaryosis, nucleolar hypertrophy and karyokinesis in CagA-treated cells (Figure 4C), compared to the control and CagA- groups.

## DISCUSSION

Infection with strains of *H pylori* that carry the *CagA*-gene is associated with gastric carcinoma. The mechanism is still not clear. Recent studies have shown that the *CagA*-gene product is delivered to gastric epithelial cells by the bacterial type IV secretion system to deregulate the SHP2 oncoprotein, which can promote gastric carcinogenesis<sup>[14-19]</sup>.

In the present study, we prepared different *H pylori* culture filtrates containing the CagA protein to treat gastric epithelial cells. We found that GES-1 cells, after being treated with *CagA*-gene-positive culture filtrates, showed dyskaryosis, nucleolar hypertrophy and karyokinesis. The growth curve showed that GES-1 cells proliferated actively, and colony formation reached 40%. The number of S-phase cells obviously increased compared to the





**Figure 4** GES-1 treated with different *H. pylori* filtrates. **A:** Control group; **B:** CagA-group; **C:** CagA+ group.

controls. These results demonstrate that CagA+ filtrates of *H. pylori* can induce carcinogenesis in gastric epithelial cells. Research has shown that reconstruction of CagA fragment induces paraplastic changes in epithelial cells *in vitro*, which supports the suggestion that CagA is involved in gastric carcinogenesis<sup>[20-21]</sup>. All these results support our findings.

Ladeira *et al.*<sup>[25]</sup> have used the comet assay or single-cell gel electrophoresis to investigate the status of DNA damage in gastric epithelial cells from the antrum and corpus in patients with *H. pylori* infection, and gastritis of varying degrees. They have demonstrated that the level of DNA damage in *H. pylori*-infected individuals is significantly higher than that in non-infected individuals, with the levels of DNA damage significantly higher in those aged  $\geq 50$  years.

The DNA repair enzyme human oxoguanine glycosylase 1 (hOGG1) is known to be responsible for the repair of the 8-hydroxy-deoxyguanosine (8-OHdG) region. Of the hOGG1 polymorphisms identified, a Ser $\rightarrow$ Cys polymorphism at position 326 has been found to interact with atrophic gastritis, but not with antioxidant dietary or nutrient intakes. It is likely that this makes patients with atrophic gastritis, who also have the hOGG1 Cys allele, more susceptible to gastric cancer<sup>[26]</sup>. The concentrations of 8-OHdG, inducible nitric oxide synthase (iNOS), nuclear factor  $\kappa$ B, myeloid cell leukemia-1, and inhibitor of apoptosis protein are significantly higher in patients with *H. pylori* infection, and in those with stage 3 and 4 gastric cancer. This points to the pivotal role that oxygen-free-radical-mediated DNA damage caused by *H. pylori* infection plays in the development of gastric carcinoma from chronic gastritis<sup>[27]</sup>. Single-cell gel electrophoresis is a simple and sensitive method for detecting DNA damage in cells. In this study, we found 41.2% of CagA+ cells formed comet tails. This shows that CagA+ culture broth filtrate has the ability to cause DNA breakage or damage, which may lead to the transformation of gastric epithelial cells.

*H. pylori*-associated inflammation related to DNA damage is indicated by increased levels of oxidative DNA damage, increased occurrence of apoptosis and proliferation, as well as increased expression of iNOS, which seems to provide the mechanistic link between

*H. pylori* infection and gastric carcinogenesis<sup>[28,29]</sup>. Upregulation of iNOS expression might contribute to the oxidative DNA damage observed during *H. pylori* infection<sup>[30]</sup>. Yabuki *et al.*<sup>[31]</sup> have investigated cell proliferation and DNA damage in ablation samples from 35 cases of gastric carcinoma, and have indicated that cell damage in *H. pylori*-infected human gastric mucosa increases cell proliferation.

We found that CagA+ culture broth filtrate induced DNA damage in human gastric epithelial cells *in vitro*, which suggests that CagA is involved in DNA damage in gastric epithelial cells. All results showed that CagA+ broth culture filtrates of *H. pylori* could accelerate human gastric epithelial cell growth and alter their morphology. It is not clear that there are other factors apart from CagA involved in the transformation of gastric epithelial cells induced by CagA+ culture broth filtrate.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

It is widely accepted that there is a strong association between *Helicobacter pylori* (*H. pylori* infection) and gastric cancer, but the exact molecular mechanism of the pathogen in gastric carcinogenesis has not yet been clarified. DNA damage is involved in the carcinogenesis process. Single-cell gel electrophoresis is a simple and sensitive method for detecting DNA damage in cells.

### Research frontiers

There has been considerable interest in recent years in virulence factors, such as CagA protein, which predispose individuals to develop gastric carcinoma. Understanding DNA damage in gastric epithelial cells will provide us with a new strategy for effective prevention of gastric cancer induced by *H. pylori* infection. It is also helpful to clarify CagA protein carcinogenesis.

### Innovations and breakthroughs

In this study, the mechanism of *H. pylori* infection in gastric carcinogenesis was explored by studying its effects on DNA damage in gastric epithelial cells *in vitro*. The results suggest that *H. pylori* can induce DNA damage in cultured gastric epithelial cells, and the effect of CagA+ strains was more significant than CagA- strains.

## Applications

This study emphasizes the close relationship between *H pylori*, especially CagA+ strains, and gastric carcinoma. It provides a new approach to elucidating the mechanism of *H pylori* gastric carcinogenesis. It also implies that compounds able to repair DNA damage in *H pylori*-infected cells may be used to create new strategies for the prevention and/or treatment of human gastric malignancy.

## Peer review

This study seems innovative and interesting. Products of DNA damage have been observed in various pathological processes of the digestive tract, including gastric cancer. To the best of our knowledge, there are still many aspects of the mechanism of *H pylori* infection in gastric carcinogenesis to explore, by studying its effects on DNA damage in gastric epithelial cells *in vitro*.

## REFERENCES

- 1 **Nomura A**, Stemmermann GN, Chyou PH, Kato I, Perez-Perez GI, Blaser MJ. Helicobacter pylori infection and gastric carcinoma among Japanese Americans in Hawaii. *N Engl J Med* 1991; **325**: 1132-1136
- 2 **Eksstrom AM**, Held M, Hansson LE, Engstrand L, Nyren O. Helicobacter pylori in gastric cancer established by CagA immunoblot as a marker of past infection. *Gastroenterology* 2001; **121**: 784-791
- 3 **An international association between Helicobacter pylori infection and gastric cancer**. The EUROGAST Study Group. *Lancet* 1993; **341**: 1359-1362
- 4 **Camargo MC**, Piazuelo MB, Mera RM, Fontham ET, Delgado AG, Yepez MC, Ceron C, Bravo LE, Bravo JC, Correa P. Effect of smoking on failure of *H. pylori* therapy and gastric histology in a high gastric cancer risk area of Colombia. *Acta Gastroenterol Latinoam* 2007; **37**: 238-245
- 5 **Sun LP**, Gong YH, Wang L, Gong W, Yuan Y. Follow-up study on a high risk population of gastric cancer in north China by serum pepsinogen assay. *J Dig Dis* 2008; **9**: 20-26
- 6 **Ogura K**, Hirata Y, Yanai A, Shibata W, Ohmae T, Mitsuno Y, Maeda S, Watabe H, Yamaji Y, Okamoto M, Yoshida H, Kawabe T, Omata M. The effect of Helicobacter pylori eradication on reducing the incidence of gastric cancer. *J Clin Gastroenterol* 2008; **42**: 279-283
- 7 **Schistosomes, liver flukes and Helicobacter pylori**. IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994. *IARC Monogr Eval Carcinog Risks Hum* 1994; **61**: 177-241
- 8 **Amieva MR**, El-Omar EM. Host-bacterial interactions in Helicobacter pylori infection. *Gastroenterology* 2008; **134**: 306-323
- 9 **Lambert R**, Hainaut P. The multidisciplinary management of gastrointestinal cancer. *Epidemiology of oesophagogastric cancer. Best Pract Res Clin Gastroenterol* 2007; **21**: 921-945
- 10 **Vauhkonen H**, Heino S, Myllykangas S, Lindholm PM, Savola S, Knuutila S. Etiology of specific molecular alterations in human malignancies. *Cytogenet Genome Res* 2007; **118**: 277-283
- 11 **Huang JQ**, Zheng GF, Sumanac K, Irvine EJ, Hunt RH. Meta-analysis of the relationship between cagA seropositivity and gastric cancer. *Gastroenterology* 2003; **125**: 1636-1644
- 12 **Jackson CB**, Judd LM, Menheniott TR, Kronborg I, Dow C, Yeomans ND, Boussioutas A, Robb L, Giraud AS. Augmented gp130-mediated cytokine signalling accompanies human gastric cancer progression. *J Pathol* 2007; **213**: 140-151
- 13 **Loh JT**, Torres VJ, Cover TL. Regulation of Helicobacter pylori cagA expression in response to salt. *Cancer Res* 2007; **67**: 4709-4715
- 14 **Tsutsumi R**, Higashi H, Higuchi M, Okada M, Hatakeyama M. Attenuation of Helicobacter pylori CagA x SHP-2 signaling by interaction between CagA and C-terminal Src kinase. *J Biol Chem* 2003; **278**: 3664-3670
- 15 **Mimuro H**, Suzuki T, Tanaka J, Asahi M, Haas R, Sasakawa C. Grb2 is a key mediator of helicobacter pylori CagA protein activities. *Mol Cell* 2002; **10**: 745-755
- 16 **Churin Y**, Al-Ghoul L, Kepp O, Meyer TF, Birchmeier W, Naumann M. Helicobacter pylori CagA protein targets the c-Met receptor and enhances the motogenic response. *J Cell Biol* 2003; **161**: 249-255
- 17 **Penta R**, De Falco M, Iaquinto G, De Luca A. Helicobacter pylori and gastric epithelial cells: from gastritis to cancer. *J Exp Clin Cancer Res* 2005; **24**: 337-345
- 18 **Lee YC**. [Pathogenesis of Helicobacter pylori infection] *Korean J Gastroenterol* 2005; **46**: 159-165
- 19 **Chen Y**, Wang Y, Xu W, Zhang Z. Analysis on the mechanism of Helicobacter pylori-induced apoptosis in gastric cancer cell line BGC-823. *Int J Mol Med* 2005; **16**: 741-745
- 20 **Sommi P**, Ricci V, Fiocca R, Romano M, Ivey KJ, Cova E, Solcia E, Ventura U. Significance of ammonia in the genesis of gastric epithelial lesions induced by Helicobacter pylori: an *in vitro* study with different bacterial strains and urea concentrations. *Digestion* 1996; **57**: 299-304
- 21 **Stein M**, Bagnoli F, Halenbeck R, Rappuoli R, Fantl WJ, Covacci A. c-Src/Lyn kinases activate Helicobacter pylori CagA through tyrosine phosphorylation of the EPIYA motifs. *Mol Microbiol* 2002; **43**: 971-980
- 22 **Azuma T**, Yamakawa A, Yamazaki S, Ohtani M, Ito Y, Muramatsu A, Suto H, Yamazaki Y, Keida Y, Higashi H, Hatakeyama M. Distinct diversity of the cag pathogenicity island among Helicobacter pylori strains in Japan. *J Clin Microbiol* 2004; **42**: 2508-2517
- 23 **Singh NP**, McCoy MT, Tice RR, Schneider EL. A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res* 1988; **175**: 184-191
- 24 **Klaude M**, Eriksson S, Nygren J, Ahnstrom G. The comet assay: mechanisms and technical considerations. *Mutat Res* 1996; **363**: 89-96
- 25 **Ladeira MS**, Rodrigues MA, Salvadori DM, Queiroz DM, Freire-Maia DV. DNA damage in patients infected by Helicobacter pylori. *Cancer Epidemiol Biomarkers Prev* 2004; **13**: 631-637
- 26 **Tsukino H**, Hanaoka T, Otani T, Iwasaki M, Kobayashi M, Hara M, Natsukawa S, Shaura K, Koizumi Y, Kasuga Y, Tsugane S. hOGG1 Ser326Cys polymorphism, interaction with environmental exposures, and gastric cancer risk in Japanese populations. *Cancer Sci* 2004; **95**: 977-983
- 27 **Chang CS**, Chen WN, Lin HH, Wu CC, Wang CJ. Increased oxidative DNA damage, inducible nitric oxide synthase, nuclear factor kappaB expression and enhanced antiapoptosis-related proteins in Helicobacter pylori-infected non-cardiac gastric adenocarcinoma. *World J Gastroenterol* 2004; **10**: 2232-2240
- 28 **Selbach M**, Moese S, Hauck CR, Meyer TF, Backert S. Src is the kinase of the Helicobacter pylori CagA protein *in vitro* and *in vivo*. *J Biol Chem* 2002; **277**: 6775-6778
- 29 **Azuma T**. Helicobacter pylori CagA protein variation associated with gastric cancer in Asia. *J Gastroenterol* 2004; **39**: 97-103
- 30 **Argent RH**, Kidd M, Owen RJ, Thomas RJ, Limb MC, Atherton JC. Determinants and consequences of different levels of CagA phosphorylation for clinical isolates of Helicobacter pylori. *Gastroenterology* 2004; **127**: 514-523
- 31 **Yabuki N**, Sasano H, Tobita M, Imatani A, Hoshi T, Kato K, Ohara S, Asaki S, Toyota T, Nagura H. Analysis of cell damage and proliferation in Helicobacter pylori-infected human gastric mucosa from patients with gastric adenocarcinoma. *Am J Pathol* 1997; **151**: 821-829



RAPID COMMUNICATION

## Clinical value of serum CA19-9 levels in evaluating resectability of pancreatic carcinoma

Shun Zhang, Yi-Ming Wang, Chuan-Dong Sun, Yun Lu, Li-Qun Wu

Shun Zhang, Yi-Ming Wang, Chuan-Dong Sun, Yun Lu, Li-Qun Wu, Department of Hepatobiliary Surgery, Affiliated Hospital of Medical College, Qingdao University, Qingdao 266003, Shandong Province, China

Author contributions: Zhang S designed the study; Wang YM collected and analyzed the data; Wang YM wrote the paper; Sun CD, Lu Y and Wu LQ revised the paper.

Correspondence to: Shun Zhang, Department of Hepatobiliary Surgery, Affiliated Hospital of Medical College, Qingdao University, No. 16 Jiangsu Road, Qingdao 266003, Shandong Province, China. [wym0066@sina.com](mailto:wym0066@sina.com)

Telephone: +86-532-82911369 Fax: +86-532-82911999

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**Peer reviewers:** Dr. Bernd Sido, Department of General and Abdominal Surgery, Teaching Hospital of the University of Regensburg, Hospital Barmherzige Brüder, Prüfeninger Strasse 86, Regensburg D-93049, Germany; Giuseppe Tisone, Professor, Department of Surgery, University of Rome Tor Vergata, Ospedale S.Eugenio, Piazzale dell'Umanesimo 10, Rome 00144, Italy

Zhang S, Wang YM, Sun CD, Lu Y, Wu LQ. Clinical value of serum CA19-9 levels in evaluating resectability of pancreatic carcinoma. *World J Gastroenterol* 2008; 14(23): 3750-3753  
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### Abstract

**AIM:** To evaluate the clinical value of serum CA19-9 levels in predicting the resectability of pancreatic carcinoma according to receiver operating characteristic (ROC) curve analysis.

**METHODS:** Serum CA19-9 levels were measured in 104 patients with pancreatic cancer which were possible to be resected according to the imaging. ROC curve was plotted for the CA19-9 levels. The point closest to the upper left-hand corner of the graph were chosen as the cut-off point. The sensitivity, specificity, positive and negative predictive values of CA19-9 at this cut-off point were calculated.

**RESULTS:** Resectable pancreatic cancer was detected in 58 (55.77%) patients and unresectable pancreatic cancer was detected in 46 (44.23%) patients. The area under the ROC curve was 0.918 and 95% CI was 0.843-0.992. The CA19-9 level was 353.15 U/mL, and the sensitivity and specificity of CA19-9 at this cut-off point were 93.1% and 78.3%, respectively. The positive and negative predictive value was 84.38% and 90%, respectively.

**CONCLUSION:** Preoperative serum CA19-9 level is a useful marker for further evaluating the resectability of pancreatic cancer. Obviously increased serum levels of CA19-9 (> 353.15 U/mL) can be regarded as an ancillary parameter for unresectable pancreatic cancer.

### INTRODUCTION

The prognosis of pancreatic cancer is extremely poor and its early diagnosis is difficult<sup>[1,2]</sup>. Surgical resection offers the best chance of cure. However, local vascular involvement, nodal and distant metastases are frequently found at the time of diagnosis, thus losing the opportunity of operation<sup>[3]</sup>. At present, the best way for preoperative staging of pancreatic cancer is bolus-contrast, and triple-phase helical computed tomography, which has been shown to be almost 100% accurate in predicting unresectable disease<sup>[4-6]</sup>. However, approximately 25%-50% of patients with resectable disease on computed tomography are found to have unresectable lesions at laparotomy<sup>[7]</sup>.

CA19-9 is the most widely used pancreatic cancer serum marker. Serum CA19-9 level has been shown to correlate with the thyroid node metastasis (TNM) staging, and tumor size in patients with pancreatic cancer<sup>[8]</sup>. However, little is known about the value of serum CA19-9 level in evaluating the resectability of pancreatic carcinoma.

Receiver operating characteristic (ROC) curve has been widely accepted as the standard method for describing and comparing the accuracy of medical diagnostic tests<sup>[9,10]</sup>. ROC curve is an efficient way to display and assess the predictive value of cut-off points.

In this study, we evaluated the clinical value of serum CA19-9 level in predicting the resectability of pancreatic carcinoma according to ROC curve analysis.

### MATERIALS AND METHODS

We retrospectively reviewed the clinical and imaging data



Table 1 Characteristics of patients (*n* = 104)

Characteristics	Data, <i>n</i> (%)
Age (yr)	59 ± 9 (mean ± SD)
Sex	
Male	72 (69.2)
Female	32 (30.8)
Location of tumors	
Head	86 (82.7)
Body	8 (7.7)
Body and tail	10 (9.6)
Type of operation	
Pancreaticoduodenectomy	48 (46.2)
Distal pancreatectomy	10 (9.6)
Exploratory laparotomy and biopsy	46 (44.2)

Table 2 CA19-9 levels in patients with resectable and unresectable pancreatic cancer

Group	<i>n</i>	CA19-9 (U/mL)			Wilcoxon	
		<i>Q</i> <sub>1</sub>	<i>Q</i> <sub>2</sub>	<i>Q</i> <sub>3</sub>	<i>Z</i>	<i>P</i>
Resectable	58	15.57	130.10	270.25	-5.132	0.000
Unresectable	46	361.30	656.20	1780.00		

including preoperative CA19-9 level in 104 patients with pancreatic cancer who underwent surgical resection at the Affiliated Hospital of Qingdao University Medical College from January 2001 to July 2007. Pancreatic adenocarcinoma was histologically confirmed. Resectability of pancreatic cancer was evaluated at least by preoperative bolus-contrast, triple-phase helical computer tomography (CT) scan.

Resectability was defined as a tumour limited to the pancreas with no invasion of the superior mesenteric artery and vein, portal vein and metastases (celiac lymph, peritoneum or liver).

Serum levels of CA19-9 and total serum bilirubin levels were measured before surgery (normal 0-39.0 U/mL for CA19-9, 3.4-17.1  $\mu$ mol/L for total serum bilirubin).

The data were described using *Q*<sub>1,3</sub>. Differences between groups were detected using the Wilcoxon 2-sample test. Serum CA19-9 levels were used to plot the ROC curve, and calculate the area under the curve (AUC). We chose the point closest to the upper left-hand corner of the graph as the cut-off point. The sensitivity, specificity, positive and negative predictive values of CA19-9 at this cut-off point were calculated. *P* < 0.05 was considered statistically significant.

## RESULTS

Of the 104 patients, 72 were males and 32 were females with a mean age of 59 years (range 41-75 years). The pancreatic tumor was confined to the head, body and tail of the pancreas in 86, 8, and 10 patients, respectively. Forty-eight patients underwent pancreatic-oduodenectomy, 10 patients distal pancreatectomy, and 46 only exploratory laparotomy and biopsy. The general characteristics of the patients are listed in Table 1.

The distribution of preoperative serum CA19-9 levels

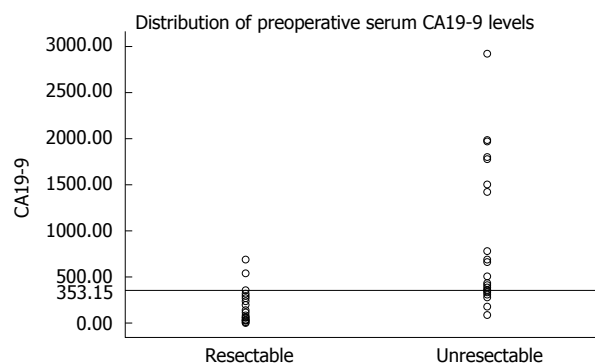


Figure 1 Distribution of preoperative serum CA19-9 levels. The horizon marker is set according to the cut-off point of CA19-9 (353.15 U/mL).

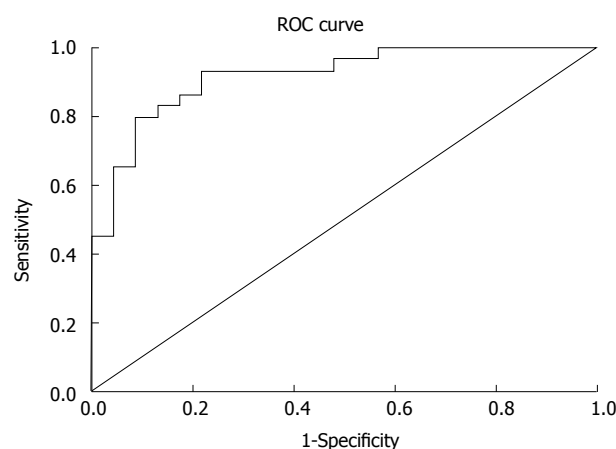


Figure 2 ROC analysis of CA19-9. Perfect discrimination has a ROC plot passing through the upper left corner (100% sensitivity, 100% specificity). The closer the ROC plot to the upper left corner, the higher the overall accuracy of the test (AUC: 0.9-1 indicating excellent; 0.8-0.9 indicating very good; 0.7-0.8 indicating good; 0.6-0.7 indicating average; 0.5-0.6 indicating poor). The AUC of CA19-9 was 0.918.

is shown in Figure 1. The *Q*<sub>2</sub> (median) preoperative serum CA19-9 level in patients with unresectable tumor was 5-fold higher than that in patients with resectable tumor (Table 2). The difference between two groups was significant (*P* < 0.01). The mean total serum bilirubin level in patients with resectable and unresectable tumor was 28.6  $\mu$ mol/L and 34.4  $\mu$ mol/L, respectively (*P* > 0.05). Therefore, the CA19-9 levels were not adjusted.

Figure 2 shows the ROC curve. The AUC was 0.918 and 95% CI was 0.843-0.992, suggesting that changes in serum CA19-9 levels may have a direct relation to resectability<sup>[11,12]</sup>. When the cut-off value of CA19-9 was 353.15 U/mL according to the point closest to the upper left-hand corner of the graph, the sensitivity and specificity were 93.1% and 78.3%, respectively. The preoperative resectability according to the cut-off point was compared with the actual operation, and the positive and negative predictive value of CA19-9 was 84.38% and 90.00%, respectively (Table 3).

## DISCUSSION

Pancreatic cancer is one of the most common causes for



**Table 3** Positive and negative predictive values of CA19-9 at the cut-off point

	Resection		Total	Predictive value
	Yes	No		
CA19-9 (U/mL)	≤ 353.15	54	10	64
	> 353.15	4	36	40
Total	58	46	104	

cancer-related death. The overall five-year survival rate ranges from 0.4% to 4%, the lowest for any cancer<sup>[1,13]</sup>. Early diagnosis of pancreatic cancer is difficult because its early symptoms are usually non-specific. Local vascular involvement, nodal and distant metastases are frequently found at the time of diagnosis<sup>[14]</sup>.

Recently, considerable improvements in radiological imaging make it possible to limit surgery for patients who will benefit<sup>[15,16]</sup>. The current methods of choice for diagnosing and staging pancreatic cancer are thin section, contrast-enhanced, and triple-phase helical computed tomography<sup>[17,18]</sup>. However, approximately 25%-50% of patients with resectable disease on computed tomography are found to have unresectable lesions at laparotomy<sup>[7,19]</sup>. Although magnetic resonance imaging is increasingly used in the evaluation of pancreatic tumor, it was reported that it offers no significant diagnostic advantage over computed tomography<sup>[20]</sup>. Endoscopic retrograde cholangio pancreatography (ERCP) is more controversial for patients with a mass on CT<sup>[21]</sup>. B-mode ultrasonography is operator-dependent and may be inaccurate due to factors such as large body habitus, presence of ascites, or overlying bowel gas. Therefore we should find other ways to further evaluate the resectability of pancreatic cancer.

CA19-9 is a tumor-associated antigen, initially described by Koprowski *et al*<sup>[22]</sup>. The sensitivity and specificity of CA19-9 for the diagnosis of pancreatic cancer are higher than those of CEA, CA50 and CA242<sup>[23-25]</sup>. CA19-9 has become a predominant tumor marker for the diagnosis of pancreatic adenocarcinoma. It was reported that CA19-9 level is useful in diagnosis and prognosis of pancreatic cancer<sup>[26,27]</sup>. However, little is known about the value of serum CA19-9 levels in evaluating the resectability of pancreatic carcinoma<sup>[28]</sup>. This study was to find whether preoperative serum CA19-9 is a useful marker for evaluating the resectability of pancreatic cancer.

In the present study, the differences between patients with resectable and unresectable pancreatic cancer were significant ( $P < 0.01$ ). The AUC was 0.918 and 95% CI was 0.843-0.992, suggesting that the preoperative serum CA19-9 level is an efficient marker for evaluating the resectability of pancreatic carcinoma. When the cut-off value of CA19-9 was 353.15 U/mL according to the point closest to the upper left-hand corner of the graph, the sensitivity, specificity, positive and negative predictive value was 93.1%, 78.3%, 84.38% and 90%, respectively, indicating that increased serum levels of CA19-9 ( $> 353.15$  U/mL) can be regarded as an ancillary parameter for the unresectable pancreatic cancer<sup>[29]</sup>. Pancreatic

cancer was resectable only in 4 patients whose preoperative serum CA19-9 level was over 353.15 U/mL (Table 3 and Figure 1).

Kilic *et al*<sup>[30]</sup> reported that the sensitivity, specificity, positive and negative predictive value are 82.4%, 92.3%, 91.4% and 83.9%, respectively, in 51 patients, and the cut-off value of CA19-9 is 256.4 U/mL. Their results are similar to our data, but the cut-off value was lower than that in our study (256.4 U/mL *vs* 353.15 U/mL). The discrepancy may be due the sample size, and the unadjusted CA19-9 level according to the bilirubin level.

In conclusion, a preoperative serum CA19-9 level is a useful marker for evaluating the resectability of pancreatic cancer. Increased serum levels of CA19-9 ( $> 353.15$  U/mL) can be regarded as an ancillary parameter for unresectable pancreatic cancer.

## COMMENTS

### Background

At present, the best way of preoperative staging of pancreatic cancer is bolus-contrast and triple-phase helical computed tomography. However, approximately 25%-50% of patients with resectable disease on computed tomography are found to have unresectable lesions at laparotomy.

### Research frontiers

CA19-9 is the most widely used serum marker of pancreatic cancer. CA19-9 has been shown to correlate with the thyroid node metastasis (TNM) staging and tumor size in patients with pancreatic cancer. However, little is known about the value of serum CA19-9 levels in evaluating the resectability of pancreatic carcinoma.

### Innovations and breakthroughs

Receiver operating characteristic (ROC) curve analysis was used to evaluate the clinical value of serum CA19-9 levels in predicting the resectability of pancreatic carcinoma.

### Applications

Preoperative serum CA19-9 level may be a useful marker for evaluating the resectability of pancreatic cancer. Increased serum level of CA19-9 ( $> 353.15$  U/mL) may be regarded as an ancillary parameter for unresectable pancreatic cancer.

### Terminology

CA19-9 is a tumor-associated antigen initially described by Koprowski *et al* and has been widely used as a serum marker of pancreatic cancer. ROC curve has been widely accepted as the standard method for describing and comparing the accuracy of medical diagnostic tests. ROC curve is an efficient way to display and assess the predictive value of cut-off points.

### Peer review

This is a very interesting study. The authors used ROC analysis as an appropriate statistical method for defining the cut-off value of serum CA19-9 to discriminate between resectable and unresectable pancreatic cancer.

## REFERENCES

- Jemal A, Siegel R, Ward E, Hao Y, Xu J, Murray T, Thun MJ. Cancer statistics, 2008. *CA Cancer J Clin* 2008; **58**: 71-96
- Wang L, Yang GH, Lu XH, Huang ZJ, Li H. Pancreatic cancer mortality in China (1991-2000). *World J Gastroenterol* 2003; **9**: 1819-1823
- Warshaw AL, Fernandez-del Castillo C. Pancreatic carcinoma. *N Engl J Med* 1992; **326**: 455-465
- Tamm EP, Silverman PM, Charnsangavej C, Evans DB. Diagnosis, staging, and surveillance of pancreatic cancer. *AJR Am J Roentgenol* 2003; **180**: 1311-1323
- Vargas R, Nino-Murcia M, Trueblood W, Jeffrey RB Jr. MDCT in Pancreatic adenocarcinoma: prediction of vascular invasion and resectability using a multiphasic technique with curved planar reformations. *AJR Am J Roentgenol* 2004; **182**: 419-425

- 6 **Wakabayashi H**, Nishiyama Y, Otani T, Sano T, Yachida S, Okano K, Izuishi K, Suzuki Y. Role of 18F-fluorodeoxyglucose positron emission tomography imaging in surgery for pancreatic cancer. *World J Gastroenterol* 2008; **14**: 64-69
- 7 **Pisters PW**, Lee JE, Vauthey JN, Charnsangavej C, Evans DB. Laparoscopy in the staging of pancreatic cancer. *Br J Surg* 2001; **88**: 325-337
- 8 **Koopmann J**, Rosenzweig CN, Zhang Z, Canto MI, Brown DA, Hunter M, Yeo C, Chan DW, Breit SN, Goggins M. Serum markers in patients with resectable pancreatic adenocarcinoma: macrophage inhibitory cytokine 1 versus CA19-9. *Clin Cancer Res* 2006; **12**: 442-446
- 9 **Zou KH**, O'Malley AJ, Mauri L. Receiver-operating characteristic analysis for evaluating diagnostic tests and predictive models. *Circulation* 2007; **115**: 654-657
- 10 **Walter SD**, Sinuff T. Studies reporting ROC curves of diagnostic and prediction data can be incorporated into meta-analyses using corresponding odds ratios. *J Clin Epidemiol* 2007; **60**: 530-534
- 11 **Altman DG**, Bland JM. Diagnostic tests 2: Predictive values. *BMJ* 1994; **309**: 102
- 12 **Altman DG**, Bland JM. Diagnostic tests 3: receiver operating characteristic plots. *BMJ* 1994; **309**: 188
- 13 **Jemal A**, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. *CA Cancer J Clin* 2003; **53**: 5-26
- 14 **Pappas S**, Federle MP, Lokshin AE, Zeh HJ 3rd. Early detection and staging of adenocarcinoma of the pancreas. *Gastroenterol Clin North Am* 2007; **36**: 413-429, x
- 15 **Takhar AS**, Palaniappan P, Dhingsa R, Lobo DN. Recent developments in diagnosis of pancreatic cancer. *BMJ* 2004; **329**: 668-673
- 16 **Misek DE**, Patwa TH, Lubman DM, Simeone DM. Early detection and biomarkers in pancreatic cancer. *J Natl Compr Canc Netw* 2007; **5**: 1034-1041
- 17 **Delbeke D**, Pinson CW. Pancreatic tumors: role of imaging in the diagnosis, staging, and treatment. *J Hepatobiliary Pancreat Surg* 2004; **11**: 4-10
- 18 **Sahani DV**, Shah ZK, Catalano OA, Boland GW, Brugge WR. Radiology of pancreatic adenocarcinoma: current status of imaging. *J Gastroenterol Hepatol* 2008; **23**: 23-33
- 19 **Karmazanovsky G**, Fedorov V, Kubyshkin V, Kotchatkov A. Pancreatic head cancer: accuracy of CT in determination of resectability. *Abdom Imaging* 2005; **30**: 488-500
- 20 **Hanbidge AE**. Cancer of the pancreas: the best image for early detection--CT, MRI, PET or US? *Can J Gastroenterol* 2002; **16**: 101-105
- 21 **Andersson R**, Vagianos C, Williamson R. Preoperative staging and evaluation of resectability in pancreatic ductal adenocarcinoma. *HPB (Oxford)* 2004; **6**: 5-12
- 22 **Koprowski H**, Steplewski Z, Mitchell K, Herlyn M, Herlyn D, Fuhrer P. Colorectal carcinoma antigens detected by hybridoma antibodies. *Somatic Cell Genet* 1979; **5**: 957-971
- 23 **Wu X**, Lu XH, Xu T, Qian JM, Zhao P, Guo XZ, Yang XO, Jiang WJ. [The diagnostic value of serum carcinoma markers, fecal K-ras and p53 gene mutation in pancreatic cancers] *Zhonghua Neike Zazhi* 2005; **44**: 741-744
- 24 **Okusaka T**, Okada S, Sato T, Wakasugi H, Saisho H, Furuse J, Ishikawa O, Matsuno S, Yokoyama S. Tumor markers in evaluating the response to radiotherapy in unresectable pancreatic cancer. *Hepatogastroenterology* 1998; **45**: 867-872
- 25 **Liao Q**, Zhao YP, Yang YC, Li LJ, Long X, Han SM. Combined detection of serum tumor markers for differential diagnosis of solid lesions located at the pancreatic head. *Hepatobiliary Pancreat Dis Int* 2007; **6**: 641-645
- 26 **Kang CM**, Kim JY, Choi GH, Kim KS, Choi JS, Lee WJ, Kim BR. The use of adjusted preoperative CA 19-9 to predict the recurrence of resectable pancreatic cancer. *J Surg Res* 2007; **140**: 31-35
- 27 **Zhao JZ**, Wu BH. Clinical significance of CA19-9 in diagnosis of digestive tract tumors. *China Nati J New Gastroenterol* 1997; **3**: 253-254
- 28 **Schlieman MG**, Ho HS, Bold RJ. Utility of tumor markers in determining resectability of pancreatic cancer. *Arch Surg* 2003; **138**: 951-955; discussion 955-956
- 29 **Zakowski L**, Seibert C, VanEyck S. Evidence-based medicine: answering questions of diagnosis. *Clin Med Res* 2004; **2**: 63-69
- 30 **Kilic M**, Gocmen E, Tez M, Ertan T, Keskek M, Koc M. Value of preoperative serum CA 19-9 levels in predicting resectability for pancreatic cancer. *Can J Surg* 2006; **49**: 241-244

S- Editor Li DL L- Editor Wang XL E- Editor Liu Y



RAPID COMMUNICATION

## Effect of fragile histidine triad gene transduction on proliferation and apoptosis of human hepatocellular carcinoma cells

Rong-Hua Xu, Liang-Yan Zheng, Dong-Lei He, Jian Tong, Li-Ping Zheng, Wu-Ping Zheng, Jin Meng, Li-Ping Xia, Cong-Jun Wang, Ji-Lin Yi

Rong-Hua Xu, Liang-Yan Zheng, Dong-Lei He, Jian Tong, Li-Ping Zheng, Wu-Ping Zheng, Jin Meng, Li-Ping Xia, The Affiliated Hospital of Hainan Medical College, Haikou 570102, Hainan Province, China

Cong-Jun Wang, Department of General Surgery, First People's Hospital Affiliated to Shanghai Jiaotong University, Shanghai 200080, China

Ji-Lin Yi, Department of General surgery, Tongji Hospital, Tongji Medical College of Huazhong University of Science and Technology, Wuhan 430030, Hubei Province, China

**Author contributions:** Xu RH and Zheng LY wrote the paper and organized the figures; Xu RH, He DL, Tong J, Zheng LP, Zheng WP, Meng J, Xia LP did cell culture, transduction, RT-PCR, Western blot and animal experiments; Wang CJ carried out the statistical analysis; Yi JL supervised the writing and organization process.

**Correspondence to:** Dr. Rong-Hua Xu, Department of Oncology Surgery, The Affiliated Hospital of Hainan Medical College, Haikou 570102, Hainan Province, China. [xu\\_ronghua2231@hotmail.com](mailto:xu_ronghua2231@hotmail.com)

Telephone: +86-898-66528115

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### Abstract

**AIM:** To evaluate the inhibitory effects of human fragile histidine triad (FHIT) gene on cell proliferation and apoptosis in human hepatocellular carcinoma line Hep3B *in vitro*.

**METHODS:** A recombinant pcDNA3.1 (+)/FHIT including the functional region of FHIT gene was constructed and transferred into human hepatocellular carcinoma cells *in vitro*. mRNA and protein expression of the FHIT gene in the transfected cells was detected by RT-PCR and Western blot, respectively. The effect of FHIT on proliferation was detected by MTT assay. Changes in cell cycle and apoptosis were assayed by flow cytometry. Five mice received subcutaneous transplantation of Hep3B-FHIT; 5 mice received subcutaneous transplantation of normal Hep3B and Hep3B-C as controls. The body weight of nude mice and tumor growth were measured.

**RESULTS:** RT-PCR and Western blot analysis showed that the expression level of FHIT-mRNA and FHIT protein was higher in Hep3B cells after infection with

pcDNA3.1 (+)/FHIT. The growth of Hep3B cells treated with pcDNA3.1 (+)/FHIT was significantly inhibited. The pcDNA3.1 (+)/FHIT-transfected Hep3B cells showed a significantly higher cell rate at G<sub>0</sub>-G<sub>1</sub> phase and increased apoptosis in comparison with controls ( $P < 0.05$ ). The growth of transplanted tumor was inhibited markedly by FHIT. Tumors arising from the Hep3B-FHIT cells occurred much later than those arising from the Hep3B and Hep3B-C cells. The growth of Hep3B-FHIT cells was slow and the tumor volume was low.

**CONCLUSION:** Transduction of FHIT gene inhibits the growth of human hepatocellular carcinoma cells and induces cell apoptosis *in vivo* and *in vitro*.

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**Key words:** Hepatocellular carcinoma; Gene therapy; Fragile histidine triad gene

**Peer reviewer:** Luis Rodrigo, Professor, Gastroenterology Service, Hospital Central de Asturias, c/ Celestino Villamil, s.n., Oviedo 33.006, Spain

Xu RH, Zheng LY, He DL, Tong J, Zheng LP, Zheng WP, Meng J, Xia LP, Wang CJ, Yi JL. Effect of fragile histidine triad gene transduction on proliferation and apoptosis of human hepatocellular carcinoma cells. *World J Gastroenterol* 2008; 14(23): 3754-3758 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3754.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3754>

### INTRODUCTION

Fragile histidine triad (FHIT) gene has been successfully cloned on chromosome 3p14.2 using an exon trapping method for gene capture<sup>[1-4]</sup>. It was reported that abnormal FHIT gene exists in majority of tumors, and is an important candidate tumor-suppressing gene<sup>[5-10]</sup>. In the hepatoma cell line Hep3B, the FHIT gene, mRNA, and protein are abnormal<sup>[11]</sup>. We constructed a recombinant pcDNA3.1 (+) /FHIT vector containing human (FHIT) gene, which was used to transfect human hepatoma Hep3B cells *in vitro* and *in vivo* to explore the

effect of *FHIT* gene on proliferation or apoptosis of hepatocellular carcinoma cells.

## MATERIALS AND METHODS

### **Bacteria, plasmids and cells**

*E. coli* DH5 $\alpha$  and eukaryotic expression vector pcDNA3.1 (+) were routinely kept in our laboratory. PBluescript SK *FHIT* plasmid with a full-length *FHIT* cDNA was kindly given by Professor Xiao-Fan Wang, Duke University, USA. Hep3B cells were purchased from the Chinese Academy of Sciences in Shanghai.

### **Main reagents**

A reverse transcription-polymerase chain reaction (RT-PCR) kit was purchased from Takara, Japan. *FHIT* antibody was purchased from Zymed, USA. A cell cycle assay kit was purchased from Becton Dickinson, USA.

### **Enzyme cutting and identification of DNA sequence**

To establish an enzyme-cutting reaction, pcDNA3.1 (or PBluescript SK *FHIT*), *Bam*H I and *Xba* I were mixed in a water bath at 37°C for 2 h. The mixture was electrophoresed, and 1.0 kb *FHIT* gene fragments, and a 5.4 kb linear pcDNA3.1 were retrieved separately with electrophoresis coagulation plastic boxes. For constructing the pcDNA3.1 (+) /*FHIT* vector, *FHIT* gene fragments, linear pcDNA3.1 and T4 ligase were mixed in an Eppendorf tube at 16°C for 12 h to establish a recombinant plasmid reaction. The pcDNA3.1 *FHIT* vector was transferred into competent bacteria DH5, and the clones were cultured and identified.

### **Cell culture and transfection**

Hep3B cells were cultured at 37°C in Dulbecco's modified Eagle's medium containing 10% fetal calf serum (Gibco), 1.0 mmol/L sodium pyruvate, 0.1 mmol/L non-indispensable amino acid and 5% CO<sub>2</sub>, at a saturated humidity. Hep3B cells with strong growth were seeded in 6-well plates. pcDNA3.1 (+) /*FHIT* was transfected into the Hep3B cells at the density of 70% with Lipofectamine 2000 (liposome transfection kit) following its manufacturer's instructions. After transfection, the cells were screened using G418 (500 mg/L) and the screening was maintained with G418 (250 mg/L). Hep3B cells transfected with *FHIT* gene and empty vector were named Hep3B-*FHIT* and Hep3B-C, respectively. Parent Hep3B cells were used as an untransfected control.

### **RT-PCR**

*FHIT* gene was amplified with primers (P1: 5'-ATGTCGTTTCAGA-3', P2: 5'-CTGAAAGTACAC-3') using the Hep3B-*FHIT* cells screened by G418<sup>[12]</sup>. Total RNA was extracted with TRIZOL method and underwent reverse-transcription PCR. The PCR products were electrophoresed on a 1% agarose gel. One  $\mu$ L of the resulting cDNA was added into the PCR reaction mixture (containing 1.0  $\mu$ L of forward primer (10 pmol/ $\mu$ L), 1.0  $\mu$ L of reverse primer (10 pmol/ $\mu$ L),

1.0  $\mu$ L of dNTPs (10 mmol/L), 0.5  $\mu$ L Taq DNA polymerase). PCR was performed for 30 cycles, each amplification cycle consisting of denaturation at 94°C for 1 min, primer annealing at 44°C for 1 min, and extension at 72°C for 45 s. The PCR products were analyzed on 10 g/L agarose gel containing ethidium bromide.

### **Western blot**

The screened Hep3B-*FHIT* cells were lysed with a cell-lysing solution. The protein concentration in the supernatant after centrifugation was measured by Bradford assay. The supernatant was electrophoresed on a 12% SDS-polyacrylamide gel. The electrophoresed proteins were transferred to nitrocellulose membrane, blocked with calf serum, combined with *FHIT* antibody and horseradish peroxidase secondary antibody, developed by enhanced chemiluminescence (ECL) and photographed.

### **Measurements of growth capacity of transfected cells by thiazolyl tetrazolium (MTT) assay**

Ten thousand cells/well were seeded in 96-well plates (200  $\mu$ L/well), with 6 wells for each cell group and 3 wells for control. MTT (5 g/L) was used as an incubation solution. DMSO was added to the cell culture for 4 h. The luminous absorbance was measured by ELISA (wavelength of 570 nm). The measurement was repeated once a day for 6 days.

### **Flow cytometry analysis of cell cycle and apoptosis**

After trypsinization, the cells were washed twice with phosphate-buffered saline (PBS), fixed with cold ethanol (70%) overnight, suspended in PBS after ethanol was purged from PBS, and stained with propidium iodide (50  $\mu$ g/mL). The samples were examined by flow cytometry.

### **Animal experiments**

After forty-eight hours, 2  $\mu$ g of pcDNA3.1 (+) and pcDNA3.1 (+)/*FHIT* plasmid was transfected with Lipofectamine 2000 into Hep3B cell lines, and clones were obtained by screening with G418 for 14 d. The clones were cultured. The study was approved by the Experimental Animal Committee of Tongji Medical College, Huazhong University of Science and Technology, and all animal experiments adhered to the Animal Welfare Committee Guidelines. Male athymic BALB/c nu/ nu mice (4-6 wk old) were obtained from the Institute of Materia Medica (Tongji Medical College, Wuhan, China) and housed in laminar-flow cabinets under specific pathogen-free (SPF) conditions. Fifteen male mice were randomized into Hep3B-*FHIT*/nude, Hep3B-C/nude and Hep3B/nude groups, 5 in each group. A suspension of Hep3B-*FHIT* or Hep3B-C or Hep3B cells (10<sup>7</sup> cells in 0.15 mL Hanks' solution) was injected into the back of mice in each group. After implantation, tumor growth was detected weekly by measuring its diameter with a Vernier caliper. Tumor volume (TV) was calculated using the following formula: TV (mm<sup>3</sup>) = d<sup>2</sup>  $\times$  D/2, where d is the shortest diameter and D



is the longest diameter. Animals were sacrificed 7 wk after implantation, and samples were harvested. The body weight of mice, and tumor growth were measured.

### Statistical analysis

The data were expressed as mean  $\pm$  SD. Student's two-sided *t*-test was used to compare the values of the test and control samples by software SPSS11.5. *P* < 0.05 was considered statistically significant.

## RESULTS

### Identification of plasmid

The sequence of PBluescript SK FHIT detected in Shanghai Biological Engineering Company was identical to that of FHIT mRNA in GenBank (Lot. Number: NM-002012). After enzyme-cut, agarose gel electrophoresis showed two DNA bands of plasmid PBluescript FHIT (1.0 kb and 2.7 kb), two DNA bands of plasmid pcDNA3.1 (+)/FHIT (1.0 kb and 5.4 kb) (Figure 1); thus, pcDNA3.1 (+)/FHIT was successfully constructed.

### FHIT-mRNA expression in transfected cells

After transfected with pcDNA3.1 (+)/FHIT, RT-PCR showed a 400 bp band of Hep3B-FHIT cells. However, the empty vector-transfected, or native cells did not show any band (Figure 2A).

### FHIT protein expression in transfected cells

Western blotting demonstrated FHIT protein (17 kDa) in the cells transfected with pcDNA3.1 (+)/FHIT, but no FHIT protein in empty vector-transfected or native cells (Figure 2B).

### Growth rates before and after transfection

Thiazolyl tetrazolium (MTT) assay showed that the growth rate of transfected cells was significantly lower than that of native or empty vector-transfected cells, especially after the logarithm growth phase (*P* < 0.05, Figure 3A).

### Changes in cell cycle and apoptosis rate

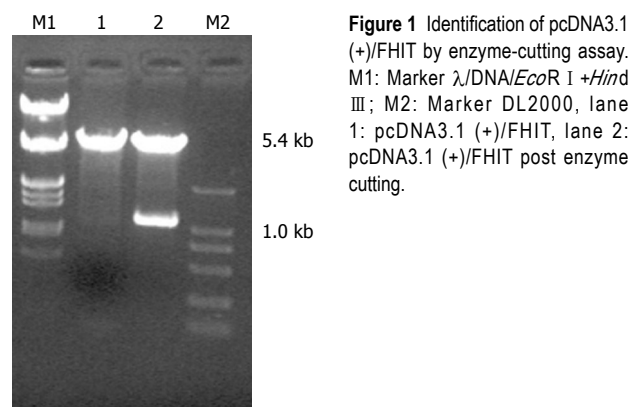
Flow cytometry analysis revealed that the number of Hep3B cells decreased in the G<sub>2</sub>/M and S phases, but increased in the G<sub>0</sub>/G<sub>1</sub> phase. The apoptosis rate was higher for transfected cells than for empty vector-transfected or native cells (*P* < 0.05, Table 1).

### Tumor growth

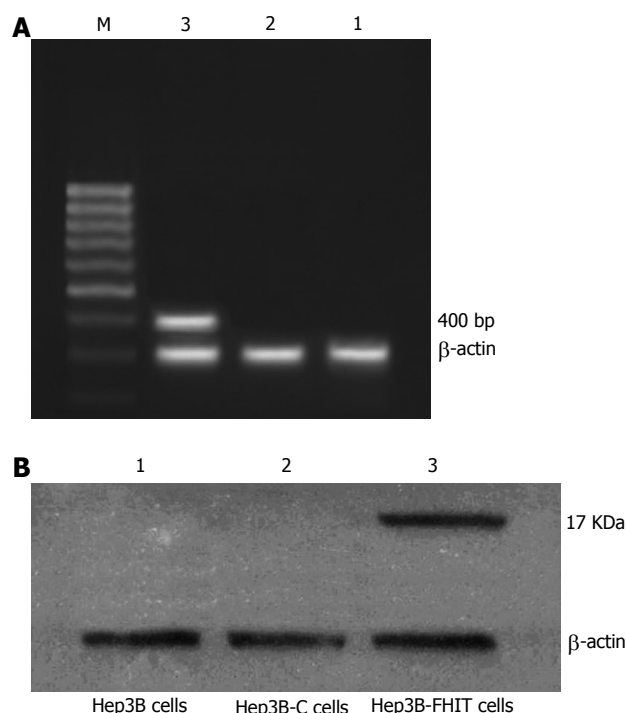
The growth of transplanted tumor was inhibited markedly by FHIT, showing that tumors arising from the Hep3B-FHIT cells occur much later than those arising from the Hep3B and Hep3B-C cells. Hep3B-FHIT cells grew slowly and their volume was small (Table 2, Figure 3B).

## DISCUSSION

It has been reported that FHIT is the first cancer-suppressive gene which links fragile sites to tumors.



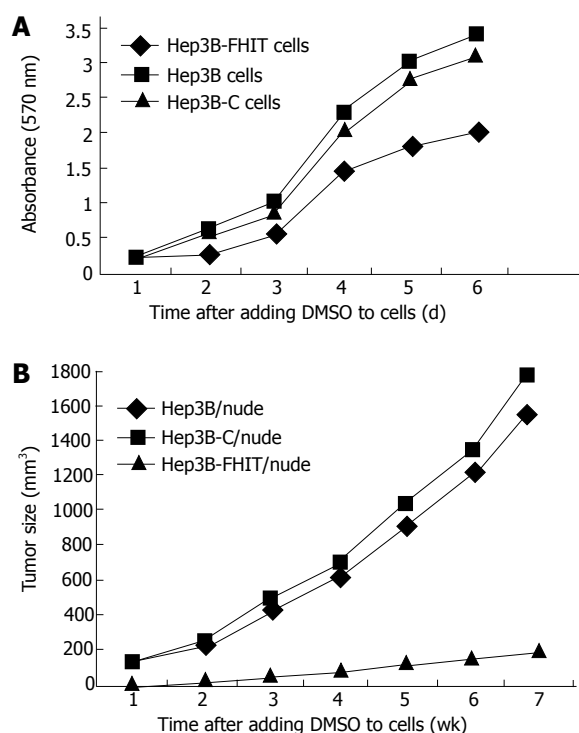
**Figure 1** Identification of pcDNA3.1 (+)/FHIT by enzyme-cutting assay. M1: Marker  $\lambda$ /DNA/EcoR I + Hind III; M2: Marker DL2000, lane 1: pcDNA3.1 (+)/FHIT, lane 2: pcDNA3.1 (+)/FHIT post enzyme cutting.



**Figure 2** Expression of FHIT mRNA and protein in Hep3B-FHIT, Hep3B-C and Hep3B cells. **A:** FHIT mRNA; **B:** FHIT protein. M: 1000 bp marker; Lane 1: Hep3B cells; lane 2: Hep3B-C cells; lane 3: Hep3B-FHIT cells.

Abnormality of the *FHIT* gene is an early event during tumor development<sup>[13-17]</sup>. Fracture at the fragile site results in mutation and inactivation of the *FHIT* gene; thus leading to abnormal cell growth<sup>[18-22]</sup>. Like many other tumors, hepatoma is characterized by highly mutated *FHIT* gene or gene loss. Yuan *et al*<sup>[11]</sup> found that down-regulation of the *FHIT* gene is detected in 64.3% cell lines (four cell lines showing mRNA down-regulation did not express FHIT protein); allelic loss of intron 5 of the *FHIT* gene was detected in 29.4% hepatomas by *in situ* hybridization; structural alterations of chromosome 3p were identified in 61.5% of hepatocellular carcinomas; expression of FHIT protein was not detectable in 50% primary tumors with immunostaining<sup>[11]</sup>.

FHIT may modulate APnA by hydrolysis of APnA to yield adenosine 5'-monophosphate (AMP). Due to mutation of FHIT, loss of APnA hydrolase activity results in elevated AP3A levels, intracellular accumulation of APnA strengthens the growth signal transduction,



**Figure 3** Growth curves. **A:** Growth curves of human hepatocellular carcinoma cells. Luminous absorbance of Hep3B-FHIT, Hep3B-C and Hep3B cells was measured by ELISA (wavelength of 570 nm) after DMSO was added. The measurement was done once a day for 6 d; **B:** Growth curves of tumors after implantation of Hep3B, Hep3B-C or Hep3B-FHIT cells in nude mice. The mice were injected sc with  $1 \times 10^7$  (0.15 mL/mouse) Hep3B, Hep3B-C or Hep3B-FHIT cells. After implantation, tumor growth was detected weekly.

and blocks growth inhibition and apoptosis, thus contributing to carcinogenesis. Additionally, the activity of FHIT on mRNA cap analogs raises the possibility that failure of a decapping function might be tumorigenic<sup>[12,23,24]</sup>.

Gramantieri *et al.*<sup>[13]</sup> examined the mRNA FHIT expression in both cancerous and matched non-cancerous tissues in 28 cases of hepatocellular carcinoma (HCC) and 10 normal livers, and abnormal FHIT transcripts were detected in 13 cases (in cancerous tissue from 11 cases and in non-cancerous tissue from 2 cases). No abnormal FHIT transcripts were found in normal livers. It was recently reported that many other factors can also induce liver carcinogenesis in rats<sup>[25-29]</sup>.

Siprashvili *et al.*<sup>[30]</sup> found that transduction of wild-type FHIT or mutative FHIT with no hydrolase activity into tumor cells lacking FHIT gene did not show any advantage to cell growth, and no difference was found in suppression of tumorigenicity, suggesting that FHIT suppresses the tumorigenicity through FHIT protein in combination with its substrate rather than through hydrolysis of APnA. Its more precise mechanism needs to be further explored.

In this study, the full-length of the FHIT cDNA eukaryotic expression vector was transfected into human hepatocellular carcinoma cell line, Hep3B, to manipulate FHIT expression, which alters the biological features of Hep3B cell line. The results indicate that successfully

**Table 1** Effect of FHIT gene on cell growth and apoptosis in human hepatocellular carcinoma (mean  $\pm$  SD)

Cell type	G <sub>0</sub> /G <sub>1</sub>	S	G <sub>2</sub> /M	Apoptosis rate (%)
Hep3B	54.36 $\pm$ 0.78	17.40 $\pm$ 1.32	28.01 $\pm$ 1.12	3.78 $\pm$ 0.36
Hep3B-C	53.17 $\pm$ 0.52	18.23 $\pm$ 2.51	28.55 $\pm$ 0.55	3.52 $\pm$ 0.33
Hep3B-FHIT <sup>a</sup>	72.23 $\pm$ 0.84	12.57 $\pm$ 0.42	15.12 $\pm$ 1.31	9.74 $\pm$ 0.43

<sup>a</sup>*P* < 0.05 vs Hep3B or Hep3B-C.

**Table 2** Mean tumor formation time and mean tumor weight in nude mice implanted with Hep3B, Hep3B-C or Hep3B-FHIT cells (*n* = 5, mean  $\pm$  SD)

Group	Mean tumor formation time (d)	Mean tumor weight (g)
Hep3B/nude	3.22 $\pm$ 0.31	3.66 $\pm$ 0.40
Hep3B-C/nude	3.08 $\pm$ 0.34	3.94 $\pm$ 0.39
Hep3B-FHIT/nude	12.17 $\pm$ 1.19	0.60 $\pm$ 0.04
Total <i>F</i> value	72.38	168.36
Total <i>P</i> value	0.00	0.00

transfected Hep3B cells could express FHIT mRNA and FHIT protein, whereas the empty-vector transfected Hep3B or native cells did not express them. Moreover, cell proliferation and differentiation were significantly decreased at the G<sub>2</sub>/M and S phases, and cell apoptosis increased at the G<sub>0</sub>/G<sub>1</sub> phases of Hep3B cells transfected with FHIT gene as compared with the control group, suggesting that FHIT gene plays an important role in blocking cell growth at the G<sub>1</sub> phase, and in inducing cell apoptosis synergized by other apoptosis-inducing factors. Furthermore, transfer of FHIT gene could inhibit the growth of human hepatocellular carcinoma cells, and induce cell apoptosis *in vivo*.

In conclusion, FHIT mRNA, and protein are expressed in FHIT-infected Hep3B cells, thus leading to low proliferation, and high apoptosis of HCC cells. Transfection of FHIT gene into human hepatocellular carcinoma cells is a promising therapeutic approach to HCC.

## COMMENTS

### Background

Abnormal fragile histidine triad (FHIT) gene, an important candidate tumor-suppressing gene, exists in a majority of tumors. In the hepatoma cell line Hep3B, the FHIT gene, mRNA, and protein is abnormal. Therefore, we constructed a recombinant pcDNA3.1 (+)/FHIT vector containing human fragile histidine triad (FHIT) gene, which was used to transfect human hepatoma Hep3B cells *in vitro* and *in vivo* to explore the effect of FHIT gene on proliferation or apoptosis of hepatocellular carcinoma cells.

### Research frontiers

Application of gene transfer technologies in treatment of cancer has led to the development of new experimental strategies like inhibition of oncogenes, restoration of tumor-suppressor genes and enzyme/prodrug therapy (GDEPT). These strategies are being evaluated for the treatment of primary and metastatic liver cancer.

### Innovations and breakthroughs

In this study, we constructed a recombinant pcDNA3.1 (+)/FHIT vector containing human FHIT gene, which can be used to transfect human hepatoma Hep3B cells (FHIT null) *in vitro* and *in vivo*. Transfection of FHIT gene could

inhibit the growth of human hepatocellular carcinoma cells, and induce cell apoptosis.

### Applications

Transfection of *FHIT* gene into human hepatocellular carcinoma cells is a promising therapeutic approach to HCC.

### Peer review

In this paper, the authors evaluated the effect of human *FHIT* gene on cell proliferation and apoptosis in hepatocellular carcinoma *in vitro* and *in vivo*. It is a very interesting paper, and the study is well designed. It provides a potential therapeutic target for hepatocellular carcinoma.

## REFERENCES

- Ohta M, Inoue H, Cotticelli MG, Kastury K, Baffa R, Palazzo J, Siprashvili Z, Mori M, McCue P, Druck T, Croce CM, Huebner K. The FHIT gene, spanning the chromosome 3p14.2 fragile site and renal carcinoma-associated t(3;8) breakpoint, is abnormal in digestive tract cancers. *Cell* 1996; **84**: 587-597
- Sozzi G, Veronese ML, Negrini M, Baffa R, Cotticelli MG, Inoue H, Tornielli S, Pilotti S, De Gregorio L, Pastorino U, Pierotti MA, Ohta M, Huebner K, Croce CM. The FHIT gene 3p14.2 is abnormal in lung cancer. *Cell* 1996; **85**: 17-26
- Negrini M, Monaco C, Vorechovsky I, Ohta M, Druck T, Baffa R, Huebner K, Croce CM. The FHIT gene at 3p14.2 is abnormal in breast carcinomas. *Cancer Res* 1996; **56**: 3173-3179
- Virgilio L, Shuster M, Gollin SM, Veronese ML, Ohta M, Huebner K, Croce CM. FHIT gene alterations in head and neck squamous cell carcinomas. *Proc Natl Acad Sci USA* 1996; **93**: 9770-9775
- Sozzi G, Pastorino U, Moiraghi L, Tagliabue E, Pezzella F, Ghirelli C, Tornielli S, Sard L, Huebner K, Pierotti MA, Croce CM, Pilotti S. Loss of FHIT function in lung cancer and preinvasive bronchial lesions. *Cancer Res* 1998; **58**: 5032-5037
- Campiglio M, Pekarsky Y, Menard S, Tagliabue E, Pilotti S, Croce CM. FHIT loss of function in human primary breast cancer correlates with advanced stage of the disease. *Cancer Res* 1999; **59**: 3866-3869
- Sorio C, Baron A, Orlandini S, Zamboni G, Pederzoli P, Huebner K, Scarpa A. The FHIT gene is expressed in pancreatic ductular cells and is altered in pancreatic cancers. *Cancer Res* 1999; **59**: 1308-1314
- Birrer MJ, Hendricks D, Farley J, Sundborg MJ, Bonome T, Walts MJ, Geradts J. Abnormal Fhit expression in malignant and premalignant lesions of the cervix. *Cancer Res* 1999; **59**: 5270-5274
- Thiagalingam S, Lisitsyn NA, Hamaguchi M, Wigler MH, Willson JK, Markowitz SD, Leach FS, Kinzler KW, Vogelstein B. Evaluation of the FHIT gene in colorectal cancers. *Cancer Res* 1996; **56**: 2936-2939
- Huebner K, Croce CM. Cancer and the FRA3B/FHIT fragile locus: it's a HIT. *Br J Cancer* 2003; **88**: 1501-1506
- Yuan BZ, Keck-Waggoner C, Zimonjic DB, Thorgeirsson SS, Popescu NC. Alterations of the FHIT gene in human hepatocellular carcinoma. *Cancer Res* 2000; **60**: 1049-1053
- Barnes LD, Garrison PN, Siprashvili Z, Guranowski A, Robinson AK, Ingram SW, Croce CM, Ohta M, Huebner K. Fhit, a putative tumor suppressor in humans, is a dinucleoside 5',5''-P1,P3-triphosphate hydrolase. *Biochemistry* 1996; **35**: 11529-11535
- Gramantieri L, Chieco P, Di Tomaso M, Masi L, Piscaglia F, Brillanti S, Gaiani S, Valgimigli M, Mazziotti A, Bolondi L. Aberrant fragile histidine triad gene transcripts in primary hepatocellular carcinoma and liver cirrhosis. *Clin Cancer Res* 1999; **5**: 3468-3475
- Zochbauer-Muller S, Wistuba II, Minna JD, Gazdar AF. Fragile histidine triad (FHIT) gene abnormalities in lung cancer. *Clin Lung Cancer* 2000; **2**: 141-145
- Ishii H, Ozawa K, Furukawa Y. Alteration of the fragile histidine triad gene early in carcinogenesis: an update. *J Exp Ther Oncol* 2003; **3**: 291-296
- Guler G, Uner A, Guler N, Han SY, Iliopoulos D, McCue P, Huebner K. Concordant loss of fragile gene expression early in breast cancer development. *Pathol Int* 2005; **55**: 471-478
- Weiske J, Albring KF, Huber O. The tumor suppressor Fhit acts as a repressor of beta-catenin transcriptional activity. *Proc Natl Acad Sci USA* 2007; **104**: 20344-20349
- Ishii H, Furukawa Y. Alterations of common chromosome fragile sites in hematopoietic malignancies. *Int J Hematol* 2004; **79**: 238-242
- Lee SH, Kim HY, Kim TJ, Park HK, Kim WH, Woo KM, Cho MH. Aberrant splicing of FHIT transcripts in human gastric cancer cell lines. *Res Commun Mol Pathol Pharmacol* 2002; **112**: 39-49
- McAvoy S, Ganapathiraju SC, Ducharme-Smith AL, Pritchett JR, Kosari F, Perez DS, Zhu Y, James CD, Smith DI. Non-random inactivation of large common fragile site genes in different cancers. *Cytogenet Genome Res* 2007; **118**: 260-269
- Smith DI, McAvoy S, Zhu Y, Perez DS. Large common fragile site genes and cancer. *Semin Cancer Biol* 2007; **17**: 31-41
- Iliopoulos D, Guler G, Han SY, Druck T, Ottey M, McCorkell KA, Huebner K. Roles of FHIT and WWOX fragile genes in cancer. *Cancer Lett* 2006; **232**: 27-36
- Zawacka-Pankau J, Podhajski AJ. Expression and simple, one-step purification of fragile histidine triad (Fhit) tumor suppressor mutant forms in *Escherichia coli* and their interaction with protoporphyrin IX. *Biotechnol Lett* 2007; **29**: 877-883
- Huang K, Frey PA. Engineering human Fhit, a diadenosine triphosphate hydrolase, into an efficient dinucleoside polyphosphate synthase. *J Am Chem Soc* 2004; **126**: 9548-9549
- Tsujiuchi T, Sasaki Y, Oka Y, Konishi Y, Tsutsumi M. Fhit gene alterations in hepatocarcinogenesis induced by a choline-deficient L-amino acid-defined diet in rats. *Mol Carcinog* 2003; **36**: 147-152
- Golebiowski F, Kowara R, Pawelczyk T. Distribution of Fhit protein in rat tissues and its intracellular localization. *Mol Cell Biochem* 2001; **226**: 49-55
- Tsujiuchi T, Sasaki Y, Kubozoe T, Tsutsumi M, Konishi Y, Nakae D. Alterations of the Fhit gene in hepatocellular carcinomas induced by N-nitrosodiethylamine in rats. *Mol Carcinog* 2002; **34**: 19-24
- Asensio AC, Rodriguez-Ferrer CR, Oaknin S, Rotllan P. Biochemical and immunochemical characterisation of human diadenosine triphosphatase provides evidence for its identification with the tumour suppressor Fhit protein. *Biochimie* 2006; **88**: 461-471
- Han SY, Iliopoulos D, Druck T, Guler G, Grubbs CJ, Pereira M, Zhang Z, You M, Lubet RA, Fong LY, Huebner K. CpG methylation in the Fhit regulatory region: relation to Fhit expression in murine tumors. *Oncogene* 2004; **23**: 3990-3998
- Siprashvili Z, Sozzi G, Barnes LD, McCue P, Robinson AK, Eryomin V, Sard L, Tagliabue E, Greco A, Fusetti L, Schwartz G, Pierotti MA, Croce CM, Huebner K. Replacement of Fhit in cancer cells suppresses tumorigenicity. *Proc Natl Acad Sci USA* 1997; **94**: 13771-13776

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## Abdominal neurenteric cyst

Radoje Čolović, Marjan Micev, Miodrag Jovanović, Slavko Matić, Nikica Grubor, Henry Dushan E Atkinson

Radoje Čolović, Marjan Micev, Miodrag Jovanović, Slavko Matić, Nikica Grubor, Clinical Center of Serbia, Institute for Digestive Diseases, Belgrade 11000, Serbia

Henry Dushan E Atkinson, Surgical Directorate, Imperial College School of Medicine, St Mary's Hospital, Praed St, London W2 1NY, United Kingdom

**Author contributions:** Čolović R took part at the operation and wrote the article; Micev M did the histology and immunohistochemistry investigation; Jovanović M operated the patient; Matić S did the literature analysis, made an original translation to English, did all the corrections of the manuscript and the correspondence; Grubor N did the literature analysis; Atkinson HDE obtained some of the cited references and corrected the final English article.

**Correspondence to:** Slavko Matić, MD, PhD, Surgeon, Assistant professor of Surgery, Clinical Center of Serbia, Institute for Digestive Diseases, K. Todorovića 6 Street, Belgrade 11000, Serbia. [slavko.matic@med.bg.ac.yu](mailto:slavko.matic@med.bg.ac.yu)

Telephone: +381-64-2181949 Fax: +381-11-3031830

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### Abstract

Neurenteric cysts are extremely rare congenital anomalies, often presenting in the first 5 years of life, and are caused by an incomplete separation of the notochord from the foregut during the third week of embryogenesis. They are frequently accompanied with spinal or gastrointestinal abnormalities, but the latter may be absent in adults. Although usually located in the thorax, neurenteric cysts may be found along the entire spine. We present a 24-year-old woman admitted for epigastric pain, nausea, vomiting, low grade fever and leucocytosis. She underwent cystogastrostomy for a loculated cyst of the distal pancreas at the age of 4 years, which recurred when she was at the age of 11 years. Ultrasound and computer tomography (CT) scan revealed a 16 cm × 15 cm cystic mass in the body and tail of pancreas, with a 6-7 mm thickened wall. Laboratory data and chest X-ray were normal and spinal radiographs did not show any structural abnormalities. The patient underwent a complete cyst excision, and after an uneventful recovery, remained symptom-free without recurrence during the 5-year follow-up. The cyst was found to contain 1200 mL of pale viscous fluid. It was covered by a primitive single-layered cuboidal epithelium, along with specialized antral glandular parenchyma and hypoplastic primitive gastric mucosa. Focal glandular groups resembling

those of the body of the stomach were also seen. In addition, ciliary respiratory epithelium, foci of squamous metaplasia and mucinous glands were present. The wall of the cyst contained a muscular layer, neuroglial tissue with plexogenic nerve fascicles, Paccini corpuscle-like structures, hyperplastic neuro ganglionar elements and occasional psammomatous bodies, as well as fibroblast-like areas of surrounding stroma. Cartilagenous tissue was not found in any part of the cyst. Immunohistochemistry confirmed the presence of neurogenic elements marked by S-100, GFAP, NF and NSE. The gastric epithelium showed mostly CK7 and EMA immunoexpression, and the respiratory epithelium revealed a CK8 and CK18 immunoprofile without CK 10/13 positive elements, though neither CEA or AFP positive cells were found. To our knowledge, this is the first reported case of an abdominally located neurenteric cyst with no associated spinal anomalies.

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**Key words:** Neurenteric cyst; Congenital; Abdomen; Pancreas; Surgical excision

**Peer reviewers:** David Adams, Professor, Liver Research Laboratories, Institute for Biomedical Research, Queen Elizabeth Hospital, University of Birmingham, Birmingham B15 2TT, United Kingdom; Werner Hohenberger, Professor, Chirurgische Klinik und Poliklinik, Krankenhausstrasse 12, Erlangen D-91054, Germany

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### INTRODUCTION

Foregut duplications can be classified into three groups: enteric cysts (lined with intestinal epithelium), bronchogenic cysts (lined with respiratory epithelium), and neurenteric cysts (where enteric cysts are associated with vertebral anomalies or communications with the nervous system)<sup>[1,2]</sup>.

Neurenteric cysts are extremely rare congenital anomalies, usually diagnosed in infancy<sup>[3,4]</sup> and tend to be located in the right upper posterior mediastinum, but can be found anywhere along the spine or even intracra-



nially<sup>[1,5,6]</sup>. They may be associated with spinal anomalies such as hemivertebrae and anterior spina bifida<sup>[5]</sup>, and esophageal atresia<sup>[7,8]</sup>, though these associated abnormalities may be absent in adults<sup>[5]</sup>.

We describe the first reported case of an abdominal neurenteric cyst, with no associated spinal anomalies.

## CASE REPORT

A 24-year-old woman presented to our unit in 2001 with epigastric pain, nausea, vomiting, low grade fever and leucocytosis.

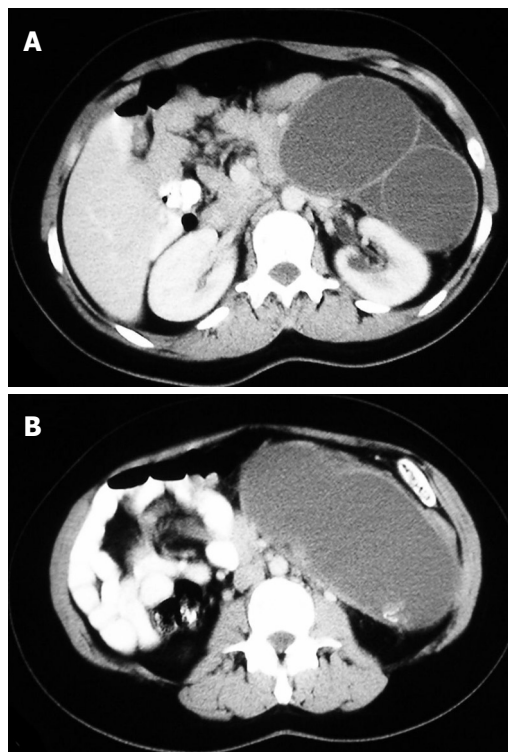
Her medical history and case notes showed that she underwent surgery for an 8 cm multi-loculated cyst in the region of the distal pancreas at the age of 4 years, and this was anastomosed to the posterior wall of the stomach. At the age of 11 years, a 2.5 cm recurrent cyst was found, though no significant enlargement was noted over the following decade, and she was largely symptom-free in almost 20 years.

On examination she was found to have a tender palpable mass in the upper left epigastrium. Ultrasonography (US) and computer tomography (CT) scan revealed a cystic mass in the region of the body and tail of the pancreas, measuring 16 cm × 15 cm, with a 6-7 mm thickened wall, and filled with dense fluid (Figure 1). Laboratory data and chest X-ray were normal and spinal radiographs did not show any structural abnormalities. Barium swallow revealed that the stomach displaced to the right and anteriorly, and a gastroscopy did not show any sign of the previous cystogastrostomy on the posterior wall of the stomach.

With a working diagnosis of a mucinous cystadenoma of the pancreas, the patient underwent laparotomy. The cystic tumor was located in the body and tail of the pancreas, and was adherent to the stomach, splenic vessels, prevertebral fascia, and spine. The pancreatic parenchyma in the region of the cyst was completely atrophied, and the previously performed anastomosis was obliterated. The cyst was completely excised, and was found to contain 1200 mL of pale viscous fluid. Laboratory analyses did not show any elevation of amylase or polymorphonuclear cells, and no growth occurred on microbiological culture.

The postoperative recovery was uneventful, the pre-operative symptoms completely resolved, and the patient remained symptom-free, without recurrence during the 5-year follow-up.

Macroscopically, the cyst wall, dark brown in color, was up to 14 mm in thickness and partly hyalinized. The inner surface of the cyst was smooth with some coarse sections, and some areas of the cyst wall also contained smaller cysts. Microscopic examination showed organoid organization resembling tissues, and organs similar to the embryonic foregut, including intestinal wall epithelial formation, partly covered by a primitive single-layered cuboidal epithelium, along with specialized antral glandular parenchyma, and hypoplastic primitive gastric mucosa. Focal glandular groups resembling those of the body of the stomach were also seen. In addition, ciliary



**Figure 1** Axial CT scan showing a large bilocular cyst in the region of the body and tail of the pancreas (A) and a second more caudal axial CT slice (B).

respiratory epithelium, foci of squamous metaplasia, and mucinous glands were present. The wall of the cyst contained a muscular layer, neuroglial tissue with plexogenic nerve fascicles, Paccini corpuscle-like structures, hyperplastic neuro ganglionic elements, and occasional psammomatous bodies, as well as fibroblast-like areas of surrounding stroma (Figures 2 and 3).

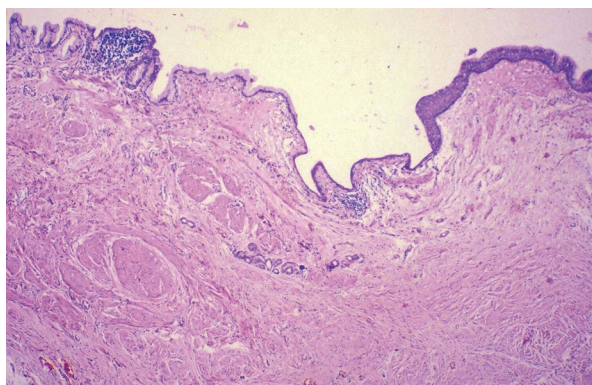
Immunohistochemistry confirmed the presence of neurogenic elements marked by S-100, GFAP, NF and NSE. The gastric epithelium showed mostly CK7 and EMA immunoreactivity, and the respiratory epithelium revealed a CK8 and CK18 immunoprofile without CK 10/13 positive elements, though neither CEA or AFP positive cells were found (Figure 4A-D).

## DISCUSSION

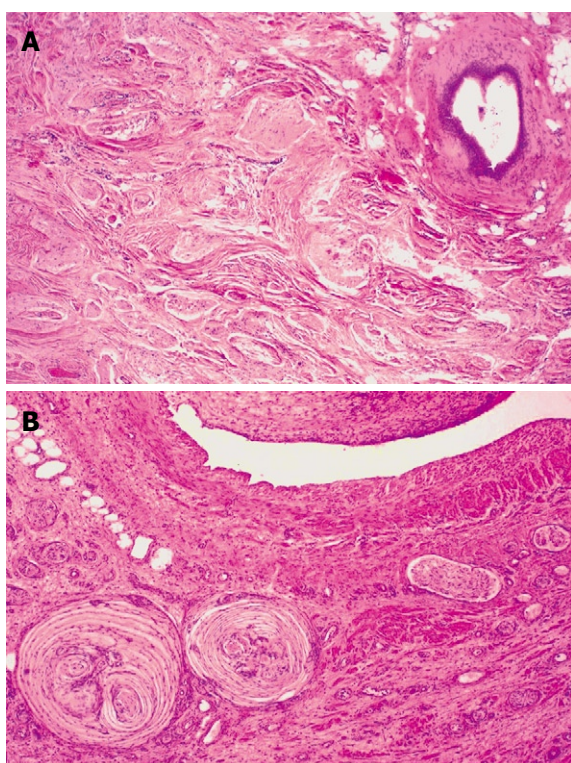
Neurenteric cysts may appear at any age, but are usually discovered during the first five years of life<sup>[4,9]</sup>. They are thought to develop early in the course of embryogenesis either due to incomplete separation of the notochord from the embryonic foregut (which are apposed), or due to herniation of the endoderm of the embryonic foregut into the dorsal ectoderm<sup>[10,11]</sup>. This attachment of the cyst to the notochord may prevent fusion of the vertebral bodies and lead to spinal anomalies<sup>[1,5]</sup>, indeed one third of patients have associated anomalies of the central nervous system or gastrointestinal tract<sup>[5]</sup>.

Neurenteric cysts may be multiloculated or septate, lined with ciliated, non-ciliated, columnar or cuboidal epithelium, and may resemble intestinal, duodenal or gastric mucosa. These cells are usually PAS-positive and





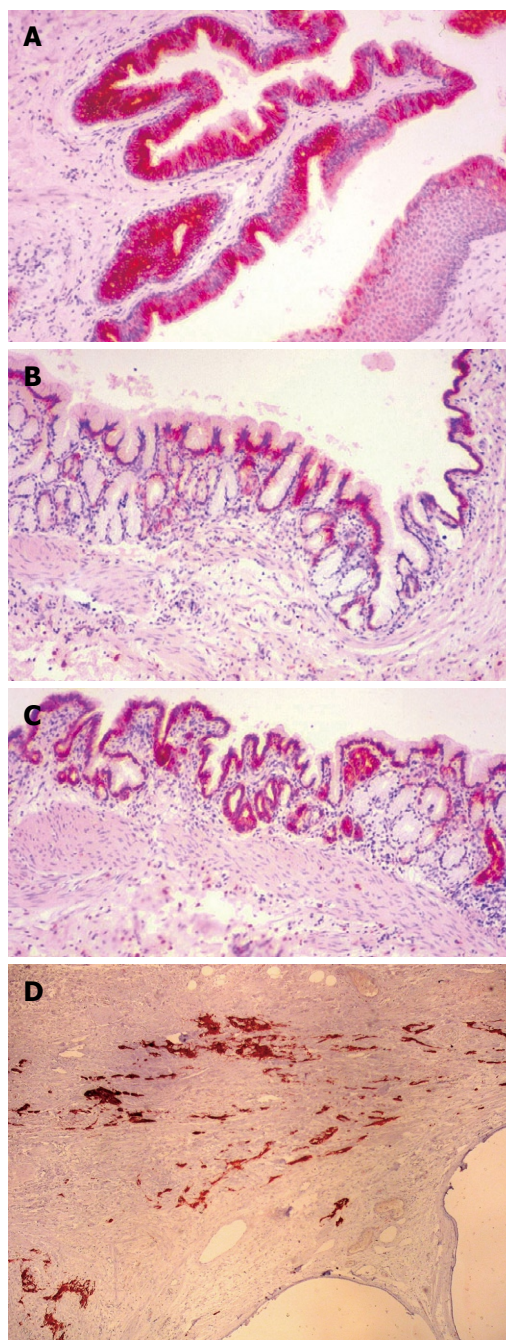
**Figure 2** Histological slides showing the full spectrum of epithelia within the cyst lining.



**Figure 3** Histological slide showing elements of an abortive muscular layer and focal irregular neuronal hyperplasia in the cyst wall (A) and a more irregular configuration of the cyst with various ganglio-neuronal elements including Paccinian corpuscles (B).

can contain mucus and globules, with occasional squamous cell metaplasia. A basal membrane is always present and the capsule consisting of fibrovascular tissue is fragile. The wall of the cyst may contain ganglionar cells, lymphatic tissue, pancreatic tissue, salivary glands or muscular tissue without serosa<sup>[1]</sup>. Cartilaginous tissue is never present<sup>[6]</sup>. The cysts usually contain clear, pale, yellowish or green viscous or mucinous fluid, depending on the presence or absence of previous hemorrhage<sup>[5]</sup>.

They are classified into Types A-C according to the histology. Type A cysts, the most described in the literature<sup>[5]</sup>, consist of simple lined cuboidal or columnar epithelium with or without cilia. Type B cysts include more complex gastrointestinal or tracheobronchial elements,



**Figure 4** Histological slides showing more specialized epithelia with consistent immunoreactivity to anti-cytokeratin 7 (A), anti-cytokeratin 18 (B), CA19-9 (C), and mixed abundant neuro-ganglionic/neuro-glial elements that were immunohistochemically verified (immunostaining is with the monoclonal antibody to glial fibrillar acidic protein) (D).

including mucous glands and smooth muscle cells in the cyst walls. In addition to these elements, type C cysts also contain ependymal and/or glial tissue<sup>[5]</sup>. Our patient had a septated type C cyst containing respiratory epithelium, gastric epithelium, and ependymal and glial tissue.

The clinical presentation usually depends on the size and location of the cyst. The patient, typically a small infant, often presents with respiratory distress, dyspnoea, stridor or a persistent cough, caused by pressure of the cyst on the lung. Respiratory distress is usually found with a mediastinal mass, and a vertebral anomaly coexisting

in more than 70% of pediatric patients with neurenteric cysts<sup>[3,9]</sup>. When located intraspinally, the cysts may cause motor and sensory neurological disturbance<sup>[9]</sup>. Chest pain, cardiac arrhythmias and dysphagia can also present, and in the rare cases of ulcer formation in ectopic gastric mucosa in the cyst wall, the patient can present with melaena or perforation of the cyst<sup>[1,12]</sup>.

The diagnosis is usually made by US, which characterizes the cyst and can evaluate for chest masses, and with the advent of high resolution US, these cysts can be detected as early as 18 wk gestation<sup>[1,5,13]</sup>. CT and nuclear magnetic resonance imaging (MRI) also have their place<sup>[14]</sup>, though the final diagnosis is based on histopathological examination.

Complete cyst excision is the recommended treatment<sup>[6,15]</sup>, with some patients requiring a simultaneous laminectomy<sup>[16]</sup>. If the cyst consists of two components, the symptomatic cyst should be excised first. If there is a combination of asymptomatic intraspinal and extraspinal cysts, the spinal cyst should be excised first in order to avoid any neurological deterioration which might occur during mediastinal cyst excision<sup>[6]</sup>.

Our patient underwent a cystogastrostomy at the age of 4 years, presumably with an underlying misdiagnosis of a pancreatic pseudocyst. However, the cyst was covered with an epithelial layer, and recurred. This case nicely demonstrates that the cyst should have previously been completely excised, and when this was finally done, the patient's symptoms resolved, with no cyst recurrence.

## REFERENCES

- 1 **Singh D**, Singh S, Kiraw R, Bhagwat SS. Neurenteric Cyst 2003. Available from URL: [http://www.bhj.org/journal/2003\\_4502\\_april/neurentericcyst\\_373.htm](http://www.bhj.org/journal/2003_4502_april/neurentericcyst_373.htm)
- 2 **Ravitch MM**. Mediastinal cysts and tumours. In: Welsch KJ, Randolph JG, Ravitch MN, O'Neill JAJ, Rowe MJ, editors. Pediatric Surgery Chicago: Year Book Medical Publishers, 1986: 606-614
- 3 **Fernandes ET**, Custer MD, Burton EM, Boulden TF, Wrenn EL Jr, Whittle AP, Edwards OP. Neurenteric cyst: surgery and diagnostic imaging. *J Pediatr Surg* 1991; **26**: 108-110
- 4 **Viladevall H**. Primitive gut morphogenesis 2004; 2006. Available from URL: <http://sprojects.mmi.mcgill.ca/embryology/gi/pgm.htm>
- 5 **Olavarria SA**, Diaz Guerrero DL, Yanis A. Neurenteric cyst 2000; 2006. Available from URL: <http://www.thefetus.net/page.php?id=230>
- 6 **Birmole BJ**, Kulkarni BK, Vaidya AS, Borwankar SS. Intrathoracic enteric foregut duplication cyst. *J Postgrad Med* 1994; **40**: 228-230
- 7 **Kapouleas GP**, Keramidas DC, Soutis M. Bochdalek's hernia combined with agenesis of the pericardium and intrathoracic solitary cyst of the liver. *Z Kinderchir* 1989; **44**: 377-378
- 8 **Hemalatha V**, Batcup G, Brereton RJ, Spitz L. Intrathoracic foregut cyst (foregut duplication) associated with esophageal atresia. *J Pediatr Surg* 1980; **15**: 178-180
- 9 **Mancini M**, Eggerstedt IM. Mediastinal Cysts 2006; 2006. Available from URL: <http://www.emedicine.com/med/topic2985.htm>
- 10 **Bourne A**. Congenital and developmental abnormalities. In: Whitehead R, editor. Gastrointestinal and esophageal pathology New York: Churchill Livingstone, 1995: 275-301
- 11 **Sen S**, Bourne AJ, Morris LL, Furness ME, Ford WD. Dorsal enteric cysts--a study of eight cases. *Aust N Z J Surg* 1988; **58**: 51-55
- 12 **Cohen SR**, Geller KA, Birns JW, Thompson JW, Meyer BW, Lindesmith GG. Foregut cysts in infants and children. Diagnosis and management. *Ann Otol Rhinol Laryngol* 1982; **91**: 622-627
- 13 **Perera GB**, Milne M. Neurenteric cyst: antenatal diagnosis by ultrasound. *Australas Radiol* 1997; **41**: 300-302
- 14 **Rattan KN**, Magu S, Rohilla S. Mediastinal foregut duplication cysts. *Indian J Pediatr* 2004; **71**: 103-105
- 15 **Cahill JF**. An unusual cause of neonatal respiratory distress. RDS in a neonate with a neuro-enteric cyst. *Anaesthesia* 1981; **36**: 790-794
- 16 **Bilik R**, Ginzberg H, Superina RA. Unconventional treatment of neuroenteric cyst in a newborn. *J Pediatr Surg* 1995; **30**: 115-117

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## Pathologic complete response confirmed by surgical resection for liver metastases of gastrointestinal stromal tumor after treatment with imatinib mesylate

Seiji Suzuki, Koji Sasajima, Masayuki Miyamoto, Hidehiro Watanabe, Tadashi Yokoyama, Hiroshi Maruyama, Takeshi Matsutani, Aimin Liu, Masaru Hosone, Shotaro Maeda, Takashi Tajiri

Seiji Suzuki, Koji Sasajima, Masayuki Miyamoto, Hidehiro Watanabe, Tadashi Yokoyama, Hiroshi Maruyama, Takeshi Matsutani, Department of Surgery, Tama-Nagayama Hospital, Nippon Medical School, 1-7-1 Nagayama, Tama, Tokyo 206-8512, Japan

Aimin Liu, Masaru Hosone, Shotaro Maeda, Department of Pathology, Tama-Nagayama Hospital, Nippon Medical School, 1-7-1 Nagayama, Tama, Tokyo 206-8512, Japan

Takashi Tajiri, Department of Surgery, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-Ku, Tokyo 113-8603, Japan

**Author contributions:** Suzuki S, Miyamoto M and Yokoyama T performed hepatectomy; Watanabe H evaluated the effect of the treatment with imatinib; Suzuki S, Maruyama H and Matsutani T treated the patient with imatinib; Liu A, Hosone M and Maeda S evaluated the histopathological findings; Suzuki S wrote the paper; Sasajima K and Tajiri T reviewed the paper.

**Correspondence to:** Seiji Suzuki, Department of Surgery, Tama-Nagayama Hospital, Nippon Medical School, 1-7-1 Nagayama, Tama, Tokyo 206-8512, Japan. [seiji@nms.ac.jp](mailto:seiji@nms.ac.jp)

Telephone: +81-42-3712111 Fax: +81-42-3727384

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**Peer reviewer:** Dr. Xin-Yuan Guan, Department of Clinical Oncology, University of Hong Kong, Room 109, Estate Building, 10 Sassoon Road, Hong Kong 852, China

Suzuki S, Sasajima K, Miyamoto M, Watanabe H, Yokoyama T, Maruyama H, Matsutani T, Liu A, Hosone M, Maeda S, Tajiri T. Pathologic complete response confirmed by surgical resection for liver metastases of gastrointestinal stromal tumor after treatment with imatinib mesylate. *World J Gastroenterol* 2008; 14(23): 3763-3767 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3763.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3763>

### INTRODUCTION

Gastrointestinal stromal tumor (GIST) is the most common soft tissue tumor of the gastrointestinal tract. It was reported that GIST derived from interstitial cells of Cajal is characterized by the expression of CD34 and c-kit (CD117). Immunohistochemical positivity for c-kit gene product-CD117, a tyrosine kinase receptor, reflects the presence of gained function of c-kit gene mutation<sup>[1]</sup>. Imatinib mesylate (IM) (Glivec; Novartis Pharmaceuticals, Basel, Switzerland) is a small-molecule tyrosine kinase inhibitor that suppresses the mutated c-kit product<sup>[2]</sup>. Clinical trials<sup>[3,4]</sup> for recurrent or metastatic GIST have demonstrated that the partial response (PR) rate is 47% to 54% based on radiographic evaluation. However, a complete response (CR) is rarely reported. Pathologically verified cases showing therapeutic efficacy have been rarely reported. Up to present, only seven cases of locally advanced or metastatic GIST with a pathologic CR to IM treatment have been reported in the literature<sup>[5-10]</sup>.

The initial treatment for a metastatic GIST is to use IM, and then surgical treatment directing toward complete resection is to be considered when the tumor has responded and reduced in size<sup>[11-13]</sup>. However, neither the adequate intervals between the start of treatment with IM and operation, nor the significance of surgical resection for the patients with metastatic GIST who have been treated with IM, has been completely elucidated.

In this paper, we present a case of GIST with meta-

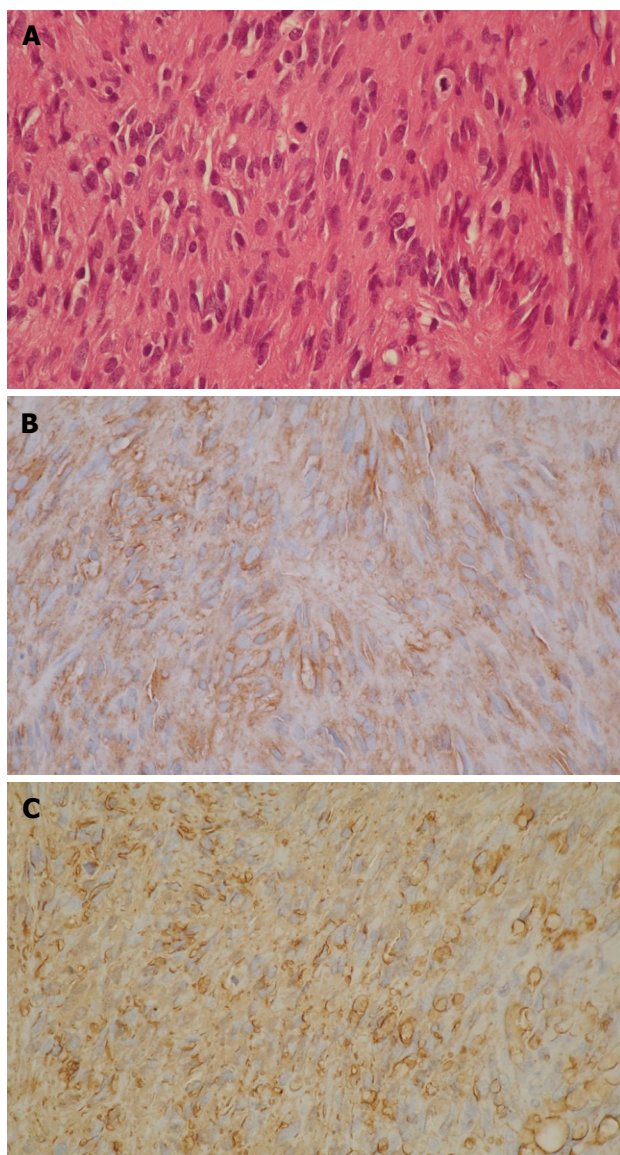
### Abstract

A 39-year-old male underwent distal gastrectomy for a high grade gastrointestinal stromal tumor (GIST). Computed tomography (CT) and magnetic resonance imaging (MRI) 107 mo after the operation, revealed a cystic mass (14 cm in diameter) and a solid mass (9 cm in diameter) in the right and left lobes of the liver, respectively. A biopsy specimen of the solid mass showed a liver metastasis of GIST. The patient received imatinib mesylate (IM) treatment, 400 mg/day orally. Following the IM treatment for a period of 35 mo, the patient underwent partial hepatectomy (S4 + S5). The effect of IM on the metastatic lesions was interpreted as pathologic complete response (CR). Pathologically verified cases showing therapeutic efficacy of IM have been rarely reported.

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**Key words:** Gastrointestinal stromal tumor; Liver metastasis; Imatinib mesylate; Pathologic complete response



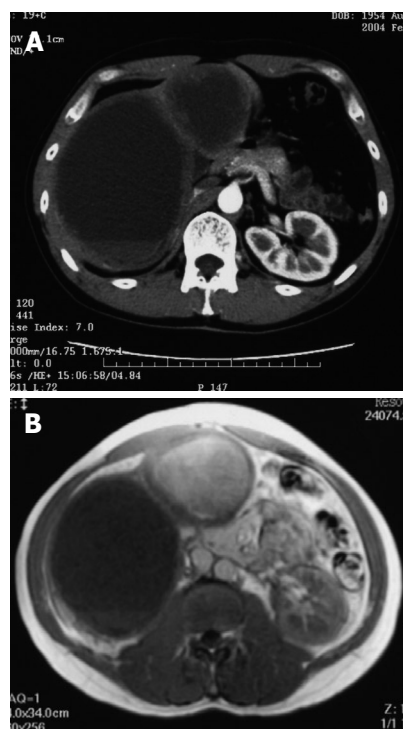


**Figure 1** Histological study showing spindle cells with mitoses (HE,  $\times 200$ ) (A) and immunohistochemistry findings revealing positive staining for CD117 (B) and CD34 ( $\times 200$ ) (C) in primary GIST of the stomach.

chronous liver metastasis treated with IM, and describe confirmed the therapeutic efficacy of such a molecular targeting drug as IM, which was confirmed by virtue of pathologic CR following complete surgical resection.

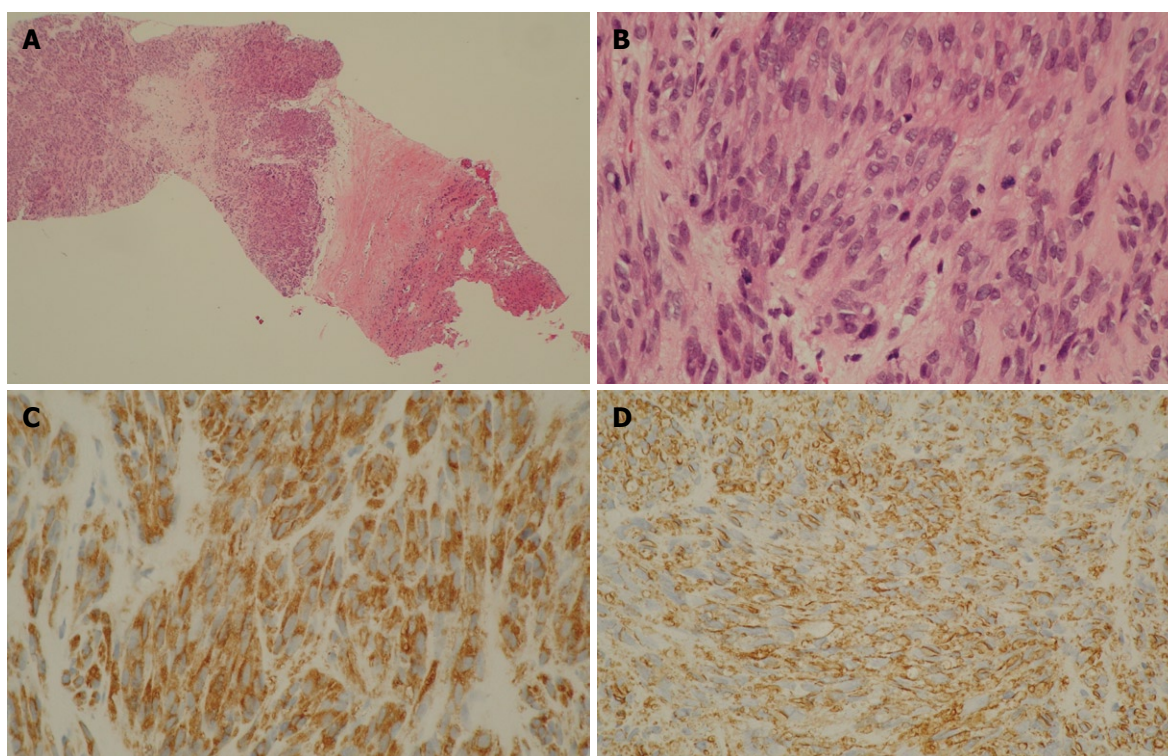
## CASE REPORT

A 39-year-old male complaining of epigastralgia was found to have a 3 cm  $\times$  2 cm submucosal tumor on the anterior surface of the body along the lesser curvature of the stomach, and underwent partial gastrectomy. Pathological examination of the surgical specimen revealed a high grade leiomyosarcoma showing spindle cells with 20 mitoses/10HPF and 17% in the MIB-1 index. The patient was subsequently diagnosed as having an uncommitted type of high grade GIST, since he was immunohistochemically positive for CD34 and CD117 (Figure 1). One hundred and seven months after the initial operation, the patient developed right upper

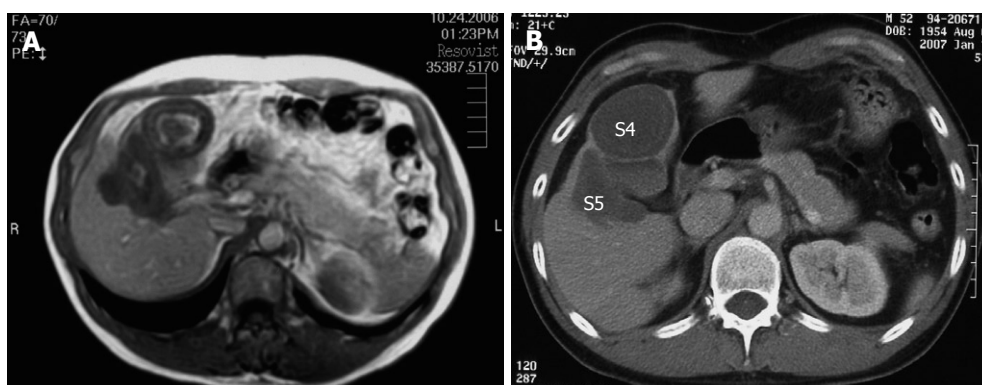


**Figure 2** Contrast-enhanced CT scan (A) and MRI (B) on T1-weighted image 107 mo after the initial operation.

quadrant pain during exercise. Physical examination revealed marked hepatomegaly and the lower margin of the liver could be palpated at five-finger widths below the costal margin. Computed tomography (CT) and magnetic resonance imaging (MRI) showed a cystic mass (14 cm in diameter) and a solid mass (9 cm in diameter) in the right and left lobes of the liver, respectively (Figure 2). Open biopsy was attempted and specimens were obtained from the solid tumor mass in the left lobe of the liver. Pathologically, spindle cells were positive for CD34 and CD117 with 15 mitoses/10 HPF and 15% in the MIB-1 index, which was indicative of a metastatic GIST of the liver (Figure 3). Only fluid was obtained from the cystic mass in the right lobe. A drainage tube was inserted into the cystic mass through the abdominal wall. Cytological examination of the fluid showed that the cystic mass was Class II. Since the cystic and solid tumors in the liver were considered too huge to be resected entirely and curatively, molecular targeting therapy using a daily dose of 400 mg of IM was started 3 mo after the liver biopsy. The drainage tube inserted into the cystic mass was removed after a three-week treatment with IM. A follow-up abdominal CT, one month after the start of IM treatment, showed apparent reduction in size of both the cystic and solid masses. The reduction of the solid mass in the left lobe was a partial response (PR). MRI, 30 mo after the treatment with IM, showed that the contrast-enhanced wall of the solid mass became thinner and central necrosis increased in size (Figure 4A). Although CT, 34 mo after the treatment, showed a 5 cm ring-enhanced mass in the left lobe (S4) and a 6 cm enhanced mass in the right lobe (S5) of the liver, the total volume of the



**Figure 3** Scanning view of metastatic GIST (× 15) (A), histological study revealed spindle cells with mitoses (HE, × 200) (B), immunohistochemistry findings revealed positive staining for CD117 (C) and CD34 (× 200) (D) in metastatic GIST of the liver obtained from liver biopsy.



**Figure 4** MRI on T1-WI 30 mo after treatment with Imatinib (A) and contrast-enhanced CT 34 mo after the treatment with Imatinib (B). The metastatic lesions (S4 + S5) are indicated.

neoplastic masses in the liver was sufficiently reduced after the curative resection of the masses (Figure 4B). The IM treatment was interrupted after 35 mo, and then the patient underwent partial hepatectomy (S4 + S5). The cut-surface of the resected specimens from S5 and S4 showed a homogenous yellow-white hard mass and a necrotic soft mass, respectively, forming a scrollwork structure, containing hemorrhagic foci, and surrounded by a yellow-white hard layer (Figure 5). Pathologically, most of the specimens were replaced with hyaline-degenerated tissue, adjacent to which, cystic-degenerated tissue and necrotic tissue with hemorrhage and macrophages containing hemosiderin granules stained with Berlin blue were observed. Since no viable tumor cells stained with CD34 or CD117 were observed in any of the whole sections at the maximum cut surface of the resected specimen, the effect of the IM treatment on the metastatic GIST was interpreted as the pathologic CR (Figure 6).

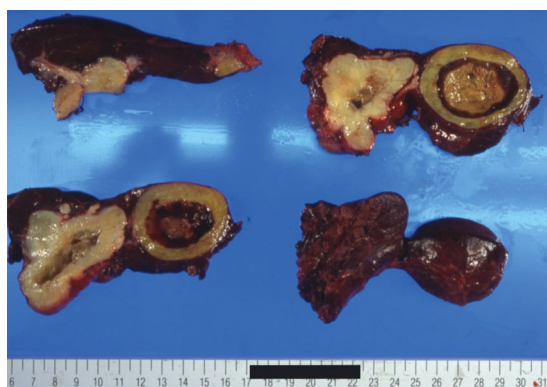
One week after the operation, oral administration of 400 mg IM daily for 12 mo was performed. Fourteen months after the partial hepatectomy at the time of writing this paper, no recurrent lesion was observed on CT and MRI examinations.

## DISCUSSION

The efficacy of aggressive surgical resection for locally advanced or metastatic GIST has been reported before the development of IM treatment<sup>[14,15]</sup>. Furthermore, clinical studies on the surgical resection after Imatinib treatment have also been reported<sup>[5,6,9-12,16,17]</sup>. Indeed, surgical resection of GIST makes it possible to elucidate the histopathologic effect of IM treatment on advanced or metastatic GIST. However, biopsy specimens from the lesion alone are usually not enough to assess the histopathologic effect of IM treatment on GIST.

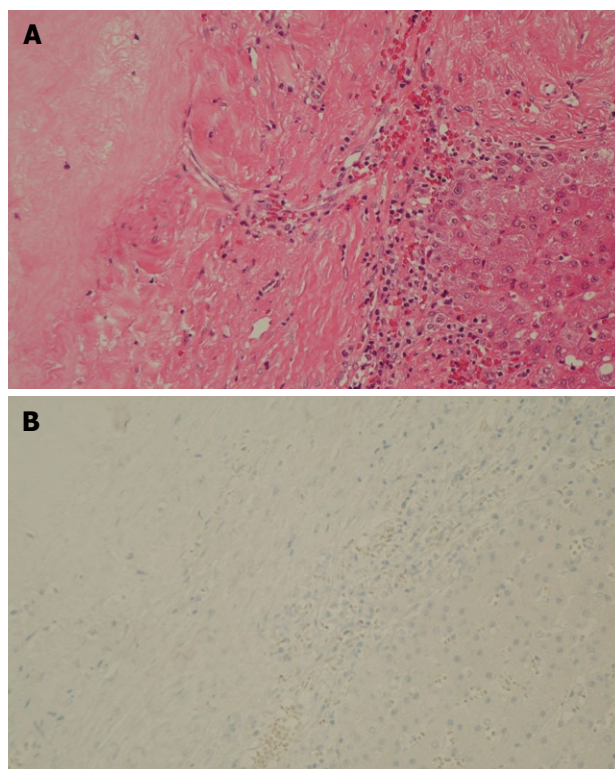
As far as we know, only six clinical reports on the





**Figure 5** Serous and cut-surface views of resected specimen obtained from partial hepatectomy (S4 + S5) after treatment with Imatinib.

pathological effect of IM treatment on locally advanced or metastatic GIST have been published<sup>[5,6,9,10,16,18]</sup>. According to Gronchi *et al*<sup>[18]</sup>, no case with a pathological CR was obtained in a series of 38 patients, although the degree of pathologic changes varied widely. Furthermore, Andtbacka *et al*<sup>[10]</sup> pointed out that radiographic and metabolic CR based upon <sup>18</sup>F-fluorodeoxyglucose positron emission tomography (FDG-PET) are not always concordant with a pathologic CR; therefore, it should be born in mind that the pathological evaluation on the surgically resected materials obtained from patients treated with IM might be indispensable for the elucidation of the therapeutic effect of IM on GIST. They also emphasized that the changes in the degree as well as the extent of contrast-enhancement, and the internal structure within the solid tumor should be carefully evaluated on CT and MRI<sup>[10]</sup>. According to their categorization, our case presented in this paper is compatible with 'initial regression then stabilization' on CT and MRI. MRI, five months prior to the operation in our case, disclosed thickening of the enhanced wall and a change of the signal intensity of the internal structure of the mass in the left lobe of the liver. In fact, the changes in internal density reflected the central necrosis on the cut-surface of the resected mass. Histopathologic changes induced by IM in GIST have been reported to be hyaline degeneration, myxoid degeneration, and appearance of scattered inflammatory cells, hemosiderin granules and foamy cells, but seldom necrosis<sup>[3,5-8]</sup>. Bauer *et al*<sup>[6]</sup> who found no necrosis in a series of twelve patients treated with IM, speculated that IM would mainly induce apoptosis, but not so much necrosis. As for the timing of surgical resection in patients with recurrent or metastatic GIST, Andtbacka *et al*<sup>[10]</sup> have reported a complete resection rate of 31.4% after IM therapy for a period of 6.9-37.5 mo (mean, 10 mo). They also emphasized that surgical resection for the IM-responsive recurrent or metastatic GIST should be considered as early as possible before the development of progression and secondary resistance to IM<sup>[10]</sup>. Surgical resection, 6-12 mo after the start of IM treatment, is recommended among responders<sup>[9]</sup>. Although the time of operation in our case was markedly



**Figure 6** Histological study showing no viable tumor cells and hyaline degenerative tissues (HE, × 200) (A) and immunohistochemistry findings revealing negative staining for CD117 (× 200) (B) in the resected specimen after treatment with Imatinib.

delayed (35 mo) in comparison with the time suggested by other investigators, it is thought to be adequate for avoidance of the secondary resistance to IM treatment.

In summary, we report a case of GIST with meta-chronous liver metastases who underwent complete surgical resection following IM treatment. The resected specimen was pathologically proven as a CR. Preoperative radiographic CT, MRI, findings and microscopic findings of the resected specimen were described from the view point of the effect of the molecular targeting therapy.

## REFERENCES

- 1 **Hirota S**, Isozaki K, Moriyama Y, Hashimoto K, Nishida T, Ishiguro S, Kawano K, Hanada M, Kurata A, Takeda M, Muhammad Tunio G, Matsuzawa Y, Kanakura Y, Shinomura Y, Kitamura Y. Gain-of-function mutations of c-kit in human gastrointestinal stromal tumors. *Science* 1998; **279**: 577-580
- 2 **Joensuu H**, Roberts PJ, Sarlomo-Rikala M, Andersson LC, Tervahartiala P, Tuveson D, Silberman S, Capdeville R, Dimitrijevic S, Druker B, Demetri GD. Effect of the tyrosine kinase inhibitor STI571 in a patient with a metastatic gastrointestinal stromal tumor. *N Engl J Med* 2001; **344**: 1052-1056
- 3 **Demetri GD**, von Mehren M, Blanke CD, Van den Abbeele AD, Eisenberg B, Roberts PJ, Heinrich MC, Tuveson DA, Singer S, Janicek M, Fletcher JA, Silverman SG, Silberman SL, Capdeville R, Kiese B, Peng B, Dimitrijevic S, Druker BJ, Corless C, Fletcher CD, Joensuu H. Efficacy and safety of imatinib mesylate in advanced gastrointestinal stromal tumors. *N Engl J Med* 2002; **347**: 472-480

- 4 **Verweij J**, Casali PG, Zalcberg J, LeCesne A, Reichardt P, Blay JY, Issels R, van Oosterom A, Hogendoorn PC, Van Glabbeke M, Bertulli R, Judson I. Progression-free survival in gastrointestinal stromal tumours with high-dose imatinib: randomised trial. *Lancet* 2004; **364**: 1127-1134
- 5 **Scaife CL**, Hunt KK, Patel SR, Benjamin RS, Burgess MA, Chen LL, Trent J, Raymond AK, Cormier JN, Pisters PW, Pollock RE, Feig BW. Is there a role for surgery in patients with "unresectable" cKIT+ gastrointestinal stromal tumors treated with imatinib mesylate? *Am J Surg* 2003; **186**: 665-669
- 6 **Bauer S**, Hartmann JT, de Wit M, Lang H, Grabellus F, Antoch G, Niebel W, Erhard J, Ebeling P, Zeth M, Taeger G, Seeber S, Flasshove M, Schutte J. Resection of residual disease in patients with metastatic gastrointestinal stromal tumors responding to treatment with imatinib. *Int J Cancer* 2005; **117**: 316-325
- 7 **Chacon M**, Roca E, Huertas E, Loria FS, Domenechini E. CASE 3. Pathologic complete remission of metastatic gastrointestinal stromal tumor after imatinib mesylate. *J Clin Oncol* 2005; **23**: 1580-1582
- 8 **Salazar M**, Barata A, Andre S, Venancio J, Francisco I, Cravo M, Nobre-Leitao C. First report of a complete pathological response of a pelvic GIST treated with imatinib as neoadjuvant therapy. *Gut* 2006; **55**: 585-586
- 9 **Bonvalot S**, Eldweny H, Pechoux CL, Vanel D, Terrier P, Cavalcanti A, Robert C, Lassau N, Cesne AL. Impact of surgery on advanced gastrointestinal stromal tumors (GIST) in the imatinib era. *Ann Surg Oncol* 2006; **13**: 1596-1603
- 10 **Andtbacka RH**, Ng CS, Scaife CL, Cormier JN, Hunt KK, Pisters PW, Pollock RE, Benjamin RS, Burgess MA, Chen LL, Trent J, Patel SR, Raymond K, Feig BW. Surgical resection of gastrointestinal stromal tumors after treatment with imatinib. *Ann Surg Oncol* 2007; **14**: 14-24
- 11 **Gold JS**, Dematteo RP. Combined surgical and molecular therapy: the gastrointestinal stromal tumor model. *Ann Surg* 2006; **244**: 176-184
- 12 **DeMatteo RP**, Maki RG, Singer S, Gonen M, Brennan MF, Antonescu CR. Results of tyrosine kinase inhibitor therapy followed by surgical resection for metastatic gastrointestinal stromal tumor. *Ann Surg* 2007; **245**: 347-352
- 13 **Soft Tissue Sarcoma**. National Comprehensive Cancer Network (NCCN) Clinical Practice Guidelines in Oncology 2006. Available from: URL: [http://www.nccn.org/professionals/physician\\_gls/PDF/sarcoma.pdf](http://www.nccn.org/professionals/physician_gls/PDF/sarcoma.pdf)
- 14 **DeMatteo RP**, Shah A, Fong Y, Jarnagin WR, Blumgart LH, Brennan MF. Results of hepatic resection for sarcoma metastatic to liver. *Ann Surg* 2001; **234**: 540-547; discussion 547-548
- 15 **Nunobe S**, Sano T, Shimada K, Sakamoto Y, Kosuge T. Surgery including liver resection for metastatic gastrointestinal stromal tumors or gastrointestinal leiomyosarcomas. *Jpn J Clin Oncol* 2005; **35**: 338-341
- 16 **Bumming P**, Andersson J, Meis-Kindblom JM, Klingensstierna H, Engstrom K, Stierner U, Wangberg B, Jansson S, Ahlman H, Kindblom LG, Nilsson B. Neoadjuvant, adjuvant and palliative treatment of gastrointestinal stromal tumours (GIST) with imatinib: a centre-based study of 17 patients. *Br J Cancer* 2003; **89**: 460-464
- 17 **Wu PC**, Langerman A, Ryan CW, Hart J, Swiger S, Posner MC. Surgical treatment of gastrointestinal stromal tumors in the imatinib (STI-571) era. *Surgery* 2003; **134**: 656-665; discussion 665-666
- 18 **Gronchi A**, Fiore M, Miselli F, Lagonigro MS, Coco P, Messina A, Pilotti S, Casali PG. Surgery of residual disease following molecular-targeted therapy with imatinib mesylate in advanced/metastatic GIST. *Ann Surg* 2007; **245**: 341-346

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**Toru Hiyama, MD, PhD**

Health Service Center, Hiroshima University, 1-7-1 Kagamiyama, Higashihiroshima 739-8521, Japan

**Ikejima Kenichi, MD, PhD**

Department of Gastroenterology, Juntendo University School of Medicine, 2-1-1, Hongo, Bunkyo-ku, 113-8421, Japan

**Peter L Lakatos, MD, PhD, Assistant Professor**

1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

**Anders E Lehmann, PhD, Associate Professor**

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**Zhe-Xiong Lian, MD, PhD, Associate Adjunct Professor**

Division of Rheumatology, Allergy and Clinical Immunology, Genome and Biomedical Sciences Facility, University of California at Davis, 451 Health Sciences Drive, Suite 6605A, Davis, CA 95616, United States

**Reza Malekzadeh, Professor, Director**

Digestive Disease Research Center, Tehran University of Medical Sciences, Shariati Hospital, Kargar Shomali Avenue, 19119 Tehran, Iran

**Kevin McGrath, MD**

Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh Medical Center, M2, C wing, PUH, 200 Lothrop St, Pittsburgh, PA 15213, United States

**Fock Kwong Ming, Professor Senior Consultant**

Department of Medicine, Changi General Hospital, 2 Simei Street 3, Singapore 529889, Singapore

**Chris JJ Mulder, Professor**

Department of Gastroenterology, VU University Medical Center, PO Box 7057, 1007 MB Amsterdam, The Netherlands

**Amado S Peña, Professor**

Department of Pathology, Immunogenetics, VU University Medical Centre, De Boelelaan 1117, PO Box 7057, Amsterdam 1007 MB, The Netherlands

**Dr. Philip Abraham, Professor**

Consultant Gastroenterologist & Hepatologist, P. D. Hinduja National Hospital & Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai 400016, India

**Carlos J Pirola, PhD, FAHA**

Instituto de Investigaciones Medicas A Lanari, Combatientes de Malvinas 3150, Buenos Aires-1427, Argentina

**Sakhawat Rahman, Mr, Consultant in HPB & Minimally Invasive Surgery**

Royal Free Hampstead NHS Trust, 133 King Henrys Road, Primrose Hill, London, NW3 3RD, United Kingdom

**Vasiliy I Reshetnyak, MD, PhD, Professor**

Scientist Secretary of the Scientific Research Institute of General Reanimatology, 25-2, Petrovka str., 107031, Moscow, Russia

**Ian C Roberts-Thomson, Professor**

Department of Gastroenterology and Hepatology, The Queen Elizabeth Hospital, 28 Woodville Road, Woodville South 5011, Australia

**Sammy Saab, MD, MPH, AGAF**

Department of Medicine and Surgery, Pflieger Liver Institute, 200 UCLA Medical Plaza, Suite 214, Box 957302, Los Angeles, CA 90095-7302, United States

**James M Scheiman, Professor**

Division of Gastroenterology, University of Michigan Medical Center, 3912 Taubman Center, Box 0362, Ann Arbor, Michigan 48109-0362, United States

**Mitsuo Shimada, Professor**

Department of Digestive and Pediatric Surgery, Tokushima University, Kuramoto 3-18-15, Tokushima 770-8503, Japan

**Wing-Kin Syn, MD**

Division of Gastroenterology, GSRB-1, Suite 1073, DUMC 3256, 595 LaSalle Street, Durham, NC27710, United States

**Akihito Tsubota, Assistant Professor**

Institute of Clinical Medicine and Research, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan

**Satoshi Yamagiwa, MD, PhD**

Division of Gastroenterology and Hepatology, Niigata University Graduate School of Medical and Dental Sciences, 757 Asahimachi-dori, Chuo-ku, Niigata, 951-8510, Japan



## Meetings

### Events Calendar 2008-2009

**FALK SYMPOSIA 2008**  
 January 24-25, Frankfurt, Germany  
 Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008  
 February 14-16, Paris, France  
 EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
 8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
 3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation  
 Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

February 29, Québec, Canada  
 Canadian Association of Gastroenterology  
 E-mail: [general@cag-acg.org](mailto:general@cag-acg.org)

March 10-13, Birmingham, UK  
 British Society of Gastroenterology Annual Meeting  
 E-mail: [BSG@mailbox.ulcc.ac.uk](mailto:BSG@mailbox.ulcc.ac.uk)

March 14-15, HangZhou, China  
 Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
 Asian Pacific Association for the Study of the Liver  
 18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
 Shanghai-Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
 OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas  
 E-mail: [robert.giuli@oeso.org](mailto:robert.giuli@oeso.org)

April 9-12, Los Angeles, USA  
 SAGES 2008 Annual Meeting - part of Surgical Spring Week  
[www.sages.org/08program/html/](http://www.sages.org/08program/html/)

April 18-22, Buenos Aires, Argentina  
 9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
 Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
 43<sup>rd</sup> Annual Meeting of the European Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary

Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA  
 Digestive Disease Week 2008

May 21-22, California, USA  
 ASGE Annual Postgraduate Course  
 Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion  
 E-mail: [education@fsg.org](mailto:education@fsg.org)

June 4-7, Helsinki, Finland  
 The 39<sup>th</sup> Nordic Meeting of Gastroenterology  
[www.congex.com/ngc2008](http://www.congex.com/ngc2008)

June 5-8, Sitges (Barcelona), Spain  
 Semana de las Enfermedades Digestivas  
 E-mail: [sepd@sepd.es](mailto:sepd@sepd.es)

June 6-8, Prague, Czech Republic  
 3<sup>rd</sup> Annual European Meeting: Perspectives in Inflammatory Bowel Diseases  
 E-mail: [meetings@imedex.com](mailto:meetings@imedex.com)

June 10-13, Istanbul, Turkey  
 ESGAR 2008 19<sup>th</sup> Annual Meeting and Postgraduate Course  
 E-mail: [fca@netvisao.pt](mailto:fca@netvisao.pt)

June 11-13, Stockholm, Sweden  
 16<sup>th</sup> International Congress of the European Association for Endoscopic Surgery  
 E-mail: [info@aes-eur.org](mailto:info@aes-eur.org)

June 13-14, Amsterdam, Netherlands  
 Falk Symposium 165: XX International Bile Acid Meeting. Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic  
 Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management  
 E-mail: [idca2008@guarant.cz](mailto:idca2008@guarant.cz)

June 25-28, Barcelona, Spain  
 10<sup>th</sup> World Congress on Gastrointestinal Cancer  
 Imedex and ESMO  
 E-mail: [meetings@imedex.com](mailto:meetings@imedex.com)

June 25-28, Lodz, Poland  
 Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatologists (IAP)  
 E-mail: [office@epc-iap2008.org](mailto:office@epc-iap2008.org)  
[www.e-p-c.org](http://www.e-p-c.org)  
[www.pancreatology.org](http://www.pancreatology.org)

June 26-28, Bratislava, Slovakia  
 5<sup>th</sup> Central European Gastroenterology Meeting  
[www.ceurgem2008.cz](http://www.ceurgem2008.cz)

July 9-12, Paris, France  
 ILTS 14<sup>th</sup> Annual International Congress  
[www.its.org](http://www.its.org)

September 10-13, Budapest, Hungary  
 11<sup>th</sup> World Congress of the International Society for Diseases of the Esophagus  
 E-mail: [isde@isde.net](mailto:isde@isde.net)

September 13-16, New Delhi, India  
 Asia Pacific Digestive Week  
 E-mail: [apdw@apdw2008.net](mailto:apdw@apdw2008.net)

III FALK GASTRO-CONFERENCE  
 September 17, Mainz, Germany

Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany  
 Falk Symposium 166:  
 GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic  
 Prague Hepatology Meeting 2008  
[www.czech-hepatology.cz/phm2008](http://www.czech-hepatology.cz/phm2008)

September 20-21, Mainz, Germany  
 Falk Symposium 167:  
 Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France  
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October 8-11, Istanbul, Turkey  
 18<sup>th</sup> World Congress of the International Association of Surgeons,  
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 16<sup>th</sup> United European Gastroenterology Week  
[www.negf.org](http://www.negf.org)  
[www.acv.at](http://www.acv.at)

October 22-25, Minnesota, USA  
 Anstralian Gastroenterology Week 2008  
 E-mail: [gesa@gesa.org.au](mailto:gesa@gesa.org.au)

October 22-25, Brisbane, Australia  
 71<sup>st</sup> Annual Colon and Rectal Surgery Conference  
 E-mail: [info@colonrectalcourse.org](mailto:info@colonrectalcourse.org)

October 31-November 4, Moscone West Convention Center, San Francisco, CA  
 59<sup>th</sup> AASLD Annual Meeting and Postgraduate Course  
 The Liver Meeting  
 Information: [www.aasld.org](http://www.aasld.org)

November 6-9, Lucerne, Switzerland  
 Neurogastroenterology & Motility Joint International Meeting 2008  
 E-mail: [ngm2008@mci-group.com](mailto:ngm2008@mci-group.com)  
[www.ngm2008.com](http://www.ngm2008.com)

November 12, Santiago de Chile, Chile  
 Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt  
 1<sup>st</sup> Hepatology and Gastroenterology Post Graduate Course  
[www.egyptgastrohep.com](http://www.egyptgastrohep.com)

December 7-9, Seoul, Korea  
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<sup>[1]</sup>Passed away on October 20, 2007

<sup>[2]</sup>Passed away on June 11, 2007



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## Multidisciplinary management of gastric and gastroesophageal cancers

Markus Moehler, Orestis Lyros, Ines Gockel, Peter R Galle, Hauke Lang

Markus Moehler, Peter R Galle, First Department of Internal Medicine of Johannes Gutenberg University of Mainz, Mainz 55101, Germany

Orestis Lyros, Ines Gockel, Hauke Lang, Institute of Surgery of Johannes Gutenberg University of Mainz, Mainz 55101, Germany

Author contributions: Moehler M and Lyros O collected the data and wrote the paper; Galle PR, Gockel I and Lang H supervised and commented this work.

Correspondence to: Dr. Markus Moehler, First Department of Internal Medicine, Johannes Gutenberg University of Mainz, Langenbeckstrasse 1, Mainz 55101, Germany. [moehler@mail.uni-mainz.de](mailto:moehler@mail.uni-mainz.de)

Telephone: +49-6131-177134 Fax: +49-6131-576621

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improve locoregional failures.

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### Abstract

Carcinomas of the stomach and gastroesophageal junction are among the five top leading cancer types worldwide. In spite of radical surgical R0 resections being the basis of cure of gastric cancer, surgery alone provides long-term survival in only 30% of patients with advanced International Union Against Cancer (UICC) stages in Western countries because of the high risk of recurrence and metachronous metastases. However, recent large phase-III studies improved the diagnostic and therapeutic options in gastric cancers, indicating a more multidisciplinary management of the disease. Multimodal strategies combining different neoadjuvant and/or adjuvant protocols have clearly improved the gastric cancer prognosis when combined with surgery with curative intention. In particular, the perioperative (neoadjuvant, adjuvant) chemotherapy is now a well-established new standard of care for advanced tumors. Adjuvant therapy alone should be carefully discussed after surgical resection, mainly in individual patients with large lymph node positive tumors when neoadjuvant therapy could not be done. The palliative treatment options have also been remarkably improved with new chemotherapeutic agents and will further be enhanced with targeted therapies such as different monoclonal antibodies. This article reviews the most relevant literature on the multidisciplinary management of gastric and gastroesophageal cancer, and discusses future strategies to

### INTRODUCTION

Gastric and esophageal cancers are among the leading causes of cancer-related death worldwide. Even if the incidence of distal gastric cancer has been decreasing over the past decades, the incidence of newly diagnosed proximal cancers (localized at cardia and gastro-esophageal junction), including Barrett's carcinoma, has dramatically increased<sup>[1]</sup>. Despite considerable progress in surgical resection as the primary curative treatment for gastric cancer in Japanese and Western countries, more than half of all patients with advanced UICC stage disease undergoing radical primary tumour resection relapse and die within five years<sup>[2]</sup>. The prognosis of these curatively resected cancer patients remains poor due to high rates of local recurrences as well as early lymph node and systemic metastases. Therefore, new perioperative, neoadjuvant, adjuvant and palliative chemotherapy strategies are of great importance in handling these patients.

### MULTIDISCIPLINARY STRATEGIES FOR DIAGNOSIS AND STAGING

Until recently, the standard diagnostic approach after endoscopic and histological diagnosis of localized advanced gastric adenocarcinoma was to perform only limited staging procedures with sonography and chest X-ray, followed nearly always by attempted surgical

resection. These limited diagnostic tools resulted in a non-optimal description of the local tumor extension and the detection of regional and distant metastases, often not allowing an optimal treatment selection.

It became accepted during the last decade that the lack of co-operation between different medical disciplines prevented an improvement in available therapies. Patient care often consisted of fragmented strategies and lacked long-term planning. The multidisciplinary management of gastric and gastroesophageal cancers, in diagnosis as well as in treatment strategies, gains now even more ground after results of recent randomised studies became available. The time has come for the launch of multimodal treatments to increase the chance of better outcome, longer survival or even cure<sup>[3]</sup>. By using this team approach, all diagnostic and therapeutic disciplines, such as the gastroenterologist, surgeon, oncologist, radiologist and radiotherapist, will be instrumental in planning the effective administration of their treatment modalities. The diagnostic arsenal, i.e. CT scan, endoscopic ultrasound (EUS), mini-laparoscopy, MRI and PET, allows improved pre- or postoperative staging<sup>[4,5]</sup>. For endoscopically large tumors and tumors of the gastro-esophageal junction in particular, CT scan of the abdomen/thorax and EUS are mandatory for an exact preoperative tumor and node metastases staging. EUS allows the differentiation between small and large tumours as well as staging or biopsies of mediastinal and celiac lymph nodes<sup>[6,7]</sup>. In addition, mini-laparoscopy is a valuable tool, as peritoneal carcinosis is found in about 20% to 30% of all gastric cancer patients at first diagnosis<sup>[8]</sup>. As PET scan has also been shown to effectively predict clinical response in esophageal and gastric cancer, it might potentially allow better allocations and adjustments for further individualized and optimized treatment strategies<sup>[9]</sup>. Staging with PET may best be used either in patients with locally advanced disease who may benefit from curative resection, if distant metastases are not found, or in patients with high grade stenosis, where EUS is not applicable.

## CURATIVE INTENT-THE OPTIMAL RESECTION

To date, the mainstay of curative treatment of gastric cancer has been radical surgical dissection (ESMO clinical recommendations 2007). However, high rates of local recurrences, early lymph node and systemic metastases highlight the need of further efforts to standardize and optimize the surgical treatment. Thus, the type of resection (subtotal *vs* total gastrectomy) and the role of extensive lymphadenectomy have been subjects of international debates. For distal gastric cancers, subtotal gastrectomy has been shown to have an equivalent oncologic result with significantly fewer complications when compared with total gastrectomy<sup>[10]</sup>. Even if the surgical procedure of choice for proximal gastric cancers is more controversial, because both proximal gastrectomy and total gastrectomy are associated with postoperative nu-

tritional impairments, the oncologic outcome of patients with proximal gastric cancer is independent of the type of gastric resection performed<sup>[11]</sup>. Currently, total gastrectomy for proximal (cardia) tumors is recommended in Europe.

The extent of regional lymphadenectomy required for optimal results is still debated. Several prospective randomised trials examining the role of more extended lymph node dissections (D1 *vs* D2) did not find clinically relevant improvements in overall survival. However, the interest in extended lymphatic dissections (D2 and greater) has not waned. A retrospective multicentre observation study in Germany found a significant survival advantage in patients undergoing extended lymphadenectomy<sup>[1]</sup>. In contrast, however, at least two prospective European trials compared D1 with D2 dissection: one in the Netherlands, by the Dutch Gastric Cancer Group (DGCG), and one in the UK, by the Medical Research Council (MRC)<sup>[12,13]</sup>. Even though the results have been debated differently, both trials found that extended lymphadenectomy associated with significantly higher morbidity and mortality rates compared with limited lymphadenectomy. Likewise, splenectomy and pancreatectomy were associated with a significantly increased risk of operative mortality. Interestingly, no significant survival difference was found for either group in the final results of the DGCG study after 11 years of follow-up<sup>[14]</sup>. As defined in this study, for patients with N2 disease an extended lymph node dissection may offer cure, but it remains difficult to identify patients who have N2 disease. Morbidity and mortality are greatly influenced by the extent of lymph node dissection, pancreatectomy, splenectomy and age. Extended lymph node dissections may thus be of benefit if morbidity and mortality can be avoided. Recently, the Japan Clinical Oncology Group (JCOG) presented an ambitious trial comparing D2 lymph node dissection with more extensive lymphadenectomy<sup>[15]</sup>. Here, the mortality rate was remarkably low (1%). Thus, a surgical option that may decrease postoperative morbidity and mortality is an "over-D1" lymphadenectomy with preservation of the pancreatic tail without splenectomy<sup>[16]</sup>.

With improvements in endoscopic techniques (endoscopic mucosal resection) and minimal access surgery, there has been interest in applying these modalities to early gastric cancer. Node-negative T1 tumors are associated with a 5-year survival of more than 90%<sup>[17]</sup>. As such, there is interest in performing more limited resection for these tumors. Endoscopic resection should be accepted as the treatment of choice in most patients with high-grade intraepithelial neoplasia and mucosal carcinoma in the esophagus. Low morbidity (1%-3%) and mortality (0%) and better quality of life, due to organ preservation, are points in favor of endoscopic resection and against surgical in cases of early oesophageal carcinoma (Barrett's)<sup>[18]</sup>. Here proper patient selection is paramount. The probability of lymph node metastasis in early gastric cancer is influenced by tumor factors and is correlated with increasing tumor size, submucosal, lymphatic and vascular invasion and poorly differentiated tumors<sup>[19]</sup>.

To improve the acceptance of endoscopic treatment, further prospective trials with long-term data are necessary. Regardless of the technique used for resecting early gastric cancer, complete excision with negative margins is required.

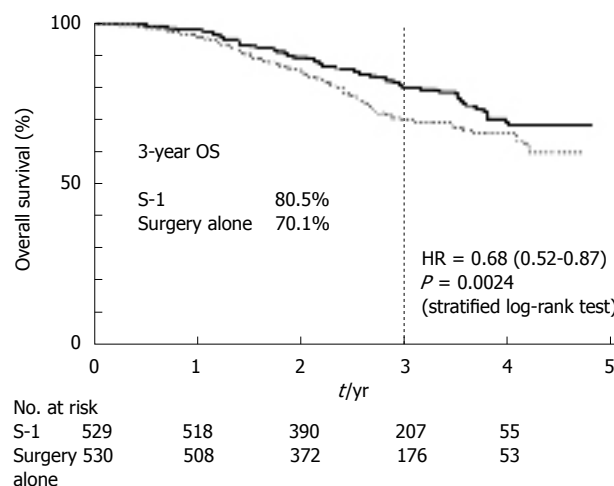
## ADJUVANT STRATEGIES

Because of the high rates of local recurrences and distant metastases, different adjuvant chemotherapy protocols have been compared with surgery alone in advanced gastric cancer in Europe, Asia and the United States. In a recent review of these studies the 5-year survival results suggested only a moderate improvement following adjuvant treatment<sup>[20]</sup>. However, the majority of the chemotherapeutic regimens used in these studies are regarded as suboptimal today.

Two recent phase III trials again support adjuvant chemotherapy. Sasako *et al* examined the adjuvant efficacy of single-agent S-1 compared with surgery alone in a study with 1059 patients with Stage II/III disease, after potentially curative D2 gastrectomy (ACTS-GC study). After 3-year follow-up and rare grade 3/4 toxicities, overall survival and relapse-free survival favored the S-1 arm, with 81.1% *vs* 70.1% ( $P = 0.0015$ ) and 72.2% *vs* 60.1% ( $P = 0.0001$ ), respectively<sup>[21]</sup> (Figure 1). The Italian Gruppo Oncologico dell'Italia Meridionale (GOIM) study found also in trend some positive results for epirubicin/etoposide/5-FU/folinic acid (FA) in > D1 resected patients<sup>[22]</sup>.

Adjuvant radiotherapy alone has failed over the last decades to improve treatment results and patient outcome. In the British Stomach Cancer Group trial, no survival advantage has been shown for 436 patients randomized between surgery only and surgery with 45 Gy-50 Gy radiotherapy or surgery with FAM chemotherapy<sup>[23]</sup>. This debate was further stimulated by the presentation of the SWOG 9008 group study, with combined radiochemotherapy in resected stage IB-IV gastric cancers<sup>[24]</sup>. After randomisation to either observation or to 2 cycles of FA/5-FU (Mayo-clinic regimen) followed by radiation + FA/5-FU and another 2 cycles FA/5-FU, a statistical significant difference in disease-free and overall survival in favor of the chemoradiation was shown. The absolute increase in median survival of 9 mo was hampered by suboptimal surgery (less than D1 in majority of patients) and radiotherapy; 35% protocol deviations. Additionally, in the multimodality arm, the local relapse rate was reduced from 90% to 29%. However, there was no difference in the risk of distant metastasis for either group. Despite positive data, several concerns have been raised concerning that in both arms the patients had high risk for relapse (more than 2/3 had T3 or T4 tumors and 85% positive lymph node metastases), the suboptimal surgery (54% of below D1) was counterbalanced by adjuvant chemoradiation and the number of patients (only 64%) who received the full schedule of chemotherapy and radiation.

In addition, Park and colleagues investigated a similar protocol in 290 patients, all of whom were curatively re-



**Figure 1** Survival of S-1 monotherapy versus surgery alone for stage II/III gastric cancer patients after curative D2 gastrectomy (ACTS-GC study)<sup>[22]</sup>.

sected with extensive D2 lymph node dissection<sup>[25]</sup>. After a median follow up of 49 mo, 43% of patients relapsed, with 67% local relapses and 36% distant metastases. The five-year overall and relapse free survival rates were 60% and 57%, better than in the SWOG trial, respectively<sup>[25]</sup>. Therefore, it is still questionable whether Japanese or European patients undergoing D2 resection may benefit of postoperative chemoradiation.

## PERIOPERATIVE MULTISCIPLINARY APPROACHES

### Neoadjuvant chemotherapy

Many reasonable rationales justify the application of neoadjuvant chemotherapy, which is particularly interesting as a short-term therapy (i.e. two cycles of 6-8 wk) given simultaneously and/or sequentially with radiochemotherapy. Possible advantages of neoadjuvant therapy +/- adjuvant strategies are: (1) Tumour vascularisation results in higher therapeutic efficacy and downstaging. (2) Excision of chemoradiated areas can result in lower long-term toxicity. (3) Early systemic therapy allows better control of tumour micrometastases. (4) Operation may not be compromised with higher morbidity and mortality.

Neoadjuvant chemotherapy aims at downstaging patients, improving curative resectability of locally advanced disease, and eventually increasing patient survival. It can also provide important information for the postoperative use of chemotherapeutic agents, by evaluating the response of the resected primary tumor, and it is also considered effective in reducing occult micrometastases. Theoretically, introducing chemotherapy at an early phase of the disease may facilitate delivery of drugs towards the primary lesion without impairing vascularization. In addition, major surgery such as total gastrectomy delays the start of postoperative systemic chemotherapy by a month or more, potentially giving microscopic residual diseases an opportunity to proliferate. On the other hand, it has been suggested that patho-

**Table 1** Ongoing important phase III clinical trials, including monoclonal antibodies and signal transduction/tyrosine kinase inhibitors

Name	Design	Indication
TOGA	XP or FP +/- trastuzumab	Advanced gastric cancer HER2-positive
AVAGAST	XP +/- bevacizumab	Metastatic gastric cancer
REAL-3	EOX +/- panitumumab	Advanced esophagogastric cancer
FFCD 03-07	ECX followed by FOLFIRI <i>vs</i> FOLFIRI followed by ECX	Advanced esophagogastric cancer
EXPAND	XP +/- Cetuximab	Advanced/Metastatic gastric cancer
MAGIC-B	Perioperative ECX +/- bevacizumab	Neo-adjuvant gastric cancer
CLASSIC	XELOX <i>vs</i> observation	Adjuvant gastric cancer

logical non-staging of the tumor could be the major disadvantage of neoadjuvant strategies. However, since modern imaging technologies such as CT, MRI and EUS plus fine-needle biopsies allow preoperative clinical staging for locoregional lymph node spread, overtreatment of patients with gastric cancer is less likely compared with earlier trials.

Patients responding to neoadjuvant treatment presented with a better performance status during their remission without compromising the subsequent operation with higher morbidity and mortality<sup>[26]</sup>. Although a number of randomised (mainly phase II) studies for neoadjuvant chemotherapy alone have suggested improved survival compared with historical controls, evidence from a randomised phase III trial were still missing<sup>[27,28]</sup>.

Apart from the newly updated version of the neoadjuvant MRC trial<sup>[29,30]</sup>, two large phase III studies have now clearly proved the preoperative concept to be beneficial for patients with gastric and gastro-oesophageal cancers<sup>[31,32]</sup>. The recently published MAGIC trial was the first large randomised study of perioperative chemotherapy to be conducted with an adequate follow-up period. It was initiated to compare surgery alone *versus* surgery with perioperative chemotherapy in which patients received three preoperative and three postoperative cycles of ECF<sup>[31]</sup>. After enrolment of 503 patients with resectable gastric (74%) or lower oesophageal cancer (26%), the proportion of patients with curative resection was larger in the chemotherapy plus surgery arm (79% *vs* 69%,  $P = 0.018$ ). After 5 years, the overall survival rate clearly favored the chemotherapy plus surgery arm over the surgery alone arm (hazard ratio for death, 0.75; 95% CI, 0.60-0.93;  $P = 0.009$ ; 5-year survival rate, 36% *vs* 23%), as did the progression-free survival rate (hazard ratio for progression, 0.66; 95% CI, 0.53-0.81;  $P < 0.001$ ).

The French FFCD (Federation Française de Cancérologie Digestive) Group trial confirmed these important data with their phase III study in which they randomized 224 patients to perioperative FUP (5-FU/cisplatin; surgery; 5-FU/cisplatin) or surgery alone. With the same postoperative mortality rates for both arms, the perioperative group presented with significantly higher R0 resection rates. In addition, 3-year disease-free survival increased by 15% (40% *vs* 25%) and 5-year survival improved (38% *vs* 24%)<sup>[32]</sup>.

To generate additional neoadjuvant data on tumors of the gastro-oesophageal junction, three randomised studies for oesophageal cancer included high percentages

of adenocarcinomas of the lower oesophagus or the cardia region<sup>[33]</sup>. In contrast to one large negative phase III trial with chemotherapy/surgery *vs* surgery alone<sup>[34]</sup>, one study showed a significant survival benefit and one study found a trend for improved 3-year survival for radio-chemotherapy<sup>[35,36]</sup>. Additionally, the recently updated MRC trial with 802 patients demonstrated a long lasting benefit in median survival (16.8 mo *vs* 13.3 mo) and the increase in 2-year survival of 9% for the chemotherapy group, with no difference in the rate of perioperative death or postoperative complications<sup>[29,30]</sup>.

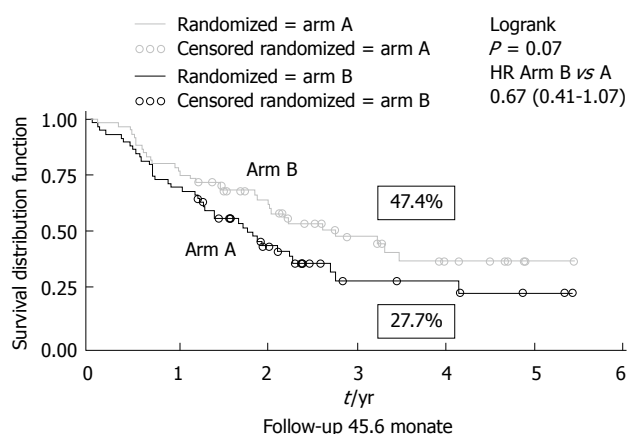
The identification of an effective chemotherapy regimen and optimal treatment schedule for locally advanced disease has been another important issue in neoadjuvant treatment for gastric cancer. There is optimism that the use of multiple targeted therapies in gastric cancer will produce further improved results. Thus, various phase I / II clinical trials, including monoclonal antibodies and signal transduction/tyrosine kinase inhibitors for EGFR, monoclonal antibodies to the HER-2/*neu* receptor and VEGF-ligand, and other novel drugs acting on intracellular signaling pathways, are under way, like bevacizumab<sup>[37]</sup> or cetuximab or panitumumab (Table 1).

### Neoadjuvant radiation

With regard to optimizing locoregional tumour control, radiotherapy in the neoadjuvant setting recently came into focus. Preoperative radio-chemotherapy has the advantage that the location of the primary cancer is known more precisely, which facilitates the planning of more accurate and effective radiation fields. In addition, the preoperative approach may allow significant time to observe high-risk patients for future growth of advanced cancers or metastases.

The German Oesophageal Cancer Study Group recently analysed the additional contribution of preoperative radiotherapy to neoadjuvant chemotherapy (POET study)<sup>[38]</sup> (Figure 2). Patients with locally advanced oesophagogastric adenocarcinomas (Stage T3-T4 NX M0 according to EUS, CT and laparoscopy) were randomised to 2.5 courses of chemotherapy (cisplatin/FA/5-FU weekly) *versus* two courses of the same chemotherapy followed by 3 wk of chemo-radiotherapy (30 Gy/cisplatin/etoposide). Despite some increased postoperative mortality after chemo-radiotherapy (five *vs* two patients), the median survival (32.8 mo) and the 3-year survival rate (43%) were





**Figure 2** Overall survival of patients with locally advanced oesophagogastric cancers with preoperative neoadjuvant chemoradiation (Arm B) vs neoadjuvant chemotherapy alone (Arm A, POET study)<sup>[38]</sup>.

significantly improved in this group compared with patients who received chemotherapy alone (21.1 mo and 27%, respectively).

Pathological responses of oesophageal cancers strongly correlated with disease-free survival after preoperative radio-chemotherapy<sup>[39,40]</sup>. Ajani *et al* investigated the effects of induction chemotherapy combined with preoperative radio-chemotherapy. Taxanes, cisplatin and 5-FU were followed by radiation with 45 Gy (25 fractions in 5 wk) plus 5-FU infusions<sup>[41]</sup>. Interestingly, pCR/pPR response rates were 64% in all operated patients, with a significantly longer median survival (64 mo *vs* 30 mo) in patients with pathological remissions. In a second multicentre study of Ajani and colleagues, the pCR and R0 resection rates were 26% and 77%, respectively. At 1 year, more patients with pCR (82%) were alive compared with those with < pCR (69%). Again, these parameters were closely associated with better progression-free and overall survival<sup>[42]</sup>. Furthermore, it has still to be determined whether any radiation escalation by hyperfractionation (more than one fraction of radiotherapy per day) or acceleration (shortening of treatment duration) may improve local control whilst maintaining a similar risk of late normal tissue damage<sup>[43]</sup>.

In addition to cisplatin/5-FU, new anticancer drugs such as taxanes, irinotecan and oxaliplatin have been reported to induce even higher objective response rates of up to 70% in recent years, and an improvement in overall median survival of up to 12 mo in palliative treatment<sup>[44]</sup>. The taxanes, docetaxel or paclitaxel promote microtubule stabilisation by increasing tubulin polymerization. As a result, they may also enhance radiosensitivity by causing cell cycle arrest in the G2/M phase<sup>[45]</sup>. These new chemotherapy regimens may be additional combinations to intensify localized multidisciplinary approaches in resectable or unresectable advanced diseases to further decrease incomplete resection rates as well as morbidity and mortality rates.

Additionally, results of other tumor entities, such

as the JCOG 9907 study for esophageal squamous cell cancers, clearly favour a combined modality approach in the neoadjuvant setting<sup>[46]</sup>. Even more, some randomized trials of chemo-radiotherapy *vs* radiotherapy alone in head and neck, oesophageal or anal cancer showed better locoregional control and overall survival rates for the multimodal protocols. Thus, direct comparisons between neoadjuvant and postoperative adjuvant strategies will be worthwhile in the near future.

## TREATMENT OF METASTATIC DISEASE (STAGE IV)

Chemotherapy has increasingly justified its role in the treatment of metastatic disease, with the survival of treated patients being significantly better than that for patients receiving best supportive care. To date, 5-FU derivatives combined with cisplatin have been accepted as the most useful form of palliative chemotherapy, often additionally modulated by combinations with other anti-cancer drugs, such as epirubicin or leucovorin (FA)<sup>[44,47]</sup>. In a Cochrane review of randomised trials in advanced gastric cancer, the best survival rates were achieved with anthracyclines, cisplatin and 5-FU, both independently and in combination<sup>[48]</sup>. Within these combinations, ECF proved to be the best tolerated. Other trials have shown improved overall survival with palliative regimens, such as docetaxel/cisplatin/5-FU<sup>[49]</sup>, oxaliplatin/FA/5-FU<sup>[50]</sup> and irinotecan/FA/5-FU<sup>[51,52]</sup>. However, continuous infusion of 5-FU is considered cumbersome because it requires the implantation of central venous catheter and the use of portable infusion pumps, which are associated with complications such as thromboses and wound infections. Capecitabine and S1, prodrugs and oral analogues of 5-FU, can mimic 5-FU continuous infusions and are at least equally effective in tumor control and less toxic than intravenous 5-FU in gastric cancer patients<sup>[53,54]</sup>. Remarkably, just recently Cunningham and colleagues evaluated capecitabine and oxaliplatin as alternatives to infused 5-FU and cisplatin for untreated advanced esophagogastric cancer and depicted at least similar effectiveness for both regimens<sup>[55]</sup>.

Moreover, the use of multiple targeted therapies renewed hope for more effective and better tolerated chemotherapy regimes in the palliative setting to further improve efficacy and survival. Pinto *et al* combined Cetuximab + FOLFIRI (FOLCETUX) in a phase II study and they demonstrated an overall response rate (ORR) of 44.1%, with a median TTP of 8 mo and a median OS time of 16 mo<sup>[56]</sup>. The major toxicity appeared to be limited to neutropenia (42.1% of grade 3-4), together with the typical side effects associated with cetuximab (skin 21.1%/grade 3-4). Two additional German AIO trials recently support the efficacy of cetuximab, favouring the analysis of standard therapy with or without EGFR inhibitors in advanced cancers<sup>[57,58]</sup>.

Additionally, response rate (65%), time to disease progression (8.3 mo), and overall survival (12.3 mo) were encouraging when bevacizumab was combined with

Irinotecan/Cisplatin in a multicenter phase II study<sup>[37]</sup>. Ongoing studies testing novel agents will further assess the potential improvement in the treatment of patients with metastatic gastric or gastroesophageal junction adenocarcinoma advanced gastric cancer.

## CONCLUSION

With respect to the new perioperative and neoadjuvant achievements in improving the treatment options for advanced gastric cancer, multidisciplinary strategies should be integrated into the daily practice of a patient's work-up<sup>[54,59]</sup>. Clinical co-operative groups of local comprehensive cancer centers and international study groups, such as the JCOG, SWOG, EORTC, MRC, AIO, FFCD and others, have shown that complex preoperative strategies can be implemented. Thus, patients should be included into the aforementioned innovative studies whenever possible. However, if the local clinical setting does not allow participation in such trials, regionally organized treating physicians, e.g. general practitioner, gastroenterologist, surgeon, radiotherapist and oncologist, should meet regularly, ideally weekly, to decide the multimodal therapeutic concepts, integrating pre-operative and post-operative strategies. With all these clinical and scientific efforts, these treatment strategies will definitely continue to further improve the outcome of gastric cancer patients.

## REFERENCES

- 1 Siewert JR, Bottcher K, Stein HJ, Roder JD. Relevant prognostic factors in gastric cancer: ten-year results of the German Gastric Cancer Study. *Ann Surg* 1998; **228**: 449-461
- 2 Sasako M. Principles of surgical treatment for curable gastric cancer. *J Clin Oncol* 2003; **21**: 274s-275s
- 3 Moehler M, Schimanski CC, Gockel I, Junginger T, Galle PR. (Neo)adjuvant strategies of advanced gastric carcinoma: time for a change? *Dig Dis* 2004; **22**: 345-350
- 4 Sotiropoulos GC, Kaiser GM, Lang H, Treckmann J, Brokalaki EI, Pottgen C, Gerken G, Paul A, Broelsch CE. Staging laparoscopy in gastric cancer. *Eur J Med Res* 2005; **10**: 88-91
- 5 Ott K, Fink U, Becker K, Stahl A, Dittler HJ, Busch R, Stein H, Lordick F, Link T, Schwaiger M, Siewert JR, Weber WA. Prediction of response to preoperative chemotherapy in gastric carcinoma by metabolic imaging: results of a prospective trial. *J Clin Oncol* 2003; **21**: 4604-4610
- 6 Pfau PR, Chak A. Endoscopic ultrasonography. *Endoscopy* 2002; **34**: 21-28
- 7 Chen CH, Yang CC, Yeh YH. Preoperative staging of gastric cancer by endoscopic ultrasound: the prognostic usefulness of ascites detected by endoscopic ultrasound. *J Clin Gastroenterol* 2002; **35**: 321-327
- 8 Denzer U, Hoffmann S, Helmreich-Becker I, Kauczor HU, Thelen M, Kanzler S, Galle PR, Lohse AW. Minilaparoscopy in the diagnosis of peritoneal tumor spread: prospective controlled comparison with computed tomography. *Surg Endosc* 2004; **18**: 1067-1070
- 9 Lordick F, Ott K, Krause BJ, Weber WA, Becker K, Stein HJ, Lorenzen S, Schuster T, Wieder H, Herrmann K, Bredenkamp R, Hofler H, Fink U, Peschel C, Schwaiger M, Siewert JR. PET to assess early metabolic response and to guide treatment of adenocarcinoma of the oesophagogastric junction: the MUNICON phase II trial. *Lancet Oncol* 2007; **8**: 797-805
- 10 Bozzetti F, Marubini E, Bonfanti G, Miceli R, Piano C, Gennari L. Subtotal versus total gastrectomy for gastric cancer: five-year survival rates in a multicenter randomized Italian trial. Italian Gastrointestinal Tumor Study Group. *Ann Surg* 1999; **230**: 170-178
- 11 Al-Refaie W, Pisters P, Chang G. 153 Proximal versus total gastrectomy for proximal gastric cancer: A population-based appraisal. *J Surg Res* 2007; **137**: 216-217
- 12 Bonenkamp JJ, Hermans J, Sasako M, van de Velde CJ, Welvaart K, Songun I, Meyer S, Plukker JT, Van Elk P, Obertop H, Gouma DJ, van Lanschot JJ, Taat CW, de Graaf PW, von Meyenfeldt MF, Tilanus H. Extended lymph-node dissection for gastric cancer. *N Engl J Med* 1999; **340**: 908-914
- 13 Cuschieri A, Weeden S, Fielding J, Bancewicz J, Craven J, Joypaul V, Sydes M, Fayers P. Patient survival after D1 and D2 resections for gastric cancer: long-term results of the MRC randomized surgical trial. Surgical Co-operative Group. *Br J Cancer* 1999; **79**: 1522-1530
- 14 Hartgrink HH, van de Velde CJ, Putter H, Bonenkamp JJ, Klein Kranenbarg E, Songun I, Welvaart K, van Krieken JH, Meijer S, Plukker JT, van Elk PJ, Obertop H, Gouma DJ, van Lanschot JJ, Taat CW, de Graaf PW, von Meyenfeldt MF, Tilanus H, Sasako M. Extended lymph node dissection for gastric cancer: who may benefit? Final results of the randomized Dutch gastric cancer group trial. *J Clin Oncol* 2004; **22**: 2069-2077
- 15 Sano T, Sasako M, Yamamoto S, Nashimoto A, Kurita A, Hiratsuka M, Tsujinaka T, Kinoshita T, Arai K, Yamamura Y, Okajima K. Gastric cancer surgery: morbidity and mortality results from a prospective randomized controlled trial comparing D2 and extended para-aortic lymphadenectomy--Japan Clinical Oncology Group study 9501. *J Clin Oncol* 2004; **22**: 2767-2773
- 16 Jansen EP, Boot H, Verheij M, van de Velde CJ. Optimal locoregional treatment in gastric cancer. *J Clin Oncol* 2005; **23**: 4509-4517
- 17 Kooby DA, Suriawinata A, Klimstra DS, Brennan MF, Karpeh MS. Biologic predictors of survival in node-negative gastric cancer. *Ann Surg* 2003; **237**: 828-835; discussion 835-837
- 18 Pech O, May A, Rabenstein T, Ell C. Endoscopic resection of early oesophageal cancer. *Gut* 2007; **56**: 1625-1634
- 19 Hyung WJ, Cheong JH, Kim J, Chen J, Choi SH, Noh SH. Application of minimally invasive treatment for early gastric cancer. *J Surg Oncol* 2004; **85**: 181-185; discussion 186
- 20 Falcone A. Future strategies and adjuvant treatment of gastric cancer. *Ann Oncol* 2003; **14** Suppl 2: ii45-ii47
- 21 Sakuramoto S, Sasako M, Yamaguchi T, Kinoshita T, Fujii M, Nashimoto A, Furukawa H, Nakajima T, Ohashi Y, Imamura H, Higashino M, Yamamura Y, Kurita A, Arai K. Adjuvant chemotherapy for gastric cancer with S-1, an oral fluoropyrimidine. *N Engl J Med* 2007; **357**: 1810-1820
- 22 De Vita F, Giuliani F, Orditura M, Maiello E, Galizia G, Di Martino N, Montemurro F, Carteni G, Manzione L, Romito S, Gebbia V, Ciardiello F, Catalano G, Colucci G. Adjuvant chemotherapy with epirubicin, leucovorin, 5-fluorouracil and etoposide regimen in resected gastric cancer patients: a randomized phase III trial by the Gruppo Oncologico Italia Meridionale (GOIM 9602 Study). *Ann Oncol* 2007; **18**: 1354-1358
- 23 Hallissey MT, Dunn JA, Ward LC, Allum WH. The second British Stomach Cancer Group trial of adjuvant radiotherapy or chemotherapy in resectable gastric cancer: five-year follow-up. *Lancet* 1994; **343**: 1309-1312
- 24 Macdonald JS, Smalley SR, Benedetti J, Hundahl SA, Estes NC, Stemmermann GN, Haller DG, Ajani JA, Gunderson LL, Jessup JM, Martenson JA. Chemoradiotherapy after surgery compared with surgery alone for adenocarcinoma of the stomach or gastroesophageal junction. *N Engl J Med* 2001; **345**: 725-730
- 25 Park SH, Kim DY, Heo JS, Lim DH, Park CK, Lee KW, Choi SH, Sohn TS, Kim S, Noh JH, Kim YI, Park JO, Kim K, Kim WS, Jung CW, Im YH, Lee MH, Park K, Park CH, Kang WK.

- Postoperative chemoradiotherapy for gastric cancer. *Ann Oncol* 2003; **14**: 1373-1377
- 26 **Kelsen D.** Neoadjuvant therapy for upper gastrointestinal tract cancers. *Curr Opin Oncol* 1996; **8**: 321-328
  - 27 **Songun I,** Keizer HJ, Hermans J, Klementsitsch P, de Vries JE, Wils JA, van der Bijl J, van Krieken JH, van de Velde CJ. Chemotherapy for operable gastric cancer: results of the Dutch randomised FAMTX trial. The Dutch Gastric Cancer Group (DGCG). *Eur J Cancer* 1999; **35**: 558-562
  - 28 **Fujitani K,** Ajani JA, Crane CH, Feig BW, Pisters PW, Janjan N, Walsh GL, Swisher SG, Vaporciyan AA, Rice D, Welch A, Baker J, Faust J, Mansfield PF. Impact of induction chemotherapy and preoperative chemoradiotherapy on operative morbidity and mortality in patients with locoregional adenocarcinoma of the stomach or gastroesophageal junction. *Ann Surg Oncol* 2007; **14**: 2010-2017
  - 29 **Medical Research Council Oesophageal Cancer Working Group.** Surgical resection with or without preoperative chemotherapy in oesophageal cancer: a randomised controlled trial. *Lancet* 2002; **359**: 1727-1733
  - 30 **Allum WH,** Fogarty PJ, Stenning SP, Langley RE, NCRI Upper GI Cancer Clinical Studies Group. Long term results of the MRC OEO2 randomized trial of surgery with or without preoperative chemotherapy in resectable esophageal cancer. (Abstract, No. 9). 2008 Gastrointestinal Cancers Symposium; Jan 24. Available from: URL: [http://www.asco.org/ASCO/Abstracts+%26+Virtual+Meeting/Abstracts?&vmview=abst\\_detail\\_view&confID=53&abstractID=10652](http://www.asco.org/ASCO/Abstracts+%26+Virtual+Meeting/Abstracts?&vmview=abst_detail_view&confID=53&abstractID=10652)
  - 31 **Cunningham D,** Allum WH, Stenning SP, Thompson JN, Van de Velde CJ, Nicolson M, Scarffe JH, Lofts FJ, Falk SJ, Iveson TJ, Smith DB, Langley RE, Verma M, Weeden S, Chua YJ, MAGIC Trial Participants. Perioperative chemotherapy *versus* surgery alone for resectable gastroesophageal cancer. *N Engl J Med* 2006; **355**: 11-20
  - 32 **Boige V,** Pignon J, Saint-Aubert B, Lasser P, Conroy T, Bouche O, Segol P, Bedenne L, Rougier P, Ychou M. Final results of a randomized trial comparing preoperative 5-fluorouracil (F)/cisplatin (P) to surgery alone in adenocarcinoma of stomach and lower esophagus (ASLE): FNLCC ACCORD07-FFCD 9703 trial. *Journal of Clinical Oncology*, 2007 ASCO Annual Meeting Proceedings Part I. Vol 25, No. 18S (June 20 Supplement), 2007: 4510. Available from: URL: [http://www.asco.org/ASCO/Abstracts+%26+Virtual+Meeting/Abstracts?&vmview=abst\\_detail\\_view&confID=47&abstractID=33499](http://www.asco.org/ASCO/Abstracts+%26+Virtual+Meeting/Abstracts?&vmview=abst_detail_view&confID=47&abstractID=33499)
  - 33 **Lordick F,** Stein HJ, Peschel C, Siewert JR. Neoadjuvant therapy for oesophagogastric cancer. *Br J Surg* 2004; **91**: 540-551
  - 34 **Kelsen DP,** Ginsberg R, Pajak TF, Sheahan DG, Gunderson L, Mortimer J, Estes N, Haller DG, Ajani J, Kocha W, Minsky BD, Roth JA. Chemotherapy followed by surgery compared with surgery alone for localized esophageal cancer. *N Engl J Med* 1998; **339**: 1979-1984
  - 35 **Walsh TN,** Noonan N, Hollywood D, Kelly A, Keeling N, Hennessy TP. A comparison of multimodal therapy and surgery for esophageal adenocarcinoma. *N Engl J Med* 1996; **335**: 462-467
  - 36 **Urba SG,** Orringer MB, Turrisi A, Iannettoni M, Forastiere A, Strawderman M. Randomized trial of preoperative chemoradiation *versus* surgery alone in patients with locoregional esophageal carcinoma. *J Clin Oncol* 2001; **19**: 305-313
  - 37 **Shah MA,** Ramanathan RK, Ilson DH, Levnor A, D'Adamo D, O'Reilly E, Tse A, Trocola R, Schwartz L, Capanu M, Schwartz GK, Kelsen DP. Multicenter phase II study of irinotecan, cisplatin, and bevacizumab in patients with metastatic gastric or gastroesophageal junction adenocarcinoma. *J Clin Oncol* 2006; **24**: 5201-5206
  - 38 **Stahl M,** Walz MK, Stuschke M, Lehmann N, Seegenschmiedt MH, Riera Knorrenschild J, Langer P, Bieker M, Königsrainer A, Budach W, Wilke H. Preoperative chemotherapy (CTX) *versus* preoperative chemoradiotherapy (CRTX) in locally advanced esophagogastric adenocarcinomas: First results of a randomized phase III trial. *Journal of Clinical Oncology* 2007 ASCO Annual Meeting Proceedings Part I. Vol 25, No. 18S (June 20 Supplement), 2007: 4511. Available from: URL: [http://www.asco.org/ASCO/Abstracts+%26+Virtual+Meeting/Abstracts?&vmview=abst\\_detail\\_view&confID=47&abstractID=31433](http://www.asco.org/ASCO/Abstracts+%26+Virtual+Meeting/Abstracts?&vmview=abst_detail_view&confID=47&abstractID=31433)
  - 39 **Lowy AM,** Leach SD. Adjuvant/neoadjuvant chemoradiation for gastric and pancreatic cancer. *Oncology* (Williston Park) 1999; **13**: 121-130
  - 40 **Mandard AM,** Dalibard F, Mandard JC, Marnay J, Henry-Amar M, Petiot JF, Roussel A, Jacob JH, Segol P, Samama G. Pathologic assessment of tumor regression after preoperative chemoradiotherapy of esophageal carcinoma. Clinicopathologic correlations. *Cancer* 1994; **73**: 2680-2686
  - 41 **Ajani JA,** Mansfield PF, Janjan N, Morris J, Pisters PW, Lynch PM, Feig B, Myerson R, Nivers R, Cohen DS, Gunderson LL. Multi-institutional trial of preoperative chemoradiotherapy in patients with potentially resectable gastric carcinoma. *J Clin Oncol* 2004; **22**: 2774-2780
  - 42 **Ajani JA,** Winter K, Okawara GS, Donohue JH, Pisters PW, Crane CH, Greskovich JF, Anne PR, Bradley JD, Willett C, Rich TA. Phase II trial of preoperative chemoradiation in patients with localized gastric adenocarcinoma (RTOG 9904): quality of combined modality therapy and pathologic response. *J Clin Oncol* 2006; **24**: 3953-3958
  - 43 **Fu KK,** Pajak TF, Trotti A, Jones CU, Spencer SA, Phillips TL, Garden AS, Ridge JA, Cooper JS, Ang KK. A Radiation Therapy Oncology Group (RTOG) phase III randomized study to compare hyperfractionation and two variants of accelerated fractionation to standard fractionation radiotherapy for head and neck squamous cell carcinomas: first report of RTOG 9003. *Int J Radiat Oncol Biol Phys* 2000; **48**: 7-16
  - 44 **Hohler T,** Mohler M. [New chemotherapeutic options in advanced gastric cancer] *Onkologie* 2003; **26** Suppl 7: 54-59
  - 45 **Mason KA,** Hunter NR, Milas M, Abbruzzese JL, Milas L. Docetaxel enhances tumor radioresponse in vivo. *Clin Cancer Res* 1997; **3**: 2431-2438
  - 46 **Ando N,** Kato H, Shinoda M, Ozawa S, Shimizu H, Nakamura T, Yabuzaki Y, Aoyama N, Kurita A, Fukuda H. A randomized trial of postoperative adjuvant chemotherapy with cisplatin and 5-fluorouracil *versus* neoadjuvant chemotherapy for localized squamous cell carcinoma of the thoracic esophagus (JCOG 9907). (Abstract, No.10). 2008 Gastrointestinal Cancers Symposium. Jan 24. Available from: URL: [http://www.asco.org/ASCO/Abstracts+%26+Virtual+Meeting/Abstracts?&vmview=abst\\_detail\\_view&confID=53&abstractID=10425](http://www.asco.org/ASCO/Abstracts+%26+Virtual+Meeting/Abstracts?&vmview=abst_detail_view&confID=53&abstractID=10425)
  - 47 **Van Cutsem E,** Van de Velde C, Roth A, Lordick F, Kohn CH, Cascinu S, Aapro M. Expert opinion on management of gastric and gastro-oesophageal junction adenocarcinoma on behalf of the European Organisation for Research and Treatment of Cancer (EORTC)-gastrointestinal cancer group. *Eur J Cancer* 2008; **44**: 182-194
  - 48 **Wagner AD,** Grothe W, Haerting J, Kleber G, Grothey A, Fleig WE. Chemotherapy in advanced gastric cancer: a systematic review and meta-analysis based on aggregate data. *J Clin Oncol* 2006; **24**: 2903-2909
  - 49 **Van Cutsem E,** Moiseyenko VM, Tjulandin S, Majlis A, Constenla M, Boni C, Rodrigues A, Fodor M, Chao Y, Voznyi E, Risse ML, Ajani JA. Phase III study of docetaxel and cisplatin plus fluorouracil compared with cisplatin and fluorouracil as first-line therapy for advanced gastric cancer: a report of the V325 Study Group. *J Clin Oncol* 2006; **24**: 4991-4997
  - 50 **Al-Batran SE,** Atmaca A, Hegewisch-Becker S, Jaeger D, Hahnfeld S, Rummel MJ, Seipelt G, Rost A, Orth J, Knuth A, Jaeger E. Phase II trial of biweekly infusional fluorouracil, folinic acid, and oxaliplatin in patients with advanced gastric cancer. *J Clin Oncol* 2004; **22**: 658-663
  - 51 **Moehler M,** Eimermacher A, Siebler J, Hohler T, Wein A,

- Menges M, Flieger D, Junginger T, Geer T, Gracien E, Galle PR, Heike M. Randomised phase II evaluation of irinotecan plus high-dose 5-fluorouracil and leucovorin (ILF) vs 5-fluorouracil, leucovorin, and etoposide (ELF) in untreated metastatic gastric cancer. *Br J Cancer* 2005; **92**: 2122-2128
- 52 **Dank M**, Zaluski J, Barone C, Valvere V, Yalcin S, Peschel C, Wenzl M, Goker E, Cisar L, Wang K, Bugat R. Randomized phase III study comparing irinotecan combined with 5-fluorouracil and folinic acid to cisplatin combined with 5-fluorouracil in chemotherapy naive patients with advanced adenocarcinoma of the stomach or esophagogastric junction. Abstract-No. 4003 ASCO Annual Meeting, 2005
- 53 **Ajani JA**, Lee FC, Singh DA, Haller DG, Lenz HJ, Benson AB 3rd, Yanagihara R, Phan AT, Yao JC, Strumberg D. Multicenter phase II trial of S-1 plus cisplatin in patients with untreated advanced gastric or gastroesophageal junction adenocarcinoma. *J Clin Oncol* 2006; **24**: 663-667
- 54 **Van Cutsem E**, Dicato M, Arber N, Benson A, Cunningham D, Diaz-Rubio E, Glimelius B, Goldberg R, Haller D, Haustermans K, Koo-Kang Y, Labianca R, Lang I, Minsky B, Nordlinger B, Roth A, Rougier P, Schmoll HJ, Sobrero A, Tabernero J, Szawlowski A, van de Velde C. The neo-adjuvant, surgical and adjuvant treatment of gastric adenocarcinoma. Current expert opinion derived from the Seventh World Congress on Gastrointestinal Cancer, Barcelona, 2005. *Ann Oncol* 2006; **17** Suppl 6: vi13-vi18
- 55 **Cunningham D**, Starling N, Rao S, Iveson T, Nicolson M, Coxon F, Middleton G, Daniel F, Oates J, Norman AR. Capecitabine and oxaliplatin for advanced esophagogastric cancer. *N Engl J Med* 2008; **358**: 36-46
- 56 **Pinto C**, Di Fabio F, Siena S, Cascinu S, Rojas Llimpe FL, Ceccarelli C, Mutri V, Giaquinta S, Piana E, Martoni AA. Phase II study of cetuximab plus FOLFIRI as first-line treatment in patients with unresectable/metastatic gastric or gastroesophageal junction (GEJ) adenocarcinoma (FOLCETUX study): Preliminary results. (Abstract No. 4031). *Journal of Clinical Oncology*, 2006 ASCO Annual Meeting Proceedings Part I. Vol 24, No. 18S (June 20 Supplement), 2006: 4031. Available from: URL: [http://www.asco.org/ASCO/Abstracts+%26+Virtual+Meeting/Abstracts?&vmview=abst\\_detail\\_view&confID=40&abstractID=32624](http://www.asco.org/ASCO/Abstracts+%26+Virtual+Meeting/Abstracts?&vmview=abst_detail_view&confID=40&abstractID=32624)
- 57 **Moehler MH**, Trarbach T, Seufferlein T, Kubicka S, Lordick F, Geissler M, Daum S, Kanzler S, Galle P. AIO Gastric group. Cetuximab with irinotecan/Na-Fa/5-FU as first-line treatment in advanced gastric cancer: Preliminary results of a nonrandomised multi-centre AIO phase II study. (Abstract, No. 102). 2008 Gastrointestinal Cancers Symposium, Jan 24. Available from: URL: [http://www.asco.org/ASCO/Abstracts+%26+Virtual+Meeting/Abstracts?&vmview=abst\\_detail\\_view&confID=53&abstractID=10212](http://www.asco.org/ASCO/Abstracts+%26+Virtual+Meeting/Abstracts?&vmview=abst_detail_view&confID=53&abstractID=10212)
- 58 **Lordick F**, Lorenzen S, Hegewisch-Becker S, Folprecht G, Wöll E, Decker T, Endlicher E, Röthling N, Fend F, Peschel C. Cetuximab plus weekly oxaliplatin/5FU/FA (FUF0X) in 1st line metastatic gastric cancer. Final results from a multicenter phase II study of the AIO upper GI study group. *Journal of Clinical Oncology*, 2007 ASCO Annual Meeting Proceedings Part I. Vol 25, No. 18S (June 20 Supplement), 2007: 4526. Available from: URL: [http://www.asco.org/ASCO/Abstracts+%26+Virtual+Meeting/Abstracts?&vmview=abst\\_detail\\_view&confID=47&abstractID=31912](http://www.asco.org/ASCO/Abstracts+%26+Virtual+Meeting/Abstracts?&vmview=abst_detail_view&confID=47&abstractID=31912)
- 59 **Ajani J**, Bekaii-Saab T, D'Amico TA, Fuchs C, Gibson MK, Goldberg M, Hayman JA, Ilson DH, Javle M, Kelley S, Kurtz RC, Locker GY, Meropol NJ, Minsky BD, Orringer MB, Osarogiagbon RU, Posey JA, Roth J, Sasson AR, Swisher SG, Wood DE, Yen Y. Gastric Cancer Clinical Practice Guidelines. *J Natl Compr Canc Netw* 2006; **4**: 350-366

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## Autoantibodies in primary sclerosing cholangitis

Johannes Roksund Hov, Kirsten Muri Boberg, Tom H Karlsen

Johannes Roksund Hov, Kirsten Muri Boberg, Tom H Karlsen, Medical Department, Rikshospitalet University Hospital, Oslo N-0027, Norway

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**Correspondence to:** Tom H Karlsen, MD, PhD, Medical department, Rikshospitalet University Hospital, Oslo N-0027, Norway. [t.h.karlsen@klinmed.uio.no](mailto:t.h.karlsen@klinmed.uio.no)

Telephone: +47-23-072469 Fax: +47-23-073510

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### Abstract

The aetiology of primary sclerosing cholangitis (PSC) is not known and controversy exists as to whether PSC should be denominated an autoimmune disease. A large number of autoantibodies have been detected in PSC patients, but the specificity of these antibodies is generally low, and the frequencies vary largely between different studies. The presence of autoantibodies in PSC may be the result of a nonspecific dysregulation of the immune system, but the literature in PSC points to the possible presence of specific antibody targets in the biliary epithelium and in neutrophil granulocytes. The present review aims to give an overview of the studies of autoantibodies in PSC, with a particular emphasis on the prevalence, clinical relevance and possible pathogenetic importance of each individual marker.

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**Key words:** Primary sclerosing cholangitis; Autoantibodies; Autoimmunity; Antibodies against cytoplasmic constituents of neutrophil; Tropomyosin

**Peer reviewers:** Richard A Kozarek, MD, Department of Gastroenterology, Virginia Mason Medical Center, 1100 Ninth Avenue, PO Box 900, Seattle 98111-0900, United States; Dr. Pietro Invernizzi, Division of Internal Medicine, Department of Medicine, Surgery, Dentistry, San Paolo School of Medicine, University of Milan, Via Di Rudinfi 8, 20142 Milan, Italy

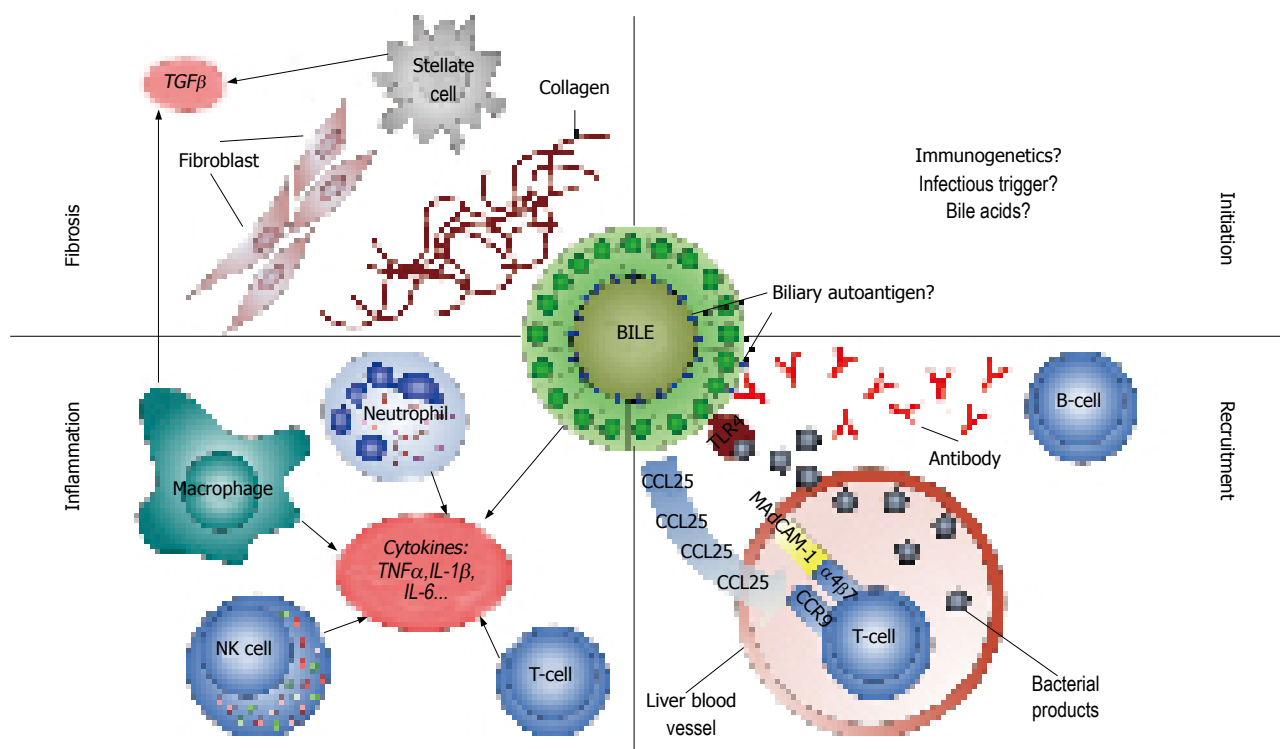
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### INTRODUCTION

Primary sclerosing cholangitis (PSC) is a chronic inflammatory disease of the intra- and extrahepatic biliary tree leading to progressive bile duct strictures and liver cirrhosis<sup>[1]</sup>. No effective medical treatment is currently available<sup>[2]</sup> and PSC is a major indication for liver transplantation<sup>[3]</sup>. The PSC population is heterogeneous, comprising subgroups of regular “large-duct” PSC, patients with “small-duct” affection only<sup>[4]</sup> and an “overlap-syndrome” between PSC and autoimmune hepatitis (AIH)<sup>[5]</sup>. Up to 80% of the PSC patients have concurrent inflammatory bowel disease (IBD)<sup>[6]</sup>. According to standard endoscopic and histological criteria, the IBD is most often classified as ulcerative colitis (UC), but there is also an association with colonic Crohn’s disease (CD)<sup>[7,8]</sup>.

The aetiology of PSC is unknown (Figure 1). Immune responses against self antigens in the bile ducts have been proposed to play an important role in the pathogenesis, although controversy exists as to whether PSC should be denominated an autoimmune or merely immune mediated disease<sup>[9]</sup>. On one side, there are several lines of evidence supporting classification of PSC as an autoimmune disease<sup>[10]</sup>. This evidence includes (1) association with other autoimmune diseases in the same individual<sup>[11]</sup> and first degree relatives<sup>[12]</sup>, (2) infiltration of T-lymphocytes in the portal tracts<sup>[13]</sup> with restriction in T cell receptor V gene usage<sup>[14]</sup>, (3) a statistical association with particular human leukocyte antigen (HLA) haplotypes<sup>[15]</sup> and (4) the presence of autoantibodies<sup>[16]</sup>. On the other side, there is no documented effect of immunosuppressants in PSC<sup>[2]</sup>, and in contrast to the female predominance of many diseases regarded as autoimmune, approximately 2/3 of PSC patients are male<sup>[17]</sup>. These notions suggest that additional pathogenetic factors may exist (e.g. bile acid toxicity<sup>[18]</sup>), and to what extent and at what disease stage autoimmune mechanisms contribute to the bile duct damage observed in PSC is not known.

In many autoimmune diseases, autoantibodies serve as markers of disease activity, may aid in the diagnosis of patients, and provide important insight into the pathogenesis. In clinical practice, a good



**Figure 1** Schematic illustration of key elements of PSC pathogenesis. Initiation (upper right): The initiating factor(s) of PSC pathogenesis are unknown. Immunogenetic factors (including the presentation of autoantigens on PSC associated HLA molecules), an infectious trigger, and toxic or immunological effects from bile acids have been proposed. Recruitment (lower right): Autoantibodies produced by B-lymphocytes bind to biliary epithelial cells (BECs), leading to inflammation when there is concomitant stimulation of toll like receptors (TLRs) by bacterial products [LPS and other pathogen-associated molecular patterns (PAMPs)] from the gut. Recruitment of gut-primed ( $\alpha 4\beta 7$ ) T-lymphocytes in inflammatory bowel disease may contribute to the inflammation because of aberrant expression of the MadCAM-1 ligand in the endothelium and production of the CCL25 chemokine by BECs. Inflammation (lower left): T-lymphocytes and natural killer (NK) cells predominate in PSC affected livers, but neutrophils and macrophages are also recruited. Together with activated BECs they are sources of the cytokines and chemokines that perpetuate the inflammation in PSC. A specific cellular component of neutrophils (tubulin beta 5 chain), has been hypothesized to serve as an autoantigen in this inflammatory process, possibly cross-reacting with the bacterial homolog FtsZ and leading to the generation of anti-neutrophil cytoplasmic antibodies (ANCA). Fibrosis (upper left): Characteristically in PSC, there is extensive fibrosis and stricturing of the bile ducts. Resulting from the inflammation and possibly concomitant bile leakage, pro-fibrotic factors [e.g. transforming growth factor beta (TGF- $\beta$ )] from macrophages and/or stellate cells are ultimately responsible for the fibrotic obliteration of the bile ducts and liver cirrhosis in PSC.

marker is sensitive and specific and yields prognostic information [e.g. anti-cyclic citrullinated proteins (anti-CCP) antibodies in rheumatoid arthritis (RA)]. In studies of pathogenetic mechanisms, a good marker is tissue specific and closely linked to other observations regarding the pathogenesis (e.g. TSH receptor antibodies in Graves' disease). In PSC patients, a large number of different autoantibodies have been reported (Table 1). Some of these autoantibodies react with biliary or colonic epithelial antigens, others with constituents of neutrophil granulocytes, and some even with various ubiquitously expressed self antigens.

One of the most consistent findings regarding the aetiology of PSC is the disease association with genetic variants within the HLA-complex on chromosome 6<sup>[15]</sup>. HLA class I and II genes encode molecules which present antigens to CD8<sup>+</sup> and CD4<sup>+</sup> T-lymphocytes, respectively, resulting in an immune response against the antigen when appropriate co-stimulation is present<sup>[19]</sup>. A relationship between particular autoantibodies and disease associated HLA variants has been detected in other autoimmune diseases<sup>[20]</sup>, but in PSC the pathogenetic importance of most of the identified

autoantibodies is poorly defined. The present editorial aims to give an overview of the studies of autoantibodies in PSC, with a particular emphasis on the prevalence, clinical relevance and possible pathogenetic importance of each individual marker.

## ANTIBODIES AGAINST BILIARY AND COLONIC EPITHELIAL ANTIGENS

The identification of antibodies against well defined biliary antigens in PSC would strongly support the hypothesis of an autoimmune aetiology. Given the high frequency of colitis among the patients, such antigens could potentially also be expressed in the colonic mucosa.

One autoantibody of this type was proposed by Das *et al*<sup>[21]</sup>, who identified an antigenic protein expressed in both colonic and biliary epithelium, in addition to eye, skin and cartilage<sup>[22]</sup>. This 40 kDa protein was identified as human tropomyosin isoform 5 (hTM5)<sup>[23-25]</sup>. A monoclonal antibody (Das-1) was developed, and serum from UC and PSC patients inhibited the binding of Das-1 to the epithelium, indicating antibodies

Table 1 Autoantibodies detected in PSC patients

Antibody	Prevalence (%)	(Median)	No. of patients	(Median)	No. of articles
Anti-BEC	63	(63)	30	(30)	1 <sup>[36]</sup>
pANCA	26-94	(68)	13-86	(30)	19 (Table 2)
AMA	0-9	(0)	15-73	(37)	10 <sup>[44,61,78,89,102,112,137-140]</sup>
Anti-LKM	0	(0)	10-80	(37)	7 <sup>[44,89,112,137,140-142]</sup>
Anti-SLA/LP	0	(0)	10-37	(25)	4 <sup>[44,89,140,142]</sup>
ANA <sup>1</sup>	8-77	(30)	13-73	(35)	13 <sup>[44,61,78,89,99,101,102,111,112,137-140]</sup>
SMA <sup>1</sup>	0-83	(17)	10-73	(36)	10 <sup>[44,61,78,89,111,112,137-140]</sup>
ASCA	44	(44)	25	(25)	1 <sup>[115]</sup>
Anti-cardiolipin	4-63	(27)	23-73	(41)	3 <sup>[61,78,87]</sup>
Rheumatoid factor	15	(15)	71	(71)	1 <sup>[78]</sup>
AECA	35	(35)	20	(20)	1 <sup>[87]</sup>
Anti-TPO	16	(16)	73	(73)	1 <sup>[78]</sup>
Anti-GBM	17	(17)	24	(24)	1 <sup>[87]</sup>
Anti-sulfite oxidase	33	(33)	39	(39)	1 <sup>[130]</sup>
Anti-GSTT1	5	(5)	58	(58)	1 <sup>[133]</sup>

PSC: Primary sclerosing cholangitis; Anti-BEC: Antibodies against biliary epithelial cells (measured with flow cytometry); PANCA: Perinuclear antineutrophil cytoplasmic antibodies; ANA: Antinuclear antibodies; SMA: Smooth muscle antibodies; ASCA: Anti *saccharomyces cerevisiae* antibodies; AMA: Anti-mitochondrial antibodies; Anti-LKM: Liver-kidney microsomal antibodies; Anti-SLA/LP: Antibodies against soluble liver antigen/liver pancreas; AECA: Anti-endothelial cell antibodies; Anti-TPO: Antibodies against thyroid peroxidase; Anti-GBM: Antibodies against the glomerular basement membrane; Anti-GSTT1: Antibodies against glutathione S transferase theta 1. <sup>1</sup>In the largest cohort investigated, ANAs and/or SMAs were detected in 22% (24/111) of the PSC patients, but the frequency of each type was not given<sup>[143]</sup>.

against hTM5 related epitope(-s) in the sera<sup>[22,26]</sup>. In the cell membrane hTM5 is found complexed with a 200 kDa colonic epithelial protein (CEP), and this complex is speculated to serve as the true target for the Das-1 antibody<sup>[27]</sup>. Antibodies against hTM5 have been detected in UC patients without PSC<sup>[28]</sup>, and anti-hTM5 in UC sera has recently been shown to induce cytotoxicity against colonic epithelial cells *in vitro*<sup>[29]</sup>. In PSC patients without concomitant UC, a single study identified antibodies against a 9-amino acid sequence from hTM (not isoform specific) in 100% (8/8) of patients as compared with 69% (33/48) of UC patients and 0/6 PBC patients<sup>[30]</sup>. The findings of Das *et al* have been partly reproduced by others<sup>[31]</sup>, but given a number of critical concerns<sup>[32-35]</sup>, further studies are required to conclusively confirm and elaborate the importance of the hTM5-CEP antigen in the pathogenesis of PSC.

A Swedish group has reported on the presence of antibodies against isolated biliary epithelial cells (BEC) at high frequencies in sera from PSC (63%) and PBC (37%) patients, *versus* 8% of controls (1/12)<sup>[36]</sup>. A 40 kDa antigenic protein was identified, but this protein did not react with tropomyosin antibodies, which implies that either the 40 kDa protein in this study is not a tropomyosin isoform or the antibody used reacts with other tropomyosin isoforms. Anti-BEC from PSC sera (and to a lesser extent PBC sera) induced isolated BEC to produce IL-6 and the adhesion molecule CD44, strongly suggesting pathogenetic importance. Recently the group also showed that sera from PSC patients with anti-BEC stimulated BEC to express toll-like receptors (TLR), leading to BEC cytokine production upon exposure to lipopolysaccharide (LPS, endotoxin) from gram negative bacteria<sup>[37]</sup>. This means that both LPS and antibodies against BEC are necessary to activate BEC and generate cytokine release. An association between the presence of the anti-BEC and PSC

associated HLA haplotypes (DR2 and DR3) was also suggested. The relevance of the Swedish findings are further strengthened by a higher frequency of acute liver transplant rejection in patients with anti-BEC prior to transplantation (all liver diseases) than in patients with no anti-BEC<sup>[38]</sup>. However, it needs to be noted that in this study there was a high prevalence of anti-BEC in all end stage liver patients (HCV 32%, PSC 56%, PBC 75%, HBV 57%, AIH 57%, and alcoholic cirrhosis 71%). This raises concerns as to the PSC specificity of the antibody, which clearly needs to be characterised prior to further studies.

Taken together, the findings of Das *et al* and the Swedish group suggest that antigens expressed in the biliary epithelium may induce self-reactive immune responses under certain conditions. Whether the antigenic epitope(s) lie within the hTM5-CEP complex or elsewhere remains to be elucidated, and the clinical significance of the corresponding autoantibodies must be established.

## ANTIBODIES AGAINST NEUTROPHILS

Antibodies against cytoplasmic constituents of neutrophils (ANCAs) were initially described in patients with glomerulonephritis and systemic vasculitis<sup>[39,40]</sup>. In UC patients, antibodies against nuclear antigens were reported by Calabresi *et al* in 1961<sup>[41]</sup> and Nielsen *et al* in 1983 (granulocyte specific-ANA)<sup>[42]</sup>. In PSC such antibodies were reported by Snook *et al* in 1989<sup>[43]</sup>. These antibodies are also present in a large proportion of patients with AIH<sup>[44]</sup> and the name ANCA was applied due to the close resemblance to ANCAs found in several of the vasculitides<sup>[45,46]</sup>. ANCA is analyzed by incubating fixated human neutrophil slides with patient serum, and subsequently with secondary antibodies conjugated to a fluorophore. The indirect immunofluorescence

Table 2 Prevalence of pANCA<sup>1</sup> in PSC patients and controls<sup>2</sup> [% ( $n^{\text{antibody positive}}/n^{\text{total population}}$ )]

Authors	PSC	PSC -IBD	PSC +IBD	UC -PSC	CD-PSC	AIH	PBC	HC	MT
Terjung <i>et al</i> <sup>[44]</sup>	94 <sup>3</sup> (33/35)					81 <sup>4</sup> (142/175)	31 (14/45)	0 (0/19)	IIF 1:10
Klein <i>et al</i> <sup>[54]</sup>	87 (26/30)			78 (18/23)	27 (16/60)			0 (0/20)	IIF 1:10
Mulder <i>et al</i> <sup>[102]</sup>	79 (19/24)	77 <sup>5</sup> (10/13)	82 <sup>5</sup> (9/11)			88 <sup>6</sup> (21/24)	28 (7/25)	5 (12/252)	IIF 1:32
Lo <i>et al</i> <sup>[56]</sup>	77 <sup>7</sup> (23/30)			33 (15/45)	0 (0/32)	33 <sup>6</sup> (1/33)	0 (0/14)	0 (0/50)	AP 1:10
Seibold <i>et al</i> <sup>[98]</sup>	775 (17/22)	40 <sup>8</sup> (2/5)	88 (15/17)	83 (38/46)	25 (20/80)	33 <sup>6</sup> (5/15)	28 (7/28)	0 (0/30)	IIF 1:10
Gur <i>et al</i> <sup>[87]</sup>	75 (15/20)	75 (3/4)	75 <sup>5</sup> (12/16)						IIF 1:20
Muratori <i>et al</i> <sup>[144]</sup>	75 (18/24)					31 <sup>4</sup> (12/39)	2 (1/51)	0 (0/18)	IIF 1:20
Seibold <i>et al</i> <sup>[84]</sup>	72 (18/25)	50 (2/4)	76 <sup>5</sup> (16/21)	62 (30/48)	4 (2/48)	35 <sup>4</sup> (8/23)	28 (6/21)	0 (0/40)	IIF 1:10
Zauli <i>et al</i> <sup>[137]</sup>	72 (33/46)								IIF - <sup>9</sup>
Hardarson <i>et al</i> <sup>[145]</sup>	69 (20/29)	75 (6/8)	67 <sup>5</sup> (14/21)	76 (16/21)	8 (2/25)	50 <sup>6</sup> (10/20)	0 (0/33)		IIF 1:40
Roozendaal <i>et al</i> <sup>[99]</sup>	67 (46/69)								IIF 1:40
Bansi <i>et al</i> <sup>[146]</sup>	66 <sup>10</sup> (57/86)					65 <sup>6</sup> (11/17)	13 (7/55)	0 (0/36)	AP 1:5
	51 <sup>10</sup> (44/86)					65 <sup>6</sup> (11/17)	11 (6/55)	0 (0/36)	IIF 1:5
Bansi <i>et al</i> <sup>[101]</sup>	65 (41/63)	29 (2/7)	70 <sup>5</sup> (39/56)	45 (38/85)				0 (0/36)	AP 1:05
Tervaert <i>et al</i> <sup>[88]</sup>	62 (8/13)					71 <sup>6</sup> (5/7)	33 (5/15)	0 (0/24)	IIF - <sup>9</sup>
Roozendaal <i>et al</i> <sup>[57]</sup>	49 (27/55)					70 <sup>11</sup> (62/88)	15 (8/53)	0 (0/78)	IIF 1:40
Claise <i>et al</i> <sup>[53]</sup>	44 (12/27)	25 (3/12)	60 (9/15)	37 (18/49)	15 (11/75)	24 <sup>11</sup> (25/105)	0 (0/30)	0 (0/50)	IIF 1:20
Vermeulen <i>et al</i> <sup>[147]</sup>	44 (16/36)			56 (56/100)	15 (15/100)	46 <sup>6</sup> (17/37)		5 (5/105)	IIF 1:40
Wilschanski <i>et al</i> <sup>[60]</sup>	29 (7/24)								IIF 1:20
Pokorny <i>et al</i> <sup>[100]</sup>	26 (10/39)	29 (5/17)	23 (5/22)			22 <sup>6</sup> (2/9)	0 (0/7)		IIF 1:20

PSC: Primary sclerosing cholangitis; IBD: Inflammatory bowel disease; UC: Ulcerative colitis; CD: Crohn's disease; AIH: Autoimmune hepatitis; PBC: Primary biliary cirrhosis; HC: Healthy controls; MT: Method and titre considered positive (cut-off); IIF: Indirect immunofluorescence; AP: Alkaline phosphatase method. <sup>1</sup>No distinction between classical/atypical; <sup>2</sup>The single largest study of autoantibodies in PSC reported ANCAs among 84% (61/73) of the patients, but this study did not apply IIF, meaning that this figure is the total of patients with any ANCA sub specificity<sup>[78]</sup>; <sup>3</sup>Atypical pANCA (as opposed to classical pANCA or cANCA); <sup>4</sup>autoimmune hepatitis type 1 and 2; <sup>5</sup>Calculated from article values; <sup>6</sup>Autoimmune hepatitis not subclassified (1 or 2); <sup>7</sup>ANCA "type 1 pattern" is interpreted as pANCA, including both IgA/IgM/IgG; <sup>8</sup>Significant difference between PSC +IBD and PSC -IBD (*P* value not given); <sup>9</sup>Details not given, correspondence, not peer-reviewed; <sup>10</sup>Calculated sum of 4 patient populations from different countries; <sup>11</sup>Autoimmune hepatitis type 1.

(IIF<sup>7</sup>) pattern is classified as cytoplasmic (cANCA) or perinuclear (pANCA)<sup>[47,48]</sup>. Billing *et al*<sup>[49]</sup> and Terjung *et al*<sup>[50-52]</sup> have made an additional contribution to this nomenclature, documenting that the main ANCA pattern in PSC, AIH and UC is "atypical". This means that the likely antigen is located in the nucleus rather than in the cytoplasm. The names anti-neutrophil nuclear antibodies (ANNAs)<sup>[51]</sup> and nuclear anti-neutrophil antibodies (NANAs) have thus been proposed<sup>[49]</sup>.

The prevalence of ANCA (subtype not specified) in PSC patients ranges from 42% to 93%<sup>[45,53-61]</sup>, and that of the pANCA subtype from 26% to 94% (Table 2). Comparable prevalences of ANCA are reported in AIH and UC (Table 2). No definite evidence links ANCA to the genetic susceptibility of PSC in terms of

particular HLA haplotypes<sup>[62]</sup>. One study has reported on an increased prevalence of ANCA in PSC relatives as compared with healthy controls<sup>[63]</sup> while another study could not confirm this<sup>[64]</sup>.

### Nuclear specificities of the neutrophil antigens

Multiple neutrophil antigens contribute to different ANCA IIF patterns (Table 3). A study published in abstract form by Terjung *et al*<sup>[65]</sup> in 2005 proposed that the main antigen of atypical pANCA in AIH, UC and PSC patients is tubulin beta 5 chain (TBB5), a nuclear membrane-associated protein present in myeloid cell lines. Further studies of anti-TBB5 are necessary to characterise the clinical and pathogenetic relevance of these findings. Other nuclear antigens have also been



**Table 3** Prevalence of antibodies against a selection of specific neutrophil antigens in PSC patients

Antibody	Frequency range % (median)	No. of patients range (median)	Number of studies
Anti-lactoferrin	4-50 (29)	12-76 (24)	10 <sup>[55,57,84,85,87,88,99,102,137,144]</sup>
Anti-myeloperoxidase	0-33 (2)	12-73 (40)	7 <sup>[57,78,84,85,87,99,102]</sup>
Anti-BPI	5-46 (29)	36-76 (69)	5 <sup>[55,57,59,78,99]</sup>
Anti-cathepsin G	0-35 (21)	14-76 (55)	5 <sup>[55,57,84,87,99]</sup>
Anti-proteinase 3	0-44 (4)	25-73 (62)	5 <sup>[57,78,87,99,102]</sup>
Anti-elastase	0-35 (9)	23-76 (69)	4 <sup>[55,87,99,102]</sup>
Anti- $\alpha$ -enolase	11-33 (27)	15-55 (36)	3 <sup>[57,89,147]</sup>
Anti-catalase	16-60 (38)	15-55 (35)	2 <sup>[57,89]</sup>
Anti- $\alpha$ -antigen	33 (33)	12 (12)	1 <sup>[85]</sup>
Anti-h-lamp-2	71 (71)	73 (73)	1 <sup>[78]</sup>
Anti-TBB5 <sup>1</sup>			

PSC: Primary sclerosing cholangitis; Anti-BPI: Antibodies against bactericidal/permeability increasing protein; Anti-h-lamp-2: Antibodies against human lysosomal-associated membrane protein 2; Anti-TBB5: Antibodies against Tubulin beta-5 chain. <sup>1</sup>No prevalence studies published.

proposed as nuclear targets of pANCA in AIH and UC, notably the high mobility group (HMG) non-histone chromosomal proteins HMG1 and HMG2<sup>[66-68]</sup> and Histone H1<sup>[69]</sup>. These have not been studied in patients with PSC.

### Cytoplasmic specificities of the neutrophil antigens

A variety of cytoplasmic proteins have also been proposed to be targets for ANCAs in PSC. In ANCA-associated small vessel vasculitis (Wegener's disease, microscopic polyangiitis and Churg-Strauss syndrome) the main proportion of specific ANCAs are directed against proteinase 3 (PR3, mainly cytoplasmic IIF pattern) and myeloperoxidase (MPO, mainly perinuclear IIF pattern)<sup>[70]</sup>. In these diseases, increased ANCA levels may predict clinical relapse, but there is limited correlation between titres and disease activity. The prevalence of anti-PR3 and anti-MPO in PSC patients is low (Table 3).

Bactericidal/permeability increasing protein (BPI) has functional domains which bind the inner core region of LPS<sup>[71]</sup>. This binding triggers anti-bacterial activity, neutralization of endotoxin and delivery of endotoxin rich particles to host cells<sup>[72]</sup>. Anti-BPI is detected in many clinical settings. In PSC, anti-BPI has been found in 5% to 46% of the patients (Table 3), which is similar to UC (3%-39%)<sup>[59,73-77]</sup>, compared with 0% to 5% of healthy controls<sup>[57,78]</sup>. Anti-BPI is also reported in RA, systemic lupus erythematosus (SLE) and systemic sclerosis<sup>[79]</sup>, and interestingly there is a high prevalence of anti-BPI in cystic fibrosis patients colonized with gram negative bacteria<sup>[80]</sup>.

Another LPS-binding ANCA target is lactoferrin, which is released from neutrophils during inflammation and has bactericidal and immune modulating effects<sup>[81]</sup>. Antibodies against lactoferrin have been detected in several autoimmune diseases including RA<sup>[82]</sup>, SLE<sup>[83]</sup>, reactive arthritis<sup>[82]</sup> and ankylosing spondylitis. The reported prevalence of anti-lactoferrin in PSC (4%-54%, Table 3) is similar to that in UC (4%-50%), and considerably higher than in CD (0%-9%)<sup>[73,75,76,84-86]</sup> and healthy controls (0%)<sup>[87,88]</sup>.

Antibodies against the proteases elastase and

cathepsin G are found in up to 35% of patients with PSC (Table 3). Catalase prevents cell damage from reactive oxygen-derived free radicals, and antibodies against catalase have been detected in up to 60% of PSC patients, compared with up to 10% of healthy controls<sup>[57,89]</sup>. Finally, human lysosomal-associated membrane protein 2 (h-lamp-2) is a target of ANCA in vasculitides<sup>[90]</sup>. In a single study, anti-h-lamp-2 was detected in a large proportion of PSC patients (71%) *versus* only 15% of healthy controls<sup>[78]</sup>. No disease controls were investigated. This finding has not yet been reproduced.

### Pathogenetic role of ANCAs

The large range of different ANCAs in PSC (Table 3) has been critically interpreted as the ANCAs serving as nonspecific epiphenomena of an immune response against dying neutrophils at an inflammatory site<sup>[91,92]</sup>. ANCAs (i.e. anti-MPO and anti-PR3) may, however, activate neutrophils<sup>[70]</sup>, and anti-BPI may inhibit clearance of LPS<sup>[93]</sup>. Also, widely and even ubiquitously expressed antigens sometimes serve as antigens in tissue specific autoimmunity [e.g. anti-mitochondrial antibodies (AMAs) in PBC].

Another possibility is related to the predominant theory on UC and CD, which involves an aberrant response to gut luminal antigens in genetically susceptible hosts<sup>[94]</sup>. A series of antibodies against bacterial antigens have been detected in IBD patients, and ANCAs may represent such antibodies<sup>[94]</sup>. One study from 1995 indicated that colonic lamina propria B-cells in UC produce pANCA<sup>[95]</sup>. In another study, absorption of human pANCA-positive sera with enteric bacterial antigens reduced or abolished the specific perinuclear staining<sup>[96]</sup>. The targets of these pANCAs are not known, but a study published in abstract form in 2006 indicates that antibodies giving rise to the atypical pANCA pattern have dual reactivity against both TBB5 and the microbial tubulin FtsZ<sup>[97]</sup>. How these cross-reacting antibodies may lead to hepatobiliary pathology can only be speculated upon.

### Diagnostic and clinical relevance of ANCAs

The sensitivity of ANCA in PSC is high in some studies,

whereas specificity is low. In one study of the diagnostic precision of autoantibodies in liver diseases, atypical pANCA with cut-off titre 1:40 had a specificity of 78% and sensitivity of 61% for PSC (AUC, 0.69; 95%CI, 0.61-0.77)<sup>[44]</sup>. Identification of the principal antigenic target of ANCAs in PSC would allow prospective studies to define this diagnostic role further. Currently, ANCA does not contribute diagnostically or during the clinical follow-up of PSC patients.

In terms of correlation between ANCA and particular clinical characteristics of PSC, no clear interpretation can be made from available data. If ANCAs were to represent markers for intestinal affection in PSC, a higher ANCA prevalence should be detected in PSC patients with IBD than in patients without IBD. This has only been shown in one small study by Seibold *et al*<sup>[98]</sup>. In another study, anti-lactoferrin was more prevalent in PSC with UC than without<sup>[99]</sup>. A few papers relate ANCA positivity to biliary tract complications like biliary calculi or cholangiocarcinoma<sup>[100]</sup>, or more extensive involvement of the biliary tree (both intra- and extrahepatic as compared with intrahepatic only)<sup>[101]</sup>. The presence of pANCA has also been found to correlate with disease stage (cirrhosis or liver transplantation)<sup>[100,102]</sup>, and in one study anti-BPI and anti-cathepsin G were more prevalent in PSC patients with cirrhosis<sup>[99]</sup>. Most other papers reported no difference in ANCA positivity between early and advanced PSC, and found no correlation between titres and disease activity<sup>[45,57,99,103]</sup>. ANCAs seem to persist after liver transplantation<sup>[98,104]</sup>, even though the titres may vary during follow-up<sup>[103]</sup>.

## AUTOANTIBODIES SPECIFIC TO LIVER DISEASES OTHER THAN PSC

AMA may be considered one of the most useful autoantibodies in the diagnosis of cholestatic liver disease, since AMAs are virtually absent in PSC patients (Table 1) compared with a 90%-95% prevalence in PBC<sup>[105]</sup>. The AMA antigens are different epitopes of the pyruvate dehydrogenase complex (PDC), especially the PDC-E2<sup>[106,107]</sup>. Mitochondrial antigens are expressed in all nucleated cells, and AMAs are classically detected by IIF. The presence of AMAs in PBC is an example of how autoimmunity against a ubiquitous antigen may be involved in the pathogenesis of a highly tissue specific disease. One of several proposed theories in PBC hypothesizes that in biliary epithelial cells the main AMA-antigen (PDC-E2) is not glutathiolated (as opposed to in other cells), causing persisting antigenicity of PDC-E2 when biliary epithelial cells undergo apoptosis<sup>[108]</sup>. Modification of AMA antigens in the liver by xenobiotics may also contribute<sup>[108]</sup>. A similar post-translational modification of proteins is known to contribute to antigenicity in several autoimmune diseases (e.g. antibodies against citrullinated proteins in RA)<sup>[109]</sup>.

Anti-liver kidney microsomes type 1 (anti-LKM1), anti-soluble liver antigen/liver pancreas antigen (anti-

SLA/LP) and anti-liver cytosolic protein type 1 (anti-LC1) are autoantibodies used in diagnosis of AIH<sup>[105]</sup>. These have not been detected in PSC patients (Table 1).

## ANTINUCLEAR (ANAS) AND SMOOTH MUSCLE ANTIBODIES (SMAS)

ANA and SMA are directed against ubiquitous antigens. ANA is the hallmark of SLE and other connective tissue diseases, but are also among the most prevalent autoantibodies in AIH<sup>[110]</sup>. ANAs may represent a large number of nuclear targets while SMAs are similarly undefined and directed against actin and other cytoplasmic filamentous proteins. ANA is reported in 8%-77% of the PSC patients (Table 1). No particular ANA subspecificities seem to predominate; anti-dsDNA has been reported in 3%-29%<sup>[61,78,87,111]</sup>, anti-ENA in 4%-12%<sup>[78,111,112]</sup>, anti SSA/B in 1%-28%<sup>[78,87]</sup> and anti-RNP, anti-SCL70, anti-Sm and anti-ssDNA in a minority of patients<sup>[87]</sup>. SMAs have been reported in 0%-83% of PSC patients (Table 1) but the prevalence is also high in AIH, various malignancies and infections<sup>[107]</sup>. ANA and SMA often co-exist, they lack organ and disease specificity, and should probably be concluded as irrelevant for the diagnostic process and pathogenesis in PSC.

## ANTIBODY AGAINST SACCHAROMYCES CEREVISIAE (ASCA)

ASCA is an antibody against baker's yeast (microbial antigens) and therefore does not represent a typical autoantibody. ASCA was first described in patients with CD in 1988<sup>[113]</sup>. The antigenic epitope of ASCA is located on the *S. cerevisiae* mannan, which is a polymer of mannose<sup>[114]</sup>. In a single study, 44% (11/25) of PSC patients were ASCA positive (57% with concurrent IBD and 39% without IBD) compared with 23% (28/123) of PBC and 18% (12/67) of AIH patients<sup>[113]</sup>. The presence of ASCA is interesting as a specific example of immune responses towards gut luminal antigens in IBD. As a serological marker in PSC, however, ASCA does not seem to contribute.

## ANTI-PHOSPHOLIPID ANTIBODIES

Anti-phospholipid antibodies are directed against phospholipids or phospholipid associated proteins, and are associated with thromboembolic disease. They are commonly detected in connective tissue disorders (e.g. SLE) but also in 1% to 5% of healthy subjects and during infections<sup>[116]</sup>. Three studies have investigated the presence of anti-cardiolipin antibodies in PSC with the prevalence ranging from 4% to 63% (Table 1). Interestingly, Angulo *et al*<sup>[78]</sup> found a positive correlation between anti-cardiolipin titres and Mayo risk score and histological disease stage, and there are anecdotal reports of an elevated risk of thrombosis in PSC patients<sup>[117]</sup>. An increased risk of hepatic artery thrombosis post liver transplantation has also been proposed<sup>[118]</sup>. Anti-

cardiolipin antibodies have also been reported at low frequencies in UC (16%-26%)<sup>[119-121]</sup> and CD (16%-27%) patients<sup>[120,121]</sup>.

## OTHER AUTOANTIBODIES

In addition to ANA and SMA, several other non-specific autoantibodies are detected in PSC. Rheumatoid factor is detected in connective tissue diseases, infections and lymphoproliferative diseases<sup>[122]</sup>, but is only found in 15% of PSC patients<sup>[78]</sup>. Anti-endothelial cell antibodies (AECAs) are directed against antigens in endothelial cells and have been reported in 35% of PSC patients in a single small study<sup>[87]</sup> but are observed in many other clinical conditions including vasculitis, SLE, systemic sclerosis and IBD<sup>[123,124]</sup>. The clinical and pathogenetic roles of AECAs are not clear<sup>[123,124]</sup>.

A few autoantibodies in PSC are probably related to co-morbidity. In one study, the prevalence of thyroid diseases in PSC patients was 8%<sup>[11]</sup>. This probably explains the elevated levels of anti-thyroid peroxidase (Table 3) and other thyroid related antibodies in PSC patients<sup>[54,78,87]</sup>. An association between PSC and celiac disease has been reported<sup>[125-127]</sup>. Recently the celiac disease related anti-tissue transglutaminase was detected in 7% of PSC patients in a large pre-transplant cohort from the Mayo Clinic (11/155), *versus* 6% (7/112) of PBC and 35% (15/43) of AIH<sup>[128]</sup> patients. This may in part be explained by shared susceptibility HLA-alleles (DQ2 and DQ8)<sup>[129]</sup>. Finally, in a single small study, antibodies against the glomerular basement membrane (anti-GBM) were detected in 17% of patients with PSC, while all healthy controls were negative<sup>[87]</sup>. The significance of this finding is not known.

Antibodies against sulfite oxidase were detected by Preuss *et al.* in 33% (13/39) of PSC patients compared with 5% (5/96) of PBC and 9% (7/77) of AIH patients<sup>[130]</sup>. Sulfite oxidase is a mitochondrial enzyme previously thought to be the antigen of anti-M4 (an AMA subtype)<sup>[131]</sup> but this does not seem to be correct<sup>[132]</sup>. The authors report lower prevalence in PSC patients treated with UDCA but the role of anti-sulfite oxidase antibodies in PSC remains to be established<sup>[130]</sup>.

Glutathione S-transferase theta 1 (GSTT1) was recently investigated as a candidate autoantigen in PSC by Ardesjö *et al* using immunoscreening<sup>[133]</sup>. This group created a cDNA library based on mRNA from human ductus choledochus<sup>[133]</sup>. The GSTT1 antigen was identified screening one single PSC patient serum for antibodies against bacteria expressing the cDNA encoded proteins. Upon testing in a larger population of PSC patients ( $n = 58$ ), antibodies against GSTT1 were only found in three patients, thus concluding GSTT1 as unlikely to serve as an important autoantigen in PSC. Nevertheless, the study points to the possible need for the application of broader screening methods in the search for autoantigens in PSC. The role of autoantibodies and B-cells in other autoimmune diseases has gained renewed interest the last few years<sup>[134]</sup>, not

only as pathogenetic factors<sup>[135]</sup>, but also as therapeutic targets (e.g. Rituximab)<sup>[136]</sup>. It is thus likely that further insight into the role of autoantibodies in PSC may be of clinical importance and further studies are warranted.

## CONCLUSION

A large number of autoantibodies have been detected in PSC patients. The specificity of these antibodies is generally low and the frequencies vary largely between different studies. Interpretation of the literature is difficult because of small patient sample sizes and variable methodology for antibody detection. The presence of autoantibodies in PSC is often attributed to a nonspecific dysregulation of the immune system, but the literature in PSC points to the possible presence of specific antibody targets both in the biliary epithelium and in neutrophils. Further characterisation of such targets would probably yield important insight into the pathogenesis of PSC. The investigation of larger populations may also further define the role of autoantibodies in PSC as diagnostic tools.

## REFERENCES

- 1 Chapman RW, Arborgh BA, Rhodes JM, Summerfield JA, Dick R, Scheuer PJ, Sherlock S. Primary sclerosing cholangitis: a review of its clinical features, cholangiography, and hepatic histology. *Gut* 1980; **21**: 870-877
- 2 Cullen SN, Chapman RW. The medical management of primary sclerosing cholangitis. *Semin Liver Dis* 2006; **26**: 52-61
- 3 Brandsaeter B, Friman S, Broome U, Isoniemi H, Olausson M, Backman L, Hansen B, Schrumpf E, Oksanen A, Ericzon BG, Hockerstedt K, Makisalo H, Kirkegaard P, Bjoro K. Outcome following liver transplantation for primary sclerosing cholangitis in the Nordic countries. *Scand J Gastroenterol* 2003; **38**: 1176-1183
- 4 Wee A, Ludwig J. Pericholangitis in chronic ulcerative colitis: primary sclerosing cholangitis of the small bile ducts? *Ann Intern Med* 1985; **102**: 581-587
- 5 Washington MK. Autoimmune liver disease: overlap and outliers. *Mod Pathol* 2007; **20** Suppl 1: S15-S30
- 6 Broome U, Bergquist A. Primary sclerosing cholangitis, inflammatory bowel disease, and colon cancer. *Semin Liver Dis* 2006; **26**: 31-41
- 7 Fausa O, Schrumpf E, Elgjo K. Relationship of inflammatory bowel disease and primary sclerosing cholangitis. *Semin Liver Dis* 1991; **11**: 31-39
- 8 Loftus EV Jr, Harewood GC, Loftus CG, Tremaine WJ, Harmsen WS, Zinsmeister AR, Jewell DA, Sandborn WJ. PSC-IBD: a unique form of inflammatory bowel disease associated with primary sclerosing cholangitis. *Gut* 2005; **54**: 91-96
- 9 Cullen S, Chapman R. Primary sclerosing cholangitis. *Autoimmun Rev* 2003; **2**: 305-312
- 10 Rose NR, Bona C. Defining criteria for autoimmune diseases (Witebsky's postulates revisited) *Immunol Today* 1993; **14**: 426-430
- 11 Saarinen S, Olerup O, Broome U. Increased frequency of autoimmune diseases in patients with primary sclerosing cholangitis. *Am J Gastroenterol* 2000; **95**: 3195-3199
- 12 Bergquist A, Lindberg G, Saarinen S, Broome U. Increased prevalence of primary sclerosing cholangitis among first-degree relatives. *J Hepatol* 2005; **42**: 252-256
- 13 Ponsioen CY, Kuiper H, Ten Kate FJ, van Milligen de Wit M, van Deventer SJ, Tytgat GN. Immunohistochemical

- analysis of inflammation in primary sclerosing cholangitis. *Eur J Gastroenterol Hepatol* 1999; **11**: 769-774
- 14 **Broome U**, Grunewald J, Scheynius A, Olerup O, Hultcrantz R. Preferential V beta3 usage by hepatic T lymphocytes in patients with primary sclerosing cholangitis. *J Hepatol* 1997; **26**: 527-534
  - 15 **Karlsen TH**, Schrumpf E, Boberg KM. Genetic epidemiology of primary sclerosing cholangitis. *World J Gastroenterol* 2007; **13**: 5421-5431
  - 16 **Terjung B**, Worman HJ. Anti-neutrophil antibodies in primary sclerosing cholangitis. *Best Pract Res Clin Gastroenterol* 2001; **15**: 629-642
  - 17 **Broome U**, Olsson R, Loof L, Bodemar G, Hultcrantz R, Danielsson A, Prytz H, Sandberg-Gertzen H, Wallerstedt S, Lindberg G. Natural history and prognostic factors in 305 Swedish patients with primary sclerosing cholangitis. *Gut* 1996; **38**: 610-615
  - 18 **O'Mahony CA**, Vierling JM. Etiopathogenesis of primary sclerosing cholangitis. *Semin Liver Dis* 2006; **26**: 3-21
  - 19 **Klein J**, Sato A. The HLA system. First of two parts. *N Engl J Med* 2000; **343**: 702-709
  - 20 **Van der Helm-van Mil AH**, Verpoort KN, Breedveld FC, Huizinga TW, Toes RE, de Vries RR. The HLA-DRB1 shared epitope alleles are primarily a risk factor for anti-cyclic citrullinated peptide antibodies and are not an independent risk factor for development of rheumatoid arthritis. *Arthritis Rheum* 2006; **54**: 1117-1121
  - 21 **Das KM**. Immunopathogenesis of primary sclerosing cholangitis: possible role of a shared colonic and biliary epithelial antigen. *J Gastroenterol Hepatol* 2004; **19**: S290
  - 22 **Das KM**, Sakamaki S, Vecchi M, Diamond B. The production and characterization of monoclonal antibodies to a human colonic antigen associated with ulcerative colitis: cellular localization of the antigen by using the monoclonal antibody. *J Immunol* 1987; **139**: 77-84
  - 23 **Das KM**, Dasgupta A, Mandal A, Geng X. Autoimmunity to cytoskeletal protein tropomyosin. A clue to the pathogenetic mechanism for ulcerative colitis. *J Immunol* 1993; **150**: 2487-2493
  - 24 **Geng X**, Biancone L, Dai HH, Lin JJ, Yoshizaki N, Dasgupta A, Pallone F, Das KM. Tropomyosin isoforms in intestinal mucosa: production of autoantibodies to tropomyosin isoforms in ulcerative colitis. *Gastroenterology* 1998; **114**: 912-922
  - 25 **Mirza ZK**, Sastri B, Lin JJ, Amenta PS, Das KM. Autoimmunity against human tropomyosin isoforms in ulcerative colitis: localization of specific human tropomyosin isoforms in the intestine and extraintestinal organs. *Inflamm Bowel Dis* 2006; **12**: 1036-1043
  - 26 **Mandal A**, Dasgupta A, Jeffers L, Squillante L, Hyder S, Reddy R, Schiff E, Das KM. Autoantibodies in sclerosing cholangitis against a shared peptide in biliary and colon epithelium. *Gastroenterology* 1994; **106**: 185-192
  - 27 **Kesari KV**, Yoshizaki N, Geng X, Lin JJ, Das KM. Externalization of tropomyosin isoform 5 in colon epithelial cells. *Clin Exp Immunol* 1999; **118**: 219-227
  - 28 **Biancone L**, Monteleone G, Marasco R, Pallone F. Autoimmunity to tropomyosin isoforms in ulcerative colitis (UC) patients and unaffected relatives. *Clin Exp Immunol* 1998; **113**: 198-205
  - 29 **Ebert EC**, Geng X, Lin J, Das KM. Autoantibodies against human tropomyosin isoform 5 in ulcerative colitis destroys colonic epithelial cells through antibody and complement-mediated lysis. *Cell Immunol* 2006; **244**: 43-49
  - 30 **Sakamaki S**, Takayanagi N, Yoshizaki N, Hayashi S, Takayama T, Kato J, Kogawa K, Yamauchi N, Takemoto N, Nobuoka A, Ayabe T, Kohgo Y, Niitsu Y. Autoantibodies against the specific epitope of human tropomyosin(s) detected by a peptide based enzyme immunoassay in sera of patients with ulcerative colitis show antibody dependent cell mediated cytotoxicity against HLA-DPw9 transfected L cells. *Gut* 2000; **47**: 236-241
  - 31 **Halstensen TS**, Das KM, Brandtzaeg P. Epithelial deposits of immunoglobulin G1 and activated complement colocalise with the M(r) 40 kD putative autoantigen in ulcerative colitis. *Gut* 1993; **34**: 650-657
  - 32 **Snook JA**, Lowes JR, Wu KC, Priddle JD, Jewell DP. Serum and tissue autoantibodies to colonic epithelium in ulcerative colitis. *Gut* 1991; **32**: 163-166
  - 33 **Cantrell M**, Prindiville T, Gershwin ME. Autoantibodies to colonic cells and subcellular fractions in inflammatory bowel disease: do they exist? *J Autoimmun* 1990; **3**: 307-320
  - 34 **Khoo UY**, Bjarnason I, Donaghy A, Williams R, Macpherson A. Antibodies to colonic epithelial cells from the serum and colonic mucosal washings in ulcerative colitis. *Gut* 1995; **37**: 63-70
  - 35 **Hamilton MI**, Bradley NJ, Srai SK, Thrasivoulou C, Pounder RE, Wakefield AJ. Autoimmunity in ulcerative colitis: tropomyosin is not the major antigenic determinant of the Das monoclonal antibody, 7E12H12. *Clin Exp Immunol* 1995; **99**: 404-411
  - 36 **Xu B**, Broome U, Ericzon BG, Sumitran-Holgersson S. High frequency of autoantibodies in patients with primary sclerosing cholangitis that bind biliary epithelial cells and induce expression of CD44 and production of interleukin 6. *Gut* 2002; **51**: 120-127
  - 37 **Karrar A**, Broome U, Sodergren T, Jaksch M, Bergquist A, Bjornstedt M, Sumitran-Holgersson S. Biliary epithelial cell antibodies link adaptive and innate immune responses in primary sclerosing cholangitis. *Gastroenterology* 2007; **132**: 1504-1514
  - 38 **Ge X**, Ericzon BG, Nowak G, oHrstrom H, Broome U, Sumitran-Holgersson S. Are preformed antibodies to biliary epithelial cells of clinical importance in liver transplantation? *Liver Transpl* 2003; **9**: 1191-1198
  - 39 **Davies DJ**, Moran JE, Niall JF, Ryan GB. Segmental necrotising glomerulonephritis with antineutrophil antibody: possible arbovirus aetiology? *Br Med J (Clin Res Ed)* 1982; **285**: 606
  - 40 **Van der Woude FJ**, Rasmussen N, Lobatto S, Wiik A, Permin H, van Es LA, van der Giessen M, van der Hem GK, The TH. Autoantibodies against neutrophils and monocytes: tool for diagnosis and marker of disease activity in Wegener's granulomatosis. *Lancet* 1985; **1**: 425-429
  - 41 **Calabresi P**, Thayer WR, Spiro HM. Demonstration of circulating antinuclear globulins in ulcerative colitis. *J Clin Invest* 1961; **40**: 2126-2133
  - 42 **Nielsen H**, Wiik A, Elmgreen J. Granulocyte specific antinuclear antibodies in ulcerative colitis. Aid in differential diagnosis of inflammatory bowel disease. *Acta Pathol Microbiol Immunol Scand [C]* 1983; **91**: 23-26
  - 43 **Snook JA**, Chapman RW, Fleming K, Jewell DP. Anti-neutrophil nuclear antibody in ulcerative colitis, Crohn's disease and primary sclerosing cholangitis. *Clin Exp Immunol* 1989; **76**: 30-33
  - 44 **Terjung B**, Bogsch F, Klein R, Sohne J, Reichel C, Wasmuth JC, Beuers U, Sauerbruch T, Spengler U. Diagnostic accuracy of atypical p-ANCA in autoimmune hepatitis using ROC- and multivariate regression analysis. *Eur J Med Res* 2004; **9**: 439-448
  - 45 **Duerr RH**, Targan SR, Landers CJ, LaRusso NF, Lindsay KL, Wiesner RH, Shanahan F. Neutrophil cytoplasmic antibodies: a link between primary sclerosing cholangitis and ulcerative colitis. *Gastroenterology* 1991; **100**: 1385-1391
  - 46 **Rump JA**, Scholmerich J, Gross V, Roth M, Helfesrieder R, Rautmann A, Ludemann J, Gross WL, Peter HH. A new type of perinuclear anti-neutrophil cytoplasmic antibody (p-ANCA) in active ulcerative colitis but not in Crohn's disease. *Immunobiology* 1990; **181**: 406-413
  - 47 **Savage J**, Dimech W, Fritzler M, Goeken J, Hagen EC, Jennette JC, McEvoy R, Pusey C, Pollock W, Trevisin M, Wiik A, Wong R. Addendum to the International Consensus Statement on testing and reporting of antineutrophil cytoplasmic antibodies. Quality control guidelines,



- comments, and recommendations for testing in other autoimmune diseases. *Am J Clin Pathol* 2003; **120**: 312-318
- 48 **Savage J**, Gillis D, Benson E, Davies D, Esnault V, Falk RJ, Hagen EC, Jayne D, Jennette JC, Paspaliaris B, Pollock W, Pusey C, Savage CO, Silvestrini R, van der Woude F, Wieslander J, Wiik A. International Consensus Statement on Testing and Reporting of Antineutrophil Cytoplasmic Antibodies (ANCA). *Am J Clin Pathol* 1999; **111**: 507-513
  - 49 **Billing P**, Tahir S, Calfin B, Gagne G, Cobb L, Targan S, Vidrich A. Nuclear localization of the antigen detected by ulcerative colitis-associated perinuclear antineutrophil cytoplasmic antibodies. *Am J Pathol* 1995; **147**: 979-987
  - 50 **Terjung B**, Herzog V, Worman HJ, Gestmann I, Bauer C, Sauerbruch T, Spengler U. Atypical antineutrophil cytoplasmic antibodies with perinuclear fluorescence in chronic inflammatory bowel diseases and hepatobiliary disorders colocalize with nuclear lamina proteins. *Hepatology* 1998; **28**: 332-340
  - 51 **Terjung B**, Spengler U, Sauerbruch T, Worman HJ. "Atypical p-ANCA" in IBD and hepatobiliary disorders react with a 50-kilodalton nuclear envelope protein of neutrophils and myeloid cell lines. *Gastroenterology* 2000; **119**: 310-322
  - 52 **Terjung B**, Worman HJ, Herzog V, Sauerbruch T, Spengler U. Differentiation of antineutrophil nuclear antibodies in inflammatory bowel and autoimmune liver diseases from antineutrophil cytoplasmic antibodies (p-ANCA) using immunofluorescence microscopy. *Clin Exp Immunol* 2001; **126**: 37-46
  - 53 **Claise C**, Johanet C, Bouhnik Y, Kapel N, Homberg JC, Poupon R. Antineutrophil cytoplasmic autoantibodies in autoimmune liver and inflammatory bowel diseases. *Liver* 1996; **16**: 28-34
  - 54 **Klein R**, Eisenburg J, Weber P, Seibold F, Berg PA. Significance and specificity of antibodies to neutrophils detected by western blotting for the serological diagnosis of primary sclerosing cholangitis. *Hepatology* 1991; **14**: 1147-1152
  - 55 **Lindgren S**, Nilsson S, Nassberger L, Verbaan H, Wieslander J. Anti-neutrophil cytoplasmic antibodies in patients with chronic liver diseases: prevalence, antigen specificity and predictive value for diagnosis of autoimmune liver disease. Swedish Internal Medicine Liver Club (SILK). *J Gastroenterol Hepatol* 2000; **15**: 437-442
  - 56 **Lo SK**, Fleming KA, Chapman RW. Prevalence of anti-neutrophil antibody in primary sclerosing cholangitis and ulcerative colitis using an alkaline phosphatase technique. *Gut* 1992; **33**: 1370-1375
  - 57 **Rooszendaal C**, de Jong MA, van den Berg AP, van Wijk RT, Limburg PC, Kallenberg CG. Clinical significance of anti-neutrophil cytoplasmic antibodies (ANCA) in autoimmune liver diseases. *J Hepatol* 2000; **32**: 734-741
  - 58 **Schwarze C**, Terjung B, Lilienweiss P, Beuers U, Herzog V, Sauerbruch T, Spengler U. IgA class antineutrophil cytoplasmic antibodies in primary sclerosing cholangitis and autoimmune hepatitis. *Clin Exp Immunol* 2003; **133**: 283-289
  - 59 **Stoffel MP**, Csernok E, Herzberg C, Johnson T, Carroll SF, Gross WL. Anti-neutrophil cytoplasmic antibodies (ANCA) directed against bactericidal/permeability increasing protein (BPI): a new seromarker for inflammatory bowel disease and associated disorders. *Clin Exp Immunol* 1996; **104**: 54-59
  - 60 **Wilschanski M**, Chait P, Wade JA, Davis L, Corey M, St Louis P, Griffiths AM, Blendis LM, Moroz SP, Scully L. Primary sclerosing cholangitis in 32 children: clinical, laboratory, and radiographic features, with survival analysis. *Hepatology* 1995; **22**: 1415-1422
  - 61 **Zachou K**, Liaskos C, Rigopoulou E, Gabeta S, Papamichalis P, Gatselis N, Georgiadou S, Dalekos GN. Presence of high avidity anticardiolipin antibodies in patients with autoimmune cholestatic liver diseases. *Clin Immunol* 2006; **119**: 203-212
  - 62 **Mehal WZ**, Lo SK, Chapman RW, Fleming KA. The immunogenetic basis for anti-neutrophil cytoplasmic antibody production in primary sclerosing cholangitis and ulcerative colitis. *J Hepatol* 1994; **21**: 910-911
  - 63 **Seibold F**, Slametschka D, Gregor M, Weber P. Neutrophil autoantibodies: a genetic marker in primary sclerosing cholangitis and ulcerative colitis. *Gastroenterology* 1994; **107**: 532-536
  - 64 **Bansi DS**, Lo S, Chapman RW, Fleming KA. Absence of antineutrophil cytoplasmic antibodies in relatives of UK patients with primary sclerosing cholangitis and ulcerative colitis. *Eur J Gastroenterol Hepatol* 1996; **8**: 111-116
  - 65 **Terjung B**, Muennich M, Gottwein J. Identification of myeloid-specific tubulin-beta isotype 5 as target antigen of antineutrophil cytoplasmic antibodies in autoimmune liver diseases. *Hepatology* 2005; **42**: 288A
  - 66 **Sobajima J**, Ozaki S, Osakada F, Uesugi H, Shirakawa H, Yoshida M, Nakao K. Novel autoantigens of perinuclear anti-neutrophil cytoplasmic antibodies (P-ANCA) in ulcerative colitis: non-histone chromosomal proteins, HMG1 and HMG2. *Clin Exp Immunol* 1997; **107**: 135-140
  - 67 **Sobajima J**, Ozaki S, Uesugi H, Osakada F, Inoue M, Fukuda Y, Shirakawa H, Yoshida M, Rokuhara A, Imai H, Kiyosawa K, Nakao K. High mobility group (HMG) non-histone chromosomal proteins HMG1 and HMG2 are significant target antigens of perinuclear anti-neutrophil cytoplasmic antibodies in autoimmune hepatitis. *Gut* 1999; **44**: 867-873
  - 68 **Sobajima J**, Ozaki S, Uesugi H, Osakada F, Shirakawa H, Yoshida M, Nakao K. Prevalence and characterization of perinuclear anti-neutrophil cytoplasmic antibodies (P-ANCA) directed against HMG1 and HMG2 in ulcerative colitis (UC). *Clin Exp Immunol* 1998; **111**: 402-407
  - 69 **Eggema M**, Cohavy O, Parseghian MH, Hamkalo BA, Clemens D, Targan SR, Gordon LK, Braun J. Identification of histone H1 as a cognate antigen of the ulcerative colitis-associated marker antibody pANCA. *J Autoimmun* 2000; **14**: 83-97
  - 70 **Kallenberg CG**, Heeringa P, Stegeman CA. Mechanisms of Disease: pathogenesis and treatment of ANCA-associated vasculitides. *Nat Clin Pract Rheumatol* 2006; **2**: 661-670
  - 71 **Gazzano-Santoro H**, Parent JB, Grinna L, Horwitz A, Parsons T, Theofan G, Elsbach P, Weiss J, Conlon PJ. High-affinity binding of the bactericidal/permeability-increasing protein and a recombinant amino-terminal fragment to the lipid A region of lipopolysaccharide. *Infect Immun* 1992; **60**: 4754-4761
  - 72 **Schultz H**, Csernok E, Schuster A, Schmitz TS, Ernst M, Gross WL. Anti-neutrophil cytoplasmic antibodies directed against the bactericidal/permeability-increasing protein (BPI) in pediatric cystic fibrosis patients do not recognize N-terminal regions important for the anti-microbial and lipopolysaccharide-binding activity of BPI. *Pediatr Allergy Immunol* 2000; **11**: 64-70
  - 73 **Rooszendaal C**, Pogany K, Horst G, Jagt TG, Kleibeuker JH, Nelis GF, Limburg PC, Kallenberg CG. Does analysis of the antigenic specificities of anti-neutrophil cytoplasmic antibodies contribute to their clinical significance in the inflammatory bowel diseases? *Scand J Gastroenterol* 1999; **34**: 1123-1131
  - 74 **Cooper T**, Savage J, Nassis L, Paspaliaris B, Neeson P, Neil J, Knight KR, Daskalakis M, Doery JC. Clinical associations and characterisation of antineutrophil cytoplasmic antibodies directed against bactericidal/permeability-increasing protein and azurocidin. *Rheumatol Int* 2000; **19**: 129-136
  - 75 **Brimnes J**, Nielsen OH, Wiik A, Heegaard NH. Autoantibodies to molecular targets in neutrophils in patients with ulcerative colitis. *Dig Dis Sci* 1999; **44**: 415-423
  - 76 **Walmsley RS**, Zhao MH, Hamilton MI, Brownlee A, Chapman P, Pounder RE, Wakefield AJ, Lockwood

- CM. Antineutrophil cytoplasm autoantibodies against bactericidal/permeability-increasing protein in inflammatory bowel disease. *Gut* 1997; **40**: 105-109
- 77 **Vecchi M**, Sinico A, Bianchi MB, Radice A, Gionchetti P, Campieri M, de Franchis R. Recognition of bactericidal/permeability-increasing protein by perinuclear anti-neutrophil cytoplasmic antibody-positive sera from ulcerative colitis patients: prevalence and clinical significance. *Scand J Gastroenterol* 1998; **33**: 1284-1288
  - 78 **Angulo P**, Peter JB, Gershwin ME, DeSotel CK, Shoenfeld Y, Ahmed AE, Lindor KD. Serum autoantibodies in patients with primary sclerosing cholangitis. *J Hepatol* 2000; **32**: 182-187
  - 79 **Khanna D**, Aggarwal A, Bhakuni DS, Dayal R, Misra R. Bactericidal/permeability-increasing protein and cathepsin G are the major antigenic targets of antineutrophil cytoplasmic autoantibodies in systemic sclerosis. *J Rheumatol* 2003; **30**: 1248-1252
  - 80 **Carlsson M**, Eriksson L, Pressler T, Kornfalt R, Mared L, Meyer P, Wiik A, Wieslander J, Segelmark M. Autoantibody response to BPI predict disease severity and outcome in cystic fibrosis. *J Cyst Fibros* 2007; **6**: 228-233
  - 81 **Baveye S**, Elasse E, Mazurier J, Spik G, Legrand D. Lactoferrin: a multifunctional glycoprotein involved in the modulation of the inflammatory process. *Clin Chem Lab Med* 1999; **37**: 281-286
  - 82 **Locht H**, Skogh T, Wiik A. Characterisation of autoantibodies to neutrophil granule constituents among patients with reactive arthritis, rheumatoid arthritis, and ulcerative colitis. *Ann Rheum Dis* 2000; **59**: 898-903
  - 83 **Chen M**, Zhao MH, Zhang YK, Wang HY. Antineutrophil cytoplasmic autoantibodies in patients with systemic lupus erythematosus recognize a novel 69 kDa target antigen of neutrophil granules. *Nephrology (Carlton)* 2005; **10**: 491-495
  - 84 **Seibold F**, Weber P, Schoning A, Mork H, Goppel S, Scheurlen M. Neutrophil antibodies (pANCA) in chronic liver disease and inflammatory bowel disease: do they react with different antigens? *Eur J Gastroenterol Hepatol* 1996; **8**: 1095-1100
  - 85 **Peen E**, Almer S, Bodemar G, Ryden BO, Sjolín C, Tejlé K, Skogh T. Anti-lactoferrin antibodies and other types of ANCA in ulcerative colitis, primary sclerosing cholangitis, and Crohn's disease. *Gut* 1993; **34**: 56-62
  - 86 **Kossa K**, Coulthart A, Ives CT, Pusey CD, Hodgson HJ. Antigen specificity of circulating anti-neutrophil cytoplasmic antibodies in inflammatory bowel disease. *Eur J Gastroenterol Hepatol* 1995; **7**: 783-789
  - 87 **Gur H**, Shen G, Sujita M, Terrberry J, Alosachie I, Barka N, Lin HC, Peter JB, Meroni PL, Kaplan M. Autoantibody profile of primary sclerosing cholangitis. *Pathobiology* 1995; **63**: 76-82
  - 88 **Tervaert JW**, Mulder AH, Horst G, Haagsma EB, Kleibeuker JH, Kallenberg CG. Antineutrophil cytoplasmic antibodies in primary sclerosing cholangitis, ulcerative colitis, and autoimmune diseases. *Gastroenterology* 1992; **102**: 1090-1091
  - 89 **Orth T**, Kellner R, Diekmann O, Faust J, Meyer zum Buschenfelde KH, Mayet WJ. Identification and characterization of autoantibodies against catalase and alpha-enolase in patients with primary sclerosing cholangitis. *Clin Exp Immunol* 1998; **112**: 507-515
  - 90 **Kain R**, Matsui K, Exner M, Binder S, Schaffner G, Sommer EM, Kerjaschki D. A novel class of autoantigens of anti-neutrophil cytoplasmic antibodies in necrotizing and crescentic glomerulonephritis: the lysosomal membrane glycoprotein h-lamp-2 in neutrophil granulocytes and a related membrane protein in glomerular endothelial cells. *J Exp Med* 1995; **181**: 585-597
  - 91 **Weismuller TJ**, Wedemeyer J, Kubicka S, Strassburg CP, Manns MP. The challenges in primary sclerosing cholangitis--aetiopathogenesis, autoimmunity, management and malignancy. *J Hepatol* 2008; **48** Suppl 1: S38-S57
  - 92 **Wiik A**. Neutrophil-specific autoantibodies in chronic inflammatory bowel diseases. *Autoimmun Rev* 2002; **1**: 67-72
  - 93 **Schultz H**. From infection to autoimmunity: a new model for induction of ANCA against the bactericidal/permeability increasing protein (BPI). *Autoimmun Rev* 2007; **6**: 223-227
  - 94 **Shih DQ**, Targan SR. Immunopathogenesis of inflammatory bowel disease. *World J Gastroenterol* 2008; **14**: 390-400
  - 95 **Targan SR**, Landers CJ, Cobb L, MacDermott RP, Vidrich A. Perinuclear anti-neutrophil cytoplasmic antibodies are spontaneously produced by mucosal B cells of ulcerative colitis patients. *J Immunol* 1995; **155**: 3262-3267
  - 96 **Seibold F**, Brandwein S, Simpson S, Terhorst C, Elson CO. pANCA represents a cross-reactivity to enteric bacterial antigens. *J Clin Immunol* 1998; **18**: 153-160
  - 97 **Terjung B**, Soehne J, Worman HJ, Sauerbruch T, Spengler U. Molecular mimicry between target antigen of ANCA and microbial protein FtsZ in autoimmune liver disorders. *Hepatology* 2006; **44**: 229A
  - 98 **Seibold F**, Weber P, Klein R, Berg PA, Wiedmann KH. Clinical significance of antibodies against neutrophils in patients with inflammatory bowel disease and primary sclerosing cholangitis. *Gut* 1992; **33**: 657-662
  - 99 **Rozenendaal C**, Van Milligen de Wit AW, Haagsma EB, Horst G, Schwarze C, Peter HH, Kleibeuker JH, Tervaert JW, Limburg PC, Kallenberg CG. Antineutrophil cytoplasmic antibodies in primary sclerosing cholangitis: defined specificities may be associated with distinct clinical features. *Am J Med* 1998; **105**: 393-399
  - 100 **Pokorny CS**, Norton ID, McCaughan GW, Selby WS. Anti-neutrophil cytoplasmic antibody: a prognostic indicator in primary sclerosing cholangitis. *J Gastroenterol Hepatol* 1994; **9**: 40-44
  - 101 **Bansi DS**, Fleming KA, Chapman RW. Importance of antineutrophil cytoplasmic antibodies in primary sclerosing cholangitis and ulcerative colitis: prevalence, titre, and IgG subclass. *Gut* 1996; **38**: 384-389
  - 102 **Mulder AH**, Horst G, Haagsma EB, Limburg PC, Kleibeuker JH, Kallenberg CG. Prevalence and characterization of neutrophil cytoplasmic antibodies in autoimmune liver diseases. *Hepatology* 1993; **17**: 411-417
  - 103 **Lo SK**, Fleming KA, Chapman RW. A 2-year follow-up study of anti-neutrophil antibody in primary sclerosing cholangitis: relationship to clinical activity, liver biochemistry and ursodeoxycholic acid treatment. *J Hepatol* 1994; **21**: 974-978
  - 104 **Haagsma EB**, Mulder AH, Gouw AS, Horst G, Meerman L, Slooff MJ, Kallenberg CG. Neutrophil cytoplasmic autoantibodies after liver transplantation in patients with primary sclerosing cholangitis. *J Hepatol* 1993; **19**: 8-14
  - 105 **Invernizzi P**, Lleo A, Podda M. Interpreting serological tests in diagnosing autoimmune liver diseases. *Semin Liver Dis* 2007; **27**: 161-172
  - 106 **Howard MJ**, Fuller C, Broadhurst RW, Perham RN, Tang JG, Quinn J, Diamond AG, Yeaman SJ. Three-dimensional structure of the major autoantigen in primary biliary cirrhosis. *Gastroenterology* 1998; **115**: 139-146
  - 107 **Czaja AJ**. Autoantibodies in autoimmune liver disease. *Adv Clin Chem* 2005; **40**: 127-164
  - 108 **Gershwin ME**, Mackay IR. The causes of primary biliary cirrhosis: Convenient and inconvenient truths. *Hepatology* 2008; **47**: 737-745
  - 109 **Eggleston P**, Haigh R, Winyard PG. Consequence of neo-antigenicity of the 'altered self'. *Rheumatology (Oxford)* 2008; **47**: 567-571
  - 110 **Terjung B**, Spengler U. Role of auto-antibodies for the diagnosis of chronic cholestatic liver diseases. *Clin Rev Allergy Immunol* 2005; **28**: 115-133
  - 111 **Zauli D**, Schrupf E, Crespi C, Cassani F, Fausa O, Aadland E. An autoantibody profile in primary sclerosing cholangitis. *J Hepatol* 1987; **5**: 14-18
  - 112 **Granito A**, Muratori P, Muratori L, Pappas G, Cassani F, Worthington J, Ferri S, Quarneri C, Cipriano V, de Molo C, Lenzi M, Chapman RW, Bianchi FB. Antibodies to SS-A/Ro-52kD and centromere in autoimmune liver disease: a

- clue to diagnosis and prognosis of primary biliary cirrhosis. *Aliment Pharmacol Ther* 2007; **26**: 831-838
- 113 **Main J**, McKenzie H, Yeaman GR, Kerr MA, Robson D, Pennington CR, Parratt D. Antibody to *Saccharomyces cerevisiae* (bakers' yeast) in Crohn's disease. *BMJ* 1988; **297**: 1105-1106
  - 114 **Sendid B**, Colombel JF, Jacquinet PM, Faille C, Fruit J, Cortot A, Lucidarme D, Camus D, Poulain D. Specific antibody response to oligomannosidic epitopes in Crohn's disease. *Clin Diagn Lab Immunol* 1996; **3**: 219-226
  - 115 **Muratori P**, Muratori L, Guidi M, Maccariello S, Pappas G, Ferrari R, Gionchetti P, Campieri M, Bianchi FB. Anti-*Saccharomyces cerevisiae* antibodies (ASCA) and autoimmune liver diseases. *Clin Exp Immunol* 2003; **132**: 473-476
  - 116 **Levine JS**, Branch DW, Rauch J. The antiphospholipid syndrome. *N Engl J Med* 2002; **346**: 752-763
  - 117 **Kirby DF**, Blei AT, Rosen ST, Vogelzang RL, Neiman HL. Primary sclerosing cholangitis in the presence of a lupus anticoagulant. *Am J Med* 1986; **81**: 1077-1080
  - 118 **Bjoro K**, Brandsaeter B, Foss A, Schrumpf E. Liver transplantation in primary sclerosing cholangitis. *Semin Liver Dis* 2006; **26**: 69-79
  - 119 **Dalekos GN**, Manoussakis MN, Goussia AC, Tsianos EV, Moutsopoulos HM. Soluble interleukin-2 receptors, antineutrophil cytoplasmic antibodies, and other autoantibodies in patients with ulcerative colitis. *Gut* 1993; **34**: 658-664
  - 120 **Aichbichler BW**, Petritsch W, Reicht GA, Wenzl HH, Eherer AJ, Hinterleitner TA, Auer-Grumbach P, Krejs GJ. Anti-cardiolipin antibodies in patients with inflammatory bowel disease. *Dig Dis Sci* 1999; **44**: 852-856
  - 121 **Koutroubakis IE**, Petinaki E, Anagnostopoulou E, Kritikos H, Mouzas IA, Kouroumalis EA, Manousos ON. Anti-cardiolipin and anti-beta2-glycoprotein I antibodies in patients with inflammatory bowel disease. *Dig Dis Sci* 1998; **43**: 2507-2512
  - 122 **Tuomi T**. Which antigen to use in the detection of rheumatoid factors? Comparison of patients with rheumatoid arthritis and subjects with 'false positive' rheumatoid factor reactions. *Clin Exp Immunol* 1989; **77**: 349-355
  - 123 **Alessandri C**, Bombardieri M, Valesini G. Pathogenic mechanisms of anti-endothelial cell antibodies (AECA): their prevalence and clinical relevance. *Adv Clin Chem* 2006; **42**: 297-326
  - 124 **Youinou P**. New target antigens for anti-endothelial cell antibodies. *Immunobiology* 2005; **210**: 789-797
  - 125 **Hay JE**, Wiesner RH, Shorter RG, LaRusso NF, Baldus WP. Primary sclerosing cholangitis and celiac disease. A novel association. *Ann Intern Med* 1988; **109**: 713-717
  - 126 **Volta U**, Rodrigo L, Granito A, Petrolini N, Muratori P, Muratori L, Linares A, Veronesi L, Fuentes D, Zauli D, Bianchi FB. Celiac disease in autoimmune cholestatic liver disorders. *Am J Gastroenterol* 2002; **97**: 2609-2613
  - 127 **Ludvigsson JF**, Elfstrom P, Broome U, Ekbom A, Montgomery SM. Celiac disease and risk of liver disease: a general population-based study. *Clin Gastroenterol Hepatol* 2007; **5**: 63-69
  - 128 **Rubio-Tapia A**, Abdulkarim AS, Wiesner RH, Moore SB, Krause PK, Murray JA. Celiac disease autoantibodies in severe autoimmune liver disease and the effect of liver transplantation. *Liver Int* 2008; **28**: 467-476
  - 129 **Rubio-Tapia A**, Murray JA. The liver in celiac disease. *Hepatology* 2007; **46**: 1650-1658
  - 130 **Preuss B**, Berg C, Altenberend F, Gregor M, Stevanovic S, Klein R. Demonstration of autoantibodies to recombinant human sulphite oxidase in patients with chronic liver disorders and analysis of their clinical relevance. *Clin Exp Immunol* 2007; **150**: 312-321
  - 131 **Klein R**, Berg PA. Anti-M4 antibodies in primary biliary cirrhosis react with sulphite oxidase, an enzyme of the mitochondrial inter-membrane space. *Clin Exp Immunol* 1991; **84**: 445-448
  - 132 **Palmer JM**, Yeaman SJ, Bassendine MF, James OF. M4 and M9 autoantigens in primary biliary cirrhosis--a negative study. *J Hepatol* 1993; **18**: 251-254
  - 133 **Ardesjo B**, Hansson CM, Bruder CE, Rorsman F, Betterle C, Dumanski JP, Kampe O, Ekwall O. Autoantibodies to glutathione S-transferase theta 1 in patients with primary sclerosing cholangitis and other autoimmune diseases. *J Autoimmun* 2008; **30**: 273-282
  - 134 **Foreman AL**, Van de Water J, Gougeon ML, Gershwin ME. B cells in autoimmune diseases: insights from analyses of immunoglobulin variable (Ig V) gene usage. *Autoimmun Rev* 2007; **6**: 387-401
  - 135 **Klareskog L**, Ronnelid J, Lundberg K, Padyukov L, Alfredsson L. Immunity to citrullinated proteins in rheumatoid arthritis. *Annu Rev Immunol* 2008; **26**: 651-675
  - 136 **Levesque MC**, St Clair EW. B cell-directed therapies for autoimmune disease and correlates of disease response and relapse. *J Allergy Clin Immunol* 2008; **121**: 13-21; quiz 22-23
  - 137 **Zauli D**, Grassi A, Cassani F, Ballardini G, Bortolotti R, Muratori L, Fusconi M, Bianchi FB. Autoimmune serology of primary sclerosing cholangitis. *Dig Liver Dis* 2001; **33**: 391-392
  - 138 **Wiesner RH**, LaRusso NF. Clinicopathologic features of the syndrome of primary sclerosing cholangitis. *Gastroenterology* 1980; **79**: 200-206
  - 139 **Boberg KM**, Aadland E, Jahnsen J, Raknerud N, Stiris M, Bell H. Incidence and prevalence of primary biliary cirrhosis, primary sclerosing cholangitis, and autoimmune hepatitis in a Norwegian population. *Scand J Gastroenterol* 1998; **33**: 99-103
  - 140 **Ballot E**, Homberg JC, Johanet C. Antibodies to soluble liver antigen: an additional marker in type 1 auto-immune hepatitis. *J Hepatol* 2000; **33**: 208-215
  - 141 **Lindgren S**, Braun HB, Michel G, Nemeth A, Nilsson S, Thome-Kromer B, Eriksson S. Absence of LKM-1 antibody reactivity in autoimmune and hepatitis-C-related chronic liver disease in Sweden. Swedish Internal Medicine Liver club. *Scand J Gastroenterol* 1997; **32**: 175-178
  - 142 **Miyakawa H**, Kawashima Y, Kitazawa E, Kawaguchi N, Kato T, Kikuchi K, Imai E, Fujikawa H, Hashimoto E, Schlumberger W. Low frequency of anti-SLA/LP autoantibody in Japanese adult patients with autoimmune liver diseases: analysis with recombinant antigen assay. *J Autoimmun* 2003; **21**: 77-82
  - 143 **Boberg KM**, Fausa O, Haaland T, Holter E, Mellbye OJ, Spurkland A, Schrumpf E. Features of autoimmune hepatitis in primary sclerosing cholangitis: an evaluation of 114 primary sclerosing cholangitis patients according to a scoring system for the diagnosis of autoimmune hepatitis. *Hepatology* 1996; **23**: 1369-1376
  - 144 **Muratori L**, Muratori P, Zauli D, Grassi A, Pappas G, Rodrigo L, Cassani F, Lenzi M, Bianchi FB. Antilactoferrin antibodies in autoimmune liver disease. *Clin Exp Immunol* 2001; **124**: 470-473
  - 145 **Hardarson S**, Labrecque DR, Mitros FA, Neil GA, Goeken JA. Antineutrophil cytoplasmic antibody in inflammatory bowel and hepatobiliary diseases. High prevalence in ulcerative colitis, primary sclerosing cholangitis, and autoimmune hepatitis. *Am J Clin Pathol* 1993; **99**: 277-281
  - 146 **Bansi DS**, Bauducci M, Bergqvist A, Boberg K, Broome U, Chapman R, Fleming K, Jorgensen R, Lindor K, Rosina F, Schrumpf E. Detection of antineutrophil cytoplasmic antibodies in primary sclerosing cholangitis: a comparison of the alkaline phosphatase and immunofluorescent techniques. *Eur J Gastroenterol Hepatol* 1997; **9**: 575-580
  - 147 **Vermeulen N**, Arijis I, Joossens S, Vermeire S, Clerens S, Van den Bergh K, Michiels G, Arckens L, Schuit F, Van Lommel L, Rutgeerts P, Bossuyt X. Anti-alpha-enolase antibodies in patients with inflammatory Bowel disease. *Clin Chem* 2008; **54**: 534-541



REVIEW

## Epithelial-mesenchymal transition mediated tumorigenesis in the gastrointestinal tract

Ammar Natalwala, Robert Spychal, Chris Tselepis

Ammar Natalwala, The Medical School, University of Birmingham, Birmingham B15 2TT, United Kingdom  
Robert Spychal, Sandwell and West Birmingham Hospitals NHS Trust, Birmingham B15 2TT, United Kingdom  
Chris Tselepis, CRUK Institute for Cancer Studies, University of Birmingham, Birmingham B15 2TT, United Kingdom  
Author contributions: Natalwala A, Spychal R and Tselepis C contributed to the writing, editing and reviewing of the manuscript.

Correspondence to: Dr. Chris Tselepis, CRUK Institute for Cancer Studies, University of Birmingham, Vincent Drive, Birmingham B15 2TH, United Kingdom. [c.tselepis@bham.ac.uk](mailto:c.tselepis@bham.ac.uk)  
Telephone: +44-121-4142972 Fax: +44-121-6272384  
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### Abstract

Epithelial-mesenchymal transition (EMT) is a highly conserved process that has been well characterised in embryogenesis. Studies have shown that the aberrant activation of EMT in adult epithelia can promote tumour metastasis by repressing cell adhesion molecules, including epithelial (E)-cadherin. Reduced intracellular adhesion may allow tumour cells to disseminate and spread throughout the body. A number of transcription proteins of the Snail superfamily have been implicated in EMT. These proteins have been shown to be over-expressed in advanced gastrointestinal (GI) tumours including oesophageal adenocarcinomas, colorectal carcinomas, gastric and pancreatic cancers, with a concomitant reduction in the expression of E-cadherin. Regulators of EMT may provide novel clinical targets to detect GI cancers early, so that cancers previously associated with a poor prognosis such as pancreatic cancer can be diagnosed before they become inoperable. Furthermore, pharmacological therapies designed to inhibit these proteins will aim to prevent local and distant tumour invasion.

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**Key words:** Epithelial-mesenchymal transition; Transcription proteins; E-cadherin; Gastrointestinal cancer

**Peer reviewer:** Francesco Feo, Professor, Dipartimento di Scienze Biomediche, Sezione di Patologia Sperimentale e

Oncologia, Università di Sassari, Via P. Manzella 4, Sassari 07100, Italy

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### INTRODUCTION

Epithelial-mesenchymal transition (EMT) is a well-characterised embryological process that has been considered to play a vital role in tumour progression<sup>[1-5]</sup>. EMT has been shown to occur during gastrulation, as well as during the development of the neural crest, heart and the musculoskeletal system<sup>[6]</sup>. In the process of EMT, epithelial cells undergo a phenotypic switch to form mesenchymal cells that are similar in appearance to fibroblasts<sup>[2,3]</sup>. The change in cell type results in the loss of polarity and also the loss of tight intracellular adhesions maintained by epithelial cells *via* adherens junctions<sup>[1-3]</sup>. This is thought to allow dynamic cellular migration and increase embryogenic diversity<sup>[5]</sup>. However, *in vitro* evidence has outlined a role for the aberrant induction of EMT in adult epithelia during tumour metastasis<sup>[6,7]</sup>. In primary tumours, the induction of EMT can lead to structural changes involving cell adhesion molecules, and in particular epithelial-cadherin (E-cadherin)<sup>[8-10]</sup>. E-cadherin is a transmembrane glycoprotein that is localised in the adherens junction typically found in epithelial cells, and plays an important role in maintaining the structural integrity of epithelial sheets<sup>[11]</sup>. The loss of E-cadherin expression has been reported in several GI cancers including advanced colorectal carcinomas, oesophageal adenocarcinomas, gastric and pancreatic cancers<sup>[12-15]</sup>. Interestingly, experiments used to silence the expression of E-cadherin not only showed a morphological shift from an epithelial to a fibroblastoid phenotype, characteristic of EMT, but also a concomitant increase in invasive cell behaviour<sup>[16]</sup>. The loss of E-cadherin has been considered to augment cellular dissemination and tumour metastasis.

The mechanisms by which E-cadherin has been shown to be inactivated include gene mutations, promoter hypermethylation, chromatin remodelling,



post-translational modification and transcriptional repression<sup>[17-19]</sup>. The major proteins implicated in the transcriptional repression of E-cadherin include the zinc finger proteins Snai1 (Snail) and Snai2 (Slug),  $\delta$ EF (ZEB-1), Smad interacting protein 1 (SIP1) or ZEB-2, and a basic helix-loop-helix (bHLH) protein called Twist<sup>[20-22]</sup>. Snail was initially described in *Drosophila melanogaster* and was shown to be essential for dorsal-ventral patterning and mesoderm formation<sup>[23]</sup>. Knockout studies revealed that mice lacking the *Snail* gene died at gastrulation due to defective EMT<sup>[24]</sup>. *Snail* was found to down-regulate epithelial markers such as claudins, occludens, desmoplakin and cytokeratins, in addition to E-cadherin, and up-regulate mesenchymal markers including fibronectin and vitronectin during EMT<sup>[25,26]</sup>. Its homologue, Slug was discovered in developing chick embryos, and was found to be abundantly expressed in cells undergoing EMT in the primitive streak, neural crest and other mesenchymal tissue<sup>[26]</sup>. Over expression of Slug in mice induced the formation of mesenchymal tumours that were mainly leukaemias and sarcomas<sup>[27]</sup>. It was discovered that both Snail and Slug are able to bind directly to E-box motifs (CANNTG) on target gene promoters, and in particular the *CDH1* gene in order to down-regulate E-cadherin expression<sup>[20]</sup>. The zinc finger proteins ZEB-1 and SIP1 were also shown to be able to repress E-cadherin by binding to similar DNA sites as Snail on the E-cadherin promoter region<sup>[28]</sup>. Furthermore, microarray analysis revealed Twist as another strong candidate for the acquisition of invasive properties of tumour cells, although its mechanism of action is less clear<sup>[22]</sup>.

## ROLE OF E-CADHERIN REPRESSORS IN CANCERS OF THE UPPER GASTROINTESTINAL TRACT

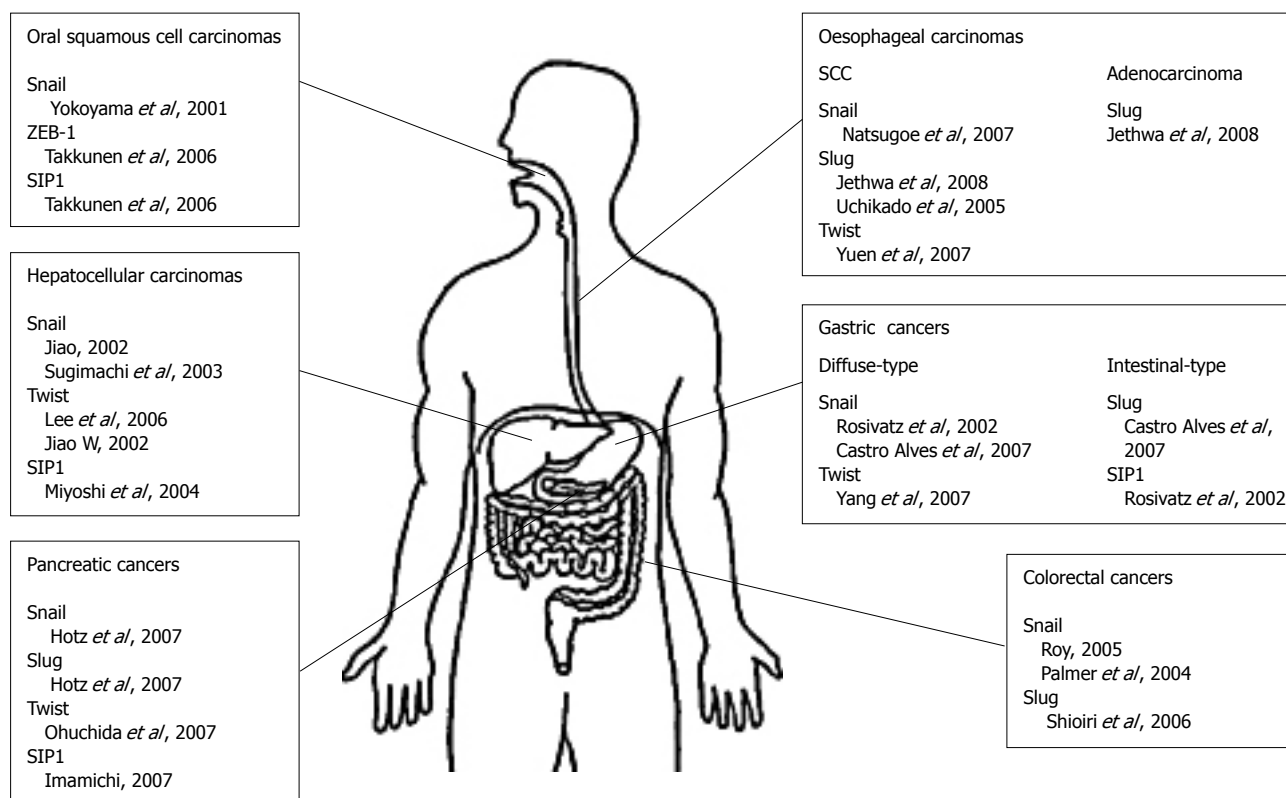
Cancers of the upper GI tract, including oral, oesophageal and gastric tumours, are associated with significant mortality worldwide<sup>[29-31]</sup>. Tumours arising in the oral cavity are predominantly squamous cell carcinomas (SCC) in origin and have a tendency to spread rapidly<sup>[32]</sup>. Oral cancer accounts for around 197 000 deaths each year, throughout the world<sup>[33]</sup>. E-cadherin expression in normal oral mucosa, compared to pre-cancerous oral lesions and primary oral SCC has been shown to be sequentially lower in each of these stages, respectively<sup>[34]</sup>. Moreover, Snail-mediated repression of E-cadherin has been confirmed in oral SCC cell lines<sup>[35]</sup>. Further analysis of primary and recurrent oral SCC, showed that the over-expression of Snail in the primary oral SCC lead to ZEB-1 and SIP1 up-regulation with a concomitant loss of E-cadherin<sup>[36]</sup>. This expression profile, now matching that of the recurrent oral SCC, may suggest that Snail is able to regulate the function of other E-cadherin repressors such as ZEB-1 and SIP1 in oral SCC.

Repression of E-cadherin has also been reported for advanced human oesophageal cancers<sup>[37]</sup>. These

tumours are known to be particularly aggressive and have a 5-year survival rate of around 8% in the United Kingdom<sup>[38]</sup>. Oesophageal cancer is histologically divided into squamous cell carcinomas and adenocarcinomas. Oesophageal SCC commonly arises in the upper third of the oesophagus and its predominant aetiological factors include alcohol and nicotine abuse<sup>[39]</sup>. Analysis of tissue samples from patients with oesophageal SCC suggests that Snail is associated with repressed E-cadherin expression in these primary tumours<sup>[40]</sup>. In addition, Slug has been shown to be over-expressed in primary oesophageal SCC, correlating with depth of tumour invasion, lymph node metastasis and poorer clinical outcome<sup>[41]</sup>. Similarly, evaluation of Twist in oesophageal SCC revealed significantly higher Twist expression relative to non-neoplastic tissue<sup>[42]</sup>.

Conversely, oesophageal adenocarcinomas arise in the lower third of the oesophagus<sup>[39]</sup>. The strongest known risk factor for the development of oesophageal adenocarcinoma is the presence of Barrett's metaplasia<sup>[43]</sup>. This is a pre-malignant state that is characterised by the replacement of native squamous oesophageal epithelium by columnar cells, and is considered to occur secondary to prolonged reflux of gastric content in the lower part of the oesophagus<sup>[44]</sup>. A recent study by Jethwa *et al* has reported an over-expression of nuclear Slug in oesophageal adenocarcinoma relative to both normal squamous and Barrett's metaplasia specimens<sup>[45]</sup>. Interestingly, no such association was observed for both Snail and Twist<sup>[45,46]</sup>.

Gastric carcinomas also form part of the upper GI cancers, and although the incidence of these tumours seems to be declining, they are still responsible for around 700 000 deaths per annum, worldwide<sup>[47]</sup>. According to Lauren's classification, gastric cancer can be subdivided into two morphologically distinct groups; diffuse and intestinal gastric cancers<sup>[48]</sup>. The aetiology of gastric cancer has been mainly linked to E-cadherin mutations, promoter hypermethylation and *H pylori* infection, but there is scarce literature on the role of E-cadherin repressors in these tumours<sup>[49,50]</sup>. Snail-regulated repression of E-cadherin has been reported for diffuse gastric cancer<sup>[25]</sup>, and over-expression of Slug has been shown in both diffuse and intestinal gastric carcinoma<sup>[51]</sup>. Previous work has also shown that in diffuse gastric carcinomas, raised Twist expression correlates with reduced E-cadherin levels, whereas in intestinal gastric cancer, SIP1 is mainly associated with reduced expression of E-cadherin<sup>[25]</sup>. Interestingly, a strong correlation between neuronal-cadherin (N-cadherin) and Twist expression has been reported in diffuse-type gastric carcinoma<sup>[25]</sup>. The up-regulation of N-cadherin has been associated with an invasive tumour phenotype and is considered to over-ride the function of E-cadherin<sup>[52]</sup>. Therefore, it is suggested that Twist may be implicated in mediating a switch from E-cadherin to the N-cadherin, in order to increase tumour cell motility<sup>[25]</sup>. A more recent study has confirmed this by using human gastric carcinoma cell lines to show that the suppression of Twist leads to a loss of cellular migration as well as N-cadherin expression<sup>[53]</sup>.



**Figure 1** Evidence for the role of EMT regulators in gastrointestinal cancer.

## COLORECTAL CANCER PROGRESSION

Colorectal cancer (CRC) is the third most common malignancy worldwide<sup>[54]</sup>. Its pathogenesis is characterised by clinical and histopathological changes known as the adenoma-carcinoma sequence, where normal colonic epithelium becomes hyper-proliferative and forms adenomatous polyps that progress to malignant disease<sup>[55]</sup>. The adenoma-carcinoma sequence is considered to occur as a result of sequential genetic changes involving defined oncogenes and tumour suppressor genes, as proposed by Fearon and Vogelstein in 1990<sup>[56]</sup>. Several studies have reported mutations of the tumour suppressor adenomatous polyposis coli (APC) gene in CRC<sup>[57-59]</sup>. These mutations are amongst the earliest genetic events found in the majority (up to 80%) of sporadic cases of CRC<sup>[60,61]</sup>. The main tumour suppressor function of APC has been shown to be in its ability to mediate the proteosomal degradation of intracellular  $\beta$ -catenin, a key member of the Wnt signalling cascade<sup>[62]</sup>. Physiologically, Wnt factors are able to induce the stabilization of cytosolic  $\beta$ -catenin, which then associates with T cell factor/lymphoid enhancer factor-1 (TCF) proteins in the nucleus to initiate the transcription of Wnt target genes<sup>[61-63]</sup>. These include genes such as c-myc, cyclin D1, Ephrin B2 and matrilysin<sup>[63]</sup>. In CRC, mutations of both APC and  $\beta$ -catenin (in 10% of cases) cause deregulation of intracellular  $\beta$ -catenin levels which leads to the nuclear accumulation of  $\beta$ -catenin<sup>[62]</sup>. This causes aberrant and constitutive expression of Wnt target genes, and thus the development of CRC<sup>[63]</sup>. Interestingly,  $\beta$ -catenin can

also interact with the cytoplasmic domain of E-cadherin, providing a link to the actin cytoskeleton *via* its binding to  $\alpha$ -catenin<sup>[64]</sup>. However, it is unclear whether the loss of this interaction with  $\beta$ -catenin, in more aggressive forms of CRC where E-cadherin is down-regulated, promotes TCF-dependent transcription<sup>[65]</sup>.

Several mechanisms of E-cadherin repression have been reported in CRC, including gene mutations and promoter hypermethylation. However, transcriptional repression of E-cadherin and associated up-regulation of Snail is also considered to play a role in the progression of CRC<sup>[66-68]</sup>. Analysis of Snail in human CRC has shown that 78% of the tumour samples examined over-expressed this protein<sup>[68]</sup>. Slug expression has been shown to be positive in 37% of cases of primary CRC, which correlated significantly with metastatic spread of the cancer<sup>[69]</sup>. Evidence for ZEB-1, SIP1 and Twist-mediated repression of E-cadherin has not yet been established in CRC<sup>[70]</sup>.

## OTHER SOLID TUMOURS OF THE GASTROINTESTINAL SYSTEM

Pancreatic cancer confers possibly the worst prognosis of the GI cancers, since it presents very late<sup>[71,72]</sup>. Evidence suggests that both Snail and Slug are over-expressed in pancreatic tumours<sup>[72]</sup>. A recent study explored Twist expression in invasive ductal carcinoma (IDC) of the pancreas and its associated pre-malignant lesion intraductal papillary mucinous neoplasia (IPMN). Although it was limited by sample size, Twist expression

was found to be significantly higher in IDC compared to matched non-tumourous and IPMN samples<sup>[73]</sup>. SIP1 expression has also been shown to be raised in pancreatic tumours<sup>[74]</sup>.

Studies have also reported the over-expression of Snail in Hepatocellular carcinomas (HCC)<sup>[75,76]</sup>. Twist expression has also been shown to be raised in HCC, which correlates with the metastatic potential of this type of tumour<sup>[77]</sup>. Miyoshi *et al* outlined the role of SIP1 in HCC by showing that transfection of SIP1 into HCC cell lines induced cellular dedifferentiation as well as E-cadherin repression. Vimentin and fibronectin, along with various matrix metalloproteinases (MMPs), were also up-regulated, and it was hypothesised that MMP up-regulation augments SIP1-induced HCC progression<sup>[78]</sup>.

## CONCLUSION

EMT is a context dependent process that is considered to be involved in the progression of GI tumours<sup>[5-7]</sup>. Snail and associated repressors of E-cadherin have been implicated in EMT, and the evidence for their role in GI cancer is summarised in Figure 1. Transcriptional repressors of E-cadherin may be useful therapeutic targets for the prevention of local invasion and distant metastasis in GI malignancies. Inhibition of these proteins may also, for the first time, allow early detection of GI cancers associated with a fatal prognosis such as pancreatic cancer to enable early intervention and avoid the situation where this type of cancer becomes inoperable. The same principle can also be applied to other pre-malignant lesions such as Barrett's metaplasia to improve the management of oesophageal adenocarcinomas. However, it is important to consider that these transcriptional repressors of E-cadherin, or EMT regulators, also have other cellular functions. Both Snail and Twist have been associated with anti-apoptotic functions and Snail has also been implicated in cell adhesion and migration<sup>[79,80]</sup>. Furthermore, some evidence suggests that the expression of E-cadherin is higher in metastatic foci, such as in CRC, thus the reverse process of EMT, or mesenchymal-epithelial transition (MET), may be required in the formation of distant metastases<sup>[8]</sup>. Therefore, the grade and location of different GI cancers will also need to be considered before commencing any pharmacological treatment targeting regulators of EMT.

Future studies should also consider the various signalling molecules that activate EMT, including the Epidermal growth factor (EGF) family members, Fibroblastic growth factors (FGF), Insulin-like growth factors (IGF), bone morphogenic proteins (BMP) and Wnt factors<sup>[79]</sup>. It is necessary to explore the interactions between these molecules, their signalling pathways, and the Snail super-family of proteins in GI tumours. This may allude to novel combined treatment regimes to improve clinical outcome. Whilst it is clear that Snail and associated regulators of EMT are implicated in GI carcinogenesis, the role of EMT in cancer is further complicated by the fact that a number of novel EMT

regulators have been identified, including molecules such as MMP-3, Met, Goosecoid, Kaiso, TGF- $\beta$ , FOXC2, GSK3 $\beta$ , Smad-3, Pez and ILK<sup>[4]</sup>. Additional research is required to support the growing literature regarding the process of EMT, in order gain full insight into its role in GI cancer progression.

## REFERENCES

- 1 **Hay ED.** The mesenchymal cell, its role in the embryo, and the remarkable signaling mechanisms that create it. *Dev Dyn* 2005; **233**: 706-720
- 2 **Thiery JP, Sleeman JP.** Complex networks orchestrate epithelial-mesenchymal transitions. *Nat Rev Mol Cell Biol* 2006; **7**: 131-142
- 3 **Shook D, Keller R.** Mechanisms, mechanics and function of epithelial-mesenchymal transitions in early development. *Mech Dev* 2003; **120**: 1351-1383
- 4 **Lee JM, Dedhar S, Kalluri R, Thompson EW.** The epithelial-mesenchymal transition: new insights in signaling, development, and disease. *J Cell Biol* 2006; **172**: 973-981
- 5 **Thiery JP.** Epithelial-mesenchymal transitions in tumour progression. *Nat Rev Cancer* 2002; **2**: 442-454
- 6 **Huber MA, Kraut N, Beug H.** Molecular requirements for epithelial-mesenchymal transition during tumor progression. *Curr Opin Cell Biol* 2005; **17**: 548-558
- 7 **Becker KF, Rosivatz E, Blechschmidt K, Kremmer E, Sarbia M, Hofler H.** Analysis of the E-cadherin repressor Snail in primary human cancers. *Cells Tissues Organs* 2007; **185**: 204-212
- 8 **Ikeguchi M, Makino M, Kaibara N.** Clinical significance of E-cadherin-catenin complex expression in metastatic foci of colorectal carcinoma. *J Surg Oncol* 2001; **77**: 201-207
- 9 **Takeichi M.** Cadherins in cancer: implications for invasion and metastasis. *Curr Opin Cell Biol* 1993; **5**: 806-811
- 10 **Birchmeier W, Behrens J.** Cadherin expression in carcinomas: role in the formation of cell junctions and the prevention of invasiveness. *Biochim Biophys Acta* 1994; **1198**: 11-26
- 11 **Kalluri R, Neilson EG.** Epithelial-mesenchymal transition and its implications for fibrosis. *J Clin Invest* 2003; **112**: 1776-1784
- 12 **Gofuku J, Shiozaki H, Tsujinaka T, Inoue M, Tamura S, Doki Y, Matsui S, Tsukita S, Kikkawa N, Monden M.** Expression of E-cadherin and alpha-catenin in patients with colorectal carcinoma. Correlation with cancer invasion and metastasis. *Am J Clin Pathol* 1999; **111**: 29-37
- 13 **Washington K, Chiappori A, Hamilton K, Shyr Y, Blanke C, Johnson D, Sawyers J, Beauchamp D.** Expression of beta-catenin, alpha-catenin, and E-cadherin in Barrett's esophagus and esophageal adenocarcinomas. *Mod Pathol* 1998; **11**: 805-813
- 14 **Oda T, Kanai Y, Oyama T, Yoshiura K, Shimoyama Y, Birchmeier W, Sugimura T, Hirohashi S.** E-cadherin gene mutations in human gastric carcinoma cell lines. *Proc Natl Acad Sci USA* 1994; **91**: 1858-1862
- 15 **Lowy AM, Knight J, Groden J.** Restoration of E-cadherin/beta-catenin expression in pancreatic cancer cells inhibits growth by induction of apoptosis. *Surgery* 2002; **132**: 141-148
- 16 **Hennig G, Behrens J, Truss M, Frisch S, Reichmann E, Birchmeier W.** Progression of carcinoma cells is associated with alterations in chromatin structure and factor binding at the E-cadherin promoter in vivo. *Oncogene* 1995; **11**: 475-484
- 17 **Rashid MG, Sanda MG, Vallorosi CJ, Rios-Doria J, Rubin MA, Day ML.** Posttranslational truncation and inactivation of human E-cadherin distinguishes prostate cancer from matched normal prostate. *Cancer Res* 2001; **61**: 489-492
- 18 **Guilford P, Hopkins J, Harraway J, McLeod M, McLeod N, Harawira P, Taite H, Scoular R, Miller A, Reeve AE.** E-cadherin germline mutations in familial gastric cancer.

- Nature* 1998; **392**: 402-405
- 19 **Hirohashi S**. Inactivation of the E-cadherin-mediated cell adhesion system in human cancers. *Am J Pathol* 1998; **153**: 333-339
  - 20 **Cano A**, Perez-Moreno MA, Rodrigo I, Locascio A, Blanco MJ, del Barrio MG, Portillo F, Nieto MA. The transcription factor snail controls epithelial-mesenchymal transitions by repressing E-cadherin expression. *Nat Cell Biol* 2000; **2**: 76-83
  - 21 **Battle E**, Sancho E, Franci C, Dominguez D, Monfar M, Baulida J, Garcia De Herreros A. The transcription factor snail is a repressor of E-cadherin gene expression in epithelial tumour cells. *Nat Cell Biol* 2000; **2**: 84-89
  - 22 **Vernon AE**, LaBonne C. Tumor metastasis: a new twist on epithelial-mesenchymal transitions. *Curr Biol* 2004; **14**: R719-R721
  - 23 **Boulay JL**, Dennefeld C, Alberga A. The Drosophila developmental gene snail encodes a protein with nucleic acid binding fingers. *Nature* 1987; **330**: 395-398
  - 24 **Carver EA**, Jiang R, Lan Y, Oram KF, Gridley T. The mouse snail gene encodes a key regulator of the epithelial-mesenchymal transition. *Mol Cell Biol* 2001; **21**: 8184-8188
  - 25 **Rosivatz E**, Becker I, Specht K, Fricke E, Lubert B, Busch R, Hofler H, Becker KF. Differential expression of the epithelial-mesenchymal transition regulators snail, SIP1, and twist in gastric cancer. *Am J Pathol* 2002; **161**: 1881-1891
  - 26 **Nieto MA**. The snail superfamily of zinc-finger transcription factors. *Nat Rev Mol Cell Biol* 2002; **3**: 155-166
  - 27 **Perez-Mancera PA**, Gonzalez-Herrero I, Maclean K, Turner AM, Yip MY, Sanchez-Martin M, Garcia JL, Robledo C, Flores T, Gutierrez-Adan A, Pintado B, Sanchez-Garcia I. SLUG (SNAIL2) overexpression in embryonic development. *Cytogenet Genome Res* 2006; **114**: 24-29
  - 28 **Comijn J**, Berx G, Vermassen P, Verschuere K, van Grunsven L, Bruyneel E, Mareel M, Huylebroeck D, van Roy F. The two-handed E box binding zinc finger protein SIP1 downregulates E-cadherin and induces invasion. *Mol Cell* 2001; **7**: 1267-1278
  - 29 **Zhong LP**, Li J, Zhang CP, Zhu HG, Sun J, Zhang ZY. Expression of E-cadherin in cervical lymph nodes from primary oral squamous cell carcinoma patients. *Arch Oral Biol* 2007; **52**: 740-747
  - 30 **Jian WG**, Darnton SJ, Jenner K, Billingham LJ, Matthews HR. Expression of E-cadherin in oesophageal carcinomas from the UK and China: disparities in prognostic significance. *J Clin Pathol* 1997; **50**: 640-644
  - 31 **Zhou YN**, Xu CP, Han B, Li M, Qiao L, Fang DC, Yang JM. Expression of E-cadherin and beta-catenin in gastric carcinoma and its correlation with the clinicopathological features and patient survival. *World J Gastroenterol* 2002; **8**: 987-993
  - 32 **Pereira MC**, Oliveira DT, Landman G, Kowalski LP. Histologic subtypes of oral squamous cell carcinoma: prognostic relevance. *J Can Dent Assoc* 2007; **73**: 339-344
  - 33 **Parkin DM**. Epidemiology of cancer: global patterns and trends. *Toxicol Lett* 1998; **102-103**: 227-234
  - 34 **Hung KF**, Chang CS, Liu CJ, Lui MT, Cheng CY, Kao SY. Differential expression of E-cadherin in metastatic lesions comparing to primary oral squamous cell carcinoma. *J Oral Pathol Med* 2006; **35**: 589-594
  - 35 **Yokoyama K**, Kamata N, Hayashi E, Hoteiya T, Ueda N, Fujimoto R, Nagayama M. Reverse correlation of E-cadherin and snail expression in oral squamous cell carcinoma cells in vitro. *Oral Oncol* 2001; **37**: 65-71
  - 36 **Takkunen M**, Grenman R, Hukkanen M, Korhonen M, Garcia de Herreros A, Virtanen I. Snail-dependent and -independent epithelial-mesenchymal transition in oral squamous carcinoma cells. *J Histochem Cytochem* 2006; **54**: 1263-1275
  - 37 **Kadowaki T**, Shiozaki H, Inoue M, Tamura S, Oka H, Doki Y, Iihara K, Matsui S, Iwazawa T, Nagafuchi A. E-cadherin and alpha-catenin expression in human esophageal cancer. *Cancer Res* 1994; **54**: 291-296
  - 38 **Office for National Statistics Cancer Statistics registrations**: Registrations of cancer diagnosed in 2002, England Series MB1 no.31. 2003, National Statistics: London
  - 39 **Siewert JR**, Ott K. Are squamous and adenocarcinomas of the esophagus the same disease? *Semin Radiat Oncol* 2007; **17**: 38-44
  - 40 **Natsugoe S**, Uchikado Y, Okumura H, Matsumoto M, Setoyama T, Tamotsu K, Kita Y, Sakamoto A, Owaki T, Ishigami S, Aikou T. Snail plays a key role in E-cadherin-preserved esophageal squamous cell carcinoma. *Oncol Rep* 2007; **17**: 517-523
  - 41 **Uchikado Y**, Natsugoe S, Okumura H, Setoyama T, Matsumoto M, Ishigami S, Aikou T. Slug Expression in the E-cadherin preserved tumors is related to prognosis in patients with esophageal squamous cell carcinoma. *Clin Cancer Res* 2005; **11**: 1174-1180
  - 42 **Yuen HF**, Chan YP, Wong ML, Kwok WK, Chan KK, Lee PY, Srivastava G, Law SY, Wong YC, Wang X, Chan KW. Upregulation of Twist in oesophageal squamous cell carcinoma is associated with neoplastic transformation and distant metastasis. *J Clin Pathol* 2007; **60**: 510-514
  - 43 **Dias Pereira A**, Suspiro A, Chaves P. Cancer risk in Barrett's oesophagus. *Eur J Gastroenterol Hepatol* 2007; **19**: 915-918
  - 44 **Jankowski JA**, Harrison RF, Perry I, Balkwill F, Tselepis C. Barrett's metaplasia. *Lancet* 2000; **356**: 2079-2085
  - 45 **Jethwa P**, Naqvi M, Hardy RG, Hotchin NA, Roberts S, Spychal R, Tselepis C. Overexpression of Slug is associated with malignant progression of esophageal adenocarcinoma. *World J Gastroenterol* 2008; **14**: 1044-1052
  - 46 **Rosivatz E**, Becker KF, Kremmer E, Schott C, Blechschmidt K, Hofler H, Sarbia M. Expression and nuclear localization of Snail, an E-cadherin repressor, in adenocarcinomas of the upper gastrointestinal tract. *Virchows Arch* 2006; **448**: 277-287
  - 47 **Forman D**, Burley VJ. Gastric cancer: global pattern of the disease and an overview of environmental risk factors. *Best Pract Res Clin Gastroenterol* 2006; **20**: 633-649
  - 48 **Vauhkonen M**, Vauhkonen H, Sipponen P. Pathology and molecular biology of gastric cancer. *Best Pract Res Clin Gastroenterol* 2006; **20**: 651-674
  - 49 **Chan AO**. E-cadherin in gastric cancer. *World J Gastroenterol* 2006; **12**: 199-203
  - 50 **Liu YC**, Shen CY, Wu HS, Chan DC, Chen CJ, Yu JC, Yu CP, Harn HJ, Shyu RY, Shih YL, Hsieh CB, Hsu HM. Helicobacter pylori infection in relation to E-cadherin gene promoter polymorphism and hypermethylation in sporadic gastric carcinomas. *World J Gastroenterol* 2005; **11**: 5174-5179
  - 51 **Castro Alves C**, Rosivatz E, Schott C, Hollweck R, Becker I, Sarbia M, Carneiro F, Becker KF. Slug is overexpressed in gastric carcinomas and may act synergistically with SIP1 and Snail in the down-regulation of E-cadherin. *J Pathol* 2007; **211**: 507-515
  - 52 **Hazan RB**, Qiao R, Keren R, Badano I, Suyama K. Cadherin switch in tumor progression. *Ann N Y Acad Sci* 2004; **1014**: 155-163
  - 53 **Yang Z**, Zhang X, Gang H, Li X, Li Z, Wang T, Han J, Luo T, Wen F, Wu X. Up-regulation of gastric cancer cell invasion by Twist is accompanied by N-cadherin and fibronectin expression. *Biochem Biophys Res Commun* 2007; **358**: 925-930
  - 54 **Early DS**, Fontana L, Davidson NO. Translational approaches to addressing complex genetic pathways in colorectal cancer. *Transl Res* 2008; **151**: 10-16
  - 55 **Lee S**, Bang S, Song K, Lee I. Differential expression in normal-adenoma-carcinoma sequence suggests complex molecular carcinogenesis in colon. *Oncol Rep* 2006; **16**: 747-754
  - 56 **Fearon ER**, Vogelstein B. A genetic model for colorectal tumorigenesis. *Cell* 1990; **61**: 759-767
  - 57 **Scott RJ**, van der Luijt R, Spycher M, Mary JL, Muller A, Hoppeler T, Haner M, Muller H, Martinoli S, Brazzola PL. Novel germline APC gene mutation in a large familial adenomatous polyposis kindred displaying variable phenotypes. *Gut* 1995; **36**: 731-736



- 58 **Sparks AB**, Morin PJ, Vogelstein B, Kinzler KW. Mutational analysis of the APC/beta-catenin/Tcf pathway in colorectal cancer. *Cancer Res* 1998; **58**: 1130-1134
- 59 **Fodde R**. The APC gene in colorectal cancer. *Eur J Cancer* 2002; **38**: 867-871
- 60 **Powell SM**, Zilz N, Beazer-Barclay Y, Bryan TM, Hamilton SR, Thibodeau SN, Vogelstein B, Kinzler KW. APC mutations occur early during colorectal tumorigenesis. *Nature* 1992; **359**: 235-237
- 61 **Schneikert J**, Behrens J. The canonical Wnt signalling pathway and its APC partner in colon cancer development. *Gut* 2007; **56**: 417-425
- 62 **Morin PJ**, Sparks AB, Korinek V, Barker N, Clevers H, Vogelstein B, Kinzler KW. Activation of beta-catenin-Tcf signaling in colon cancer by mutations in beta-catenin or APC. *Science* 1997; **275**: 1787-1790
- 63 **Behrens J**. The role of the Wnt signalling pathway in colorectal tumorigenesis. *Biochem Soc Trans* 2005; **33**: 672-675
- 64 **Hulsken J**, Birchmeier W, Behrens J. E-cadherin and APC compete for the interaction with beta-catenin and the cytoskeleton. *J Cell Biol* 1994; **127**: 2061-2069
- 65 **Kuphal F**, Behrens J. E-cadherin modulates Wnt-dependent transcription in colorectal cancer cells but does not alter Wnt-independent gene expression in fibroblasts. *Exp Cell Res* 2006; **312**: 457-467
- 66 **Efstathiou JA**, Liu D, Wheeler JM, Kim HC, Beck NE, Ilyas M, Karayiannakis AJ, Mortensen NJ, Kmiot W, Playford RJ, Pignatelli M, Bodmer WF. Mutated epithelial cadherin is associated with increased tumorigenicity and loss of adhesion and of responsiveness to the motogenic trefoil factor 2 in colon carcinoma cells. *Proc Natl Acad Sci USA* 1999; **96**: 2316-2321
- 67 **Wheeler JM**, Kim HC, Efstathiou JA, Ilyas M, Mortensen NJ, Bodmer WF. Hypermethylation of the promoter region of the E-cadherin gene (CDH1) in sporadic and ulcerative colitis associated colorectal cancer. *Gut* 2001; **48**: 367-371
- 68 **Roy HK**, Smyrk TC, Koetsier J, Victor TA, Wali RK. The transcriptional repressor SNAIL is overexpressed in human colon cancer. *Dig Dis Sci* 2005; **50**: 42-46
- 69 **Shioiri M**, Shida T, Koda K, Oda K, Seike K, Nishimura M, Takano S, Miyazaki M. Slug expression is an independent prognostic parameter for poor survival in colorectal carcinoma patients. *Br J Cancer* 2006; **94**: 1816-1822
- 70 **Rosivatz E**, Becker I, Bamba M, Schott C, Diebold J, Mayr D, Hofler H, Becker KF. Neoexpression of N-cadherin in E-cadherin positive colon cancers. *Int J Cancer* 2004; **111**: 711-719
- 71 **Koliopanos A**, Avgerinos C, Farfaras A, Manes C, Dervenis C. Radical resection of pancreatic cancer. *Hepatobiliary Pancreat Dis Int* 2008; **7**: 11-18
- 72 **Hotz B**, Arndt M, Dullat S, Bhargava S, Buhr HJ, Hotz HG. Epithelial to mesenchymal transition: expression of the regulators snail, slug, and twist in pancreatic cancer. *Clin Cancer Res* 2007; **13**: 4769-4776
- 73 **Ohuchida K**, Mizumoto K, Ohhashi S, Yamaguchi H, Konomi H, Nagai E, Yamaguchi K, Tsuneyoshi M, Tanaka M. Twist, a novel oncogene, is upregulated in pancreatic cancer: clinical implication of Twist expression in pancreatic juice. *Int J Cancer* 2007; **120**: 1634-1640
- 74 **Imamichi Y**, Konig A, Gress T, Menke A. Collagen type I-induced Smad-interacting protein 1 expression downregulates E-cadherin in pancreatic cancer. *Oncogene* 2007; **26**: 2381-2385
- 75 **Jiao W**, Miyazaki K, Kitajima Y. Inverse correlation between E-cadherin and Snail expression in hepatocellular carcinoma cell lines in vitro and in vivo. *Br J Cancer* 2002; **86**: 98-101
- 76 **Sugimachi K**, Tanaka S, Kameyama T, Taguchi K, Aishima S, Shimada M, Sugimachi K, Tsuneyoshi M. Transcriptional repressor snail and progression of human hepatocellular carcinoma. *Clin Cancer Res* 2003; **9**: 2657-2664
- 77 **Lee TK**, Poon RT, Yuen AP, Ling MT, Kwok WK, Wang XH, Wong YC, Guan XY, Man K, Chau KL, Fan ST. Twist overexpression correlates with hepatocellular carcinoma metastasis through induction of epithelial-mesenchymal transition. *Clin Cancer Res* 2006; **12**: 5369-5376
- 78 **Miyoshi A**, Kitajima Y, Sumi K, Sato K, Hagiwara A, Koga Y, Miyazaki K. Snail and SIP1 increase cancer invasion by upregulating MMP family in hepatocellular carcinoma cells. *Br J Cancer* 2004; **90**: 1265-1273
- 79 **De Craene B**, van Roy F, Berx G. Unraveling signalling cascades for the Snail family of transcription factors. *Cell Signal* 2005; **17**: 535-547
- 80 **Castanon I**, Baylies MK. A Twist in fate: evolutionary comparison of Twist structure and function. *Gene* 2002; **287**: 11-22

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## OBSERVER

Hugh James Freeman, MD, FRCPC, FACP, Series Editor

# Endoscopic stenting-Where are we now and where can we go?

Mark Terence McLoughlin, Michael Francis Byrne

Mark Terence McLoughlin, Michael Francis Byrne, UBC Division of Gastroenterology, Vancouver, British Columbia V5Z 1M9, Canada

Author contributions: McLoughlin MT and Byrne MF contributed equally to this paper.

Correspondence to: Dr. Michael Francis Byrne, MA, MD (Cantab), MRCP (UK), FRCPC, UBC Division of Gastroenterology, 5135-2775 Laurel Street, Vancouver, British Columbia V5Z 1M9, Canada. [michael.byrne@vch.ca](mailto:michael.byrne@vch.ca)

Telephone: +1-604-8755640 Fax: +1-604-8755447

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## Abstract

Self expanding metal stents (SEMS) play an important role in the management of malignant obstructing lesions in the gastrointestinal tract. Traditionally, they have been used for palliation in malignant gastric outlet and colonic obstruction and esophageal malignancy. The development of the polyflex stent, which is a removable self expanding plastic stent, allows temporary stent insertion for benign esophageal disease and possibly for patients undergoing neoadjuvant chemotherapy prior to esophagectomy. Potential complications of SEMS insertion include perforation, tumour overgrowth or ingrowth, and stent migration. Newer stents are being developed with the aim of increasing technical and clinical success rates, while reducing complication rates. Other areas of development include biodegradable stents for benign disease and radioactive or drug-eluting stents for malignant disease. It is hoped that, in the future, newer stents will improve our management of these difficult conditions and, possibly, provide prognostic as well as symptomatic benefit in the setting of malignant obstruction.

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**Key words:** Endoscopy; Stent; Palliation; Bowel obstruction; Malignancy

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## INTRODUCTION

Self expanding metal stent (SEMS) insertion has an important role in the management of malignant gastrointestinal obstruction. There are several types and sizes of SEMS on the market. Each has its own characteristics in terms of radial forces exerted, foreshortening on deployment, and flexibility. SEMS are made of either stainless steel [e.g. Z-stent (Cook)] or alloys such as Nitinol [e.g. Ultraflex (Boston Scientific), Alimaxx E (Alveolus)] or Elgiloy [e.g. Wallstent (Boston Scientific)]<sup>[1]</sup>. Stent insertion is also increasingly used in benign esophageal disease, such as non-malignant strictures and anastomotic leaks. The Polyflex stent (Boston Scientific) is a self expanding plastic stent which has been approved for use in the management of benign and malignant esophageal strictures.

Enteral SEMS, i.e. for the duodenum and colon, are generally inserted through the scope (TTS). These are deployed over a guidewire under direct vision, usually with fluoroscopic guidance. Esophageal stents are not TTS and are deployed under fluoroscopic guidance after delineating the margins of the stricture endoscopically.

In this article, we review the current state of play with respect to enteral and esophageal stents, the latest developments, and possible future directions.

## ESOPHAGEAL STENTING

SEMS have been in use for malignant dysphagia and trache-esophageal fistulae (TEF) since the early 1990s when they replaced rigid plastic stents. They are relatively easy to deploy, have a high technical success rate and provide rapid relief of dysphagia<sup>[2]</sup>. However, insertion of SEMS has a complication rate of 26%-52%<sup>[3-7]</sup> with 1 in 6 requiring further stents<sup>[8]</sup>. Procedure related mortality is 2%-3%<sup>[7,8]</sup>. Complications associated with esophageal stent insertion include perforation, bleeding,

stent migration, reflux, chest pain, recurrent dysphagia due to tumour overgrowth or ingrowth, migration, and food bolus impaction. Although SEMS insertion is still the treatment of choice for TEF, it appears not to be the safe, one-off treatment for malignant dysphagia that it was once hoped to be.

Comparisons between SEMS and brachytherapy for esophageal malignancy have shown improved dysphagia scores at 30 d with reduced complications<sup>[9]</sup> and improvements in quality of life, dysphagia, and eating scales<sup>[10]</sup> for brachytherapy. It has been suggested that, as stent re-intervention is likely to be increased for those who live longer, SEMS should be considered for those with a poorer prognosis, and chemo/radiotherapy, with temporary stent placement, for those with a longer life expectancy<sup>[12]</sup>. A Korean group inserted a removable nitinol stent in 47 patients who had concurrent radiotherapy and extracted the stent in 24 patients after 4 wk, leaving the stent in place in the remaining patients<sup>[11]</sup>. The complication and re-intervention rates were significantly lower in the group in which the stent was extracted, while the dysphagia-progression-free and overall survival rates were significantly longer. No randomized trials have yet been conducted with the Polyflex stent, which is the only removable stent licensed in the USA in this setting. Further randomized trials of SEMS in combination with other treatment modalities would help determine the optimal management strategy in terms of symptom control and overall survival. Drug-eluting and radioactive stents may also have a future role in the management of esophageal malignancy; these have been tested with success in animal models<sup>[12,13]</sup>.

In cases where the distal margin of the stent crosses the gastro-esophageal (GE) junction there are now SEMS available with an anti-reflux mechanism. Survival has been shown to be reduced in patients in whom the stent crossed the GE junction<sup>[14]</sup>. A study which compared an open stent with the Z-stent with Dua antireflux valve found that 96% of patients with the open stent had reflux symptoms, compared with 12% with the antireflux mechanism<sup>[15]</sup>. Several other SEMS with antireflux mechanisms have been manufactured. Further work will be required to determine the overall efficacy and complication rates of these stents for distal esophageal and cardia tumours.

Many of the available SEMS are covered to reduce the risk of tumour in-growth and to seal TEF. As the risk of stent migration is higher with covered stents, many have flared ends and uncovered segments at both ends to anchor on to the tissue. Fully covered SEMS may prove useful in benign disease as they are potentially removable but further experience in this area is required.

The Polyflex stent is the only stent currently licensed for benign disease but there has been an interest in the development of biodegradable stents. These would theoretically exert their effect before slowly breaking down and subsequent stent extraction, which can be stressful for the patient and physician, is avoided. A small case series from Japan had promising results when

a biodegradable stent constructed from poly-L-lactic acid monofilaments was used to treat benign esophageal stenoses<sup>[16]</sup>.

## GASTRIC OUTLET OBSTRUCTION (GOO) AND DUODENAL STENTING

Stent placement for GOO was first described in 1992<sup>[17]</sup>. Patients with GOO are generally very ill and in the terminal phase of a malignant process. Gastrojejunostomy (GJJ) has traditionally been the procedure of choice for GOO. However, insertion of a SEMS for GOO offers a relatively safe and much less invasive alternative to gastrojejunal bypass. Most trials comparing GJJ with SEMS insertion for GOO are prospective or retrospective comparative studies or case series evaluating either SEMS insertion or GJJ. A summary of the prospective and larger retrospective case series is given in Table 1. A more recent comprehensive review of stent insertion *versus* GJJ for GOO included a total of 1046 patients undergoing stent insertion and 297 undergoing GJJ<sup>[39]</sup>. There was no difference between SEMS insertion and GJJ in terms of technical success (96% *vs* 100%), early (7% *vs* 6%) and late (18% *vs* 17%) major complications, or persisting symptoms (8% *vs* 9%). Initial symptom relief was higher for SEMS (89% *vs* 72%). Recurrent obstructive symptoms were higher for SEMS (18% *vs* 1%) but hospital stay was shorter (13 d *vs* 7 d) with a mean survival of 105 d after stent placement and 164 after GJJ. These results suggest that stent placement may be the preferred option for patients with a shorter life expectancy but GJJ is preferable for patients with a more favourable prognosis.

Several stents are available for gastroduodenal use including the Wallstent Enteral, Wallflex Enteral Duodenal (Boston Scientific), Choo stent (Solco Intermed Co. Ltd. and Mi Tech Co. Ltd), and the Song stent (Stentech). The aim of stent manufacturers is to produce a SEMS which is easy to insert, is clinically effective and carries a low complication and migration rate. The use of the new Nitinol Wallflex stent was investigated by Van Hooft *et al*<sup>[40]</sup> who inserted a total of 66 Wallflex stents in 62 patients. with a clinical success rate of 85%. Median hospital stay was 6 d, and 10 of 60 patients (17%) who had follow up data for 30 d developed complications. They concluded that the new stent was effective and relatively safe.

Other recently developed stents include the Niti-S enteral stent (Taewoong Medical Co.) which has a woven rather than the usual braided design, leading to improved flexibility and reduced foreshortening and, it is hoped, reduced migration, as well as dual stents (e.g. Niti-S Comvi, Taewoong Medical Co., and the dual expandable nitinol stent, S&G Biotech). These have a covered layer to reduce tumour ingrowth and an uncovered layer to reduce migration. These newer stents have shown promising results in case series<sup>[32,33,37]</sup> but randomized comparisons with conventional stents are required to further assess their efficacy.

Table 1 Summary of case series of SEMS placement for gastric outlet obstruction (%)

Authors	Yr	Study design	n	Technical success	Clinical success	Major complications (early and late)
de Baere <i>et al</i> <sup>[18]</sup>	1997	Prospective	10	100	94	28
Bethge <i>et al</i> <sup>[19]</sup>	1998	Prospective	6	100	100	33
Jung <i>et al</i> <sup>[20]</sup>	2000	Prospective	19	95	100	26
Pinto Pabon <i>et al</i> <sup>[21]</sup>	2001	Prospective	31	100	90	10
Kim <i>et al</i> <sup>[22]</sup>	2001	Prospective	29	90	96	29
Lopera <i>et al</i> <sup>[23]</sup>	2001	Prospective	16	94	81	19
Profili <i>et al</i> <sup>[24]</sup>	2001	Prospective	15	100	93	14
Lee <i>et al</i> <sup>[25]</sup>	2001	Prospective	11	87	82	0
Espinel <i>et al</i> <sup>[26]</sup>	2001	Prospective	6	100	100	0
Jung <i>et al</i> <sup>[27]</sup>	2002	Prospective	39	97	95	36
Jeong <i>et al</i> <sup>[28]</sup>	2002	Prospective	18	100	94	28
Schiefke <i>et al</i> <sup>[29]</sup>	2003	Prospective	20	100	100	nr
Holt <i>et al</i> <sup>[30]</sup>	2004	Prospective	28	93	93	21
Huang <i>et al</i> <sup>[31]</sup>	2007	Prospective	14	100	86	14
Kim <i>et al</i> <sup>[32]</sup>	2007	Prospective	213	94	94	21
Lee <i>et al</i> <sup>[33]</sup>	2007	Prospective	11	100	91	18
Lowe <i>et al</i> <sup>[34]</sup>	2007	Prospective	87	97	87	10
Maetani <i>et al</i> <sup>[35]</sup>	2007	Prospective	37	97	94	19
Song <i>et al</i> <sup>[36]</sup>	2004	Retrospective	102	99	84	9
Telford <i>et al</i> <sup>[37]</sup>	2004	Retrospective	176	97	84	9
Bessoud <i>et al</i> <sup>[38]</sup>	2005	Retrospective	72	97	90	15

nr: Not reported.

Increasingly innovative techniques for stent insertion are also being pioneered. The development of double balloon enteroscopy has allowed us to perform therapeutic procedures in areas that were previously beyond our reach. Ross *et al* successfully inserted a SEMS in the distal duodenum for a patient with metastatic lung cancer using double balloon enteroscopy<sup>[41]</sup>. This raises the possibility of stent insertion in patients with a single point of malignant small bowel obstruction that is beyond the reach of conventional endoscopes.

## COLONIC STENTING

The use of SEMS in the palliation of malignant colonic obstruction was first described in 1991<sup>[42]</sup>. The current stents available are uncovered but there have been reports on the use of uncovered and covered esophageal stents in the colon. Overall technical success rates are generally in excess of 95% with relief of obstructive symptoms in 85%-90% for palliative stenting<sup>[1]</sup>. In a comprehensive review of 58 publications on colorectal stent publications from 1990 to 2000<sup>[43]</sup> stent insertion was successful in 551 of 598 cases (92%). There was a 4% rate of perforation, 10% migration rate and 10% re-obstruction rate. Stent migration was associated with laser pre-treatment, concurrent chemotherapy, covered stent use, and benign disease. The perforation rate was higher in the studies in which balloon pre-dilation was performed (10% *vs* 2%), suggesting that this should not be performed routinely. A variety of stents were used in the different studies but most of them were uncovered. One study, which used partially or fully covered stents, had a migration rate of 22%<sup>[44]</sup>.

Many earlier series used esophageal stents for colonic stenting and it is hoped that specifically designed

colorectal stents will have lower rates of migration. For example, in a prospective study with 44 patients<sup>[45]</sup> the nitinol Ultraflex precision colonic stent migrated in one patient (2%) who had commenced chemotherapy shortly after stent insertion. There was a technical success rate of 95% and a 6 mo clinical success rate of 81%. There have been very few comparisons between different stent types. A small retrospective study comparing the Ultraflex stent and the Wallstent found that they both provided adequate relief of obstruction but the Ultraflex had a significantly lower rate of delayed complications, need for re-intervention, and a non-significant reduction in early migration and occlusion<sup>[46]</sup>.

In recent years there has been a move towards SEMS insertion as a “bridge” to surgery for patients who present with acute malignant obstruction. In the event that a patient is subsequently deemed unsuitable for a curative resection, the stent provides palliation. 10%-30% of patients with colonic cancer present with obstructive symptoms<sup>[47]</sup> and in many centres surgical decompression remains the primary management option for such patients, either due to local preference or resources. Morbidity and mortality rates have been quoted at 32%-64% and 15%-34%, respectively, for patients who undergo emergency surgery<sup>[48-52]</sup>. Up to 40% of these patients are left with a permanent colostomy<sup>[53]</sup>. For those patients who are subsequently found to have operable disease they then need a second surgical procedure. Stent insertion is appealing as it allows these patients to have adequate rehabilitation and preparation before an elective procedure, while avoiding invasive surgery for palliative patients.

Martinez-Santos *et al*<sup>[50]</sup> performed a prospective study investigating the results of colonic stenting *versus* emergency surgery in 72 patients presenting with left



sided malignant colorectal obstruction. Forty-three patients had preoperative stent insertion followed by elective surgery (if necessary) and 29 had emergency surgical treatment. Surgical resection was subsequently found not to be indicated in 18 of the patients who had SEMS insertion and in 3 from the control group. SEMS insertion was clinically successful in 41 cases (95%). Of those patients with colonic stents who proceeded to surgery 85% had a primary anastomosis, compared to 41% in the non-stent group ( $P = 0.0025$ ), with a lower need for a colostomy (15% *vs* 59%). There were also significantly reduced severe complications, re-intervention rates, total hospital stay, and ICU stay. Overall, stent placement prevented 17 of 18 (94%) unnecessary operations. In a long-term follow up study there was no difference in 3 years (48% *vs* 50%) and 5 years (40% *vs* 44%) survival in the SEMS and emergency surgery groups respectively, but post-operative complications were significantly lower in the stent group<sup>[51]</sup>. Therefore, stent insertion as a bridge to surgery is technically and clinically successful, relatively safe, and cheaper than emergency surgery for patients who present with malignant left sided obstruction.

SEMS insertion is not currently approved for benign disease of the colon, primarily because of high failure and complication rates, as well as an inability to remove the stent endoscopically<sup>[54]</sup>. In one study there was a failure rate of 63% for 8 patients<sup>[55]</sup>. In the largest series to date 23 patients had an SEMS placed for benign colonic disease<sup>[56]</sup>. There was a 100% technical success rate and 95% (22/23) clinical success rate. Eight of the 23 patients (38%) had major complications, 7 of which (87%) occurred within the first week. Sixteen of the 19 patients who underwent a colectomy were successfully converted from an emergency procedure to an elective one; 8 patients did not require a colostomy. SEMS insertion should probably not be considered as a definitive treatment option for benign colonic strictures, in view of the high rate of complications in the limited published data available. However, in the setting of colonic obstruction it may be appropriate as a temporary measure to facilitate decompression with subsequent elective surgery, rather than proceeding to emergency colectomy.

## CONCLUSION

Endoscopic stenting remains an important tool in the management of malignant conditions of the esophagus, gastric outlet, and colon. As newer stents are developed, randomised trials are required to determine which provides the most benefit. For esophageal malignancy the evidence suggests that SEMS insertion may be appropriate for patients with a poorer prognosis, with temporary SEMS and chemoradiotherapy for those with a longer life expectancy. However, further trials are required to clarify the optimum management of these patients. For patients undergoing neo-adjuvant therapy prior to esophagectomy, a small retrospective trial reported favourable results for temporary placement

of the Polyflex stent<sup>[57]</sup>. The Polyflex stent is being increasingly used for benign esophageal strictures and has also been used with success in the management of post-operative esophageal leak<sup>[58]</sup>. Another novel use for temporary esophageal stent placement was in the management of acute esophageal variceal bleeding<sup>[59]</sup>. Although the findings of this small study were positive, a large comparative trial would be required before SEMS could replace the current therapy for bleeding varices.

Colonic stenting should be considered for palliation in malignant obstruction and as a bridge to surgery in the setting of acute obstruction. The results of further randomized controlled trials, such as the Dutch Stent-in 2 study<sup>[60]</sup>, are awaited to bolster the existing evidence. Covered stents and pre-deployment dilatation appear to increase the complication rate. The development of stents with longer delivery systems will hopefully make the right colon more accessible also. Double balloon enteroscopy may also allow stent insertion in areas that were previously beyond our reach.

As stenting devices and our skills develop, endoscopic capabilities will continue to expand. Bioabsorbable stents may allow a safe and effective method of temporary stent placement, without the need for a further procedure. Radioactive and drug-eluting esophageal stents have already been trialled in animal models; it is hoped that such stents in the future will have prognostic as well as symptomatic benefit for patients with malignant obstruction. We await these new developments with anticipation.

## REFERENCES

- 1 **Tierney W**, Chuttani R, Croffie J, DiSario J, Liu J, Mishkin DS, Shah R, Somogyi L, Petersen BT. Enteral stents. *Gastrointest Endosc* 2006; **63**: 920-926
- 2 **Dua KS**. Stents for palliating malignant dysphagia and fistula: is the paradigm shifting? *Gastrointest Endosc* 2007; **65**: 77-81
- 3 **Cwikiel W**, Tranberg KG, Cwikiel M, Lillo-Gil R. Malignant dysphagia: palliation with esophageal stents--long-term results in 100 patients. *Radiology* 1998; **207**: 513-518
- 4 **McManus K**, Khan I, McGuigan J. Self-expanding oesophageal stents: strategies for re-intervention. *Endoscopy* 2001; **33**: 601-604
- 5 **Siersema PD**, Hop WC, van Blankenstein M, van Tilburg AJ, Bac DJ, Homs MY, Kuipers EJ. A comparison of 3 types of covered metal stents for the palliation of patients with dysphagia caused by esophagogastric carcinoma: a prospective, randomized study. *Gastrointest Endosc* 2001; **54**: 145-153
- 6 **Wang MQ**, Sze DY, Wang ZP, Wang ZQ, Gao YA, Dake MD. Delayed complications after esophageal stent placement for treatment of malignant esophageal obstructions and esophagorespiratory fistulas. *J Vasc Interv Radiol* 2001; **12**: 465-474
- 7 **Shenfine J**, McNamee P, Steen N, Bond J, Griffin SM. A pragmatic randomised controlled trial of the cost-effectiveness of palliative therapies for patients with inoperable oesophageal cancer. *Health Technol Assess* 2005; **9**: iii, 1-121
- 8 **Ross WA**, Alkassab F, Lynch PM, Ayers GD, Ajani J, Lee JH, Bismar M. Evolving role of self-expanding metal stents in the treatment of malignant dysphagia and fistulas. *Gastrointest Endosc* 2007; **65**: 70-76

- 9 **Homs MY**, Steyerberg EW, Eijkenboom WM, Tilanus HW, Stalpers LJ, Bartelsman JF, van Lanschot JJ, Wijdeman HK, Mulder CJ, Reinders JG, Boot H, Aleman BM, Kuipers EJ, Siersema PD. Single-dose brachytherapy versus metal stent placement for the palliation of dysphagia from oesophageal cancer: multicentre randomised trial. *Lancet* 2004; **364**: 1497-1504
- 10 **Homs MY**, Essink-Bot ML, Borsboom GJ, Steyerberg EW, Siersema PD. Quality of life after palliative treatment for oesophageal carcinoma -- a prospective comparison between stent placement and single dose brachytherapy. *Eur J Cancer* 2004; **40**: 1862-1871
- 11 **Shin JH**, Song HY, Kim JH, Kim SB, Lee GH, Park SI, Han YM, Kang W. Comparison of temporary and permanent stent placement with concurrent radiation therapy in patients with esophageal carcinoma. *J Vasc Interv Radiol* 2005; **16**: 67-74
- 12 **Won JH**, Lee JD, Wang HJ, Kim GE, Kim BW, Yim H, Han SK, Park CH, Joh CW, Kim KH, Park KB, Shin KM. Self-expandable covered metallic esophageal stent impregnated with beta-emitting radionuclide: an experimental study in canine esophagus. *Int J Radiat Oncol Biol Phys* 2002; **53**: 1005-1013
- 13 **Guo Q**, Guo S, Wang Z. A type of esophageal stent coating composed of one 5-fluorouracil-containing EVA layer and one drug-free protective layer: in vitro release, permeation and mechanical properties. *J Control Release* 2007; **118**: 318-324
- 14 **Elphick DA**, Smith BA, Bagshaw J, Riley SA. Self-expanding metal stents in the palliation of malignant dysphagia: outcome analysis in 100 consecutive patients. *Dis Esophagus* 2005; **18**: 93-95
- 15 **Laasch HU**, Marriott A, Wilbraham L, Tunnah S, England RE, Martin DF. Effectiveness of open versus antireflux stents for palliation of distal esophageal carcinoma and prevention of symptomatic gastroesophageal reflux. *Radiology* 2002; **225**: 359-365
- 16 **Saito Y**, Tanaka T, Andoh A, Minematsu H, Hata K, Tsujikawa T, Nitta N, Murata K, Fujiyama Y. Usefulness of biodegradable stents constructed of poly-L-lactic acid monofilaments in patients with benign esophageal stenosis. *World J Gastroenterol* 2007; **13**: 3977-3980
- 17 **Kozarek RA**, Ball TJ, Patterson DJ. Metallic self-expanding stent application in the upper gastrointestinal tract: caveats and concerns. *Gastrointest Endosc* 1992; **38**: 1-6
- 18 **de Baere T**, Harry G, Ducreux M, Elias D, Briquet R, Kuoch V, Roche A. Self-expanding metallic stents as palliative treatment of malignant gastroduodenal stenosis. *AJR Am J Roentgenol* 1997; **169**: 1079-1083
- 19 **Bethge N**, Breikreutz C, Vakil N. Metal stents for the palliation of inoperable upper gastrointestinal stenoses. *Am J Gastroenterol* 1998; **93**: 643-645
- 20 **Jung GS**, Song HY, Kang SG, Huh JD, Park SJ, Koo JY, Cho YD. Malignant gastroduodenal obstructions: treatment by means of a covered expandable metallic stent-initial experience. *Radiology* 2000; **216**: 758-763
- 21 **Pinto Pabon IT**, Diaz LP, Ruiz De Adana JC, Lopez Herrero J. Gastric and duodenal stents: follow-up and complications. *Cardiovasc Intervent Radiol* 2001; **24**: 147-153
- 22 **Kim JH**, Yoo BM, Lee KJ, Hahm KB, Cho SW, Park JJ, Kim SS, Park HC, Kim JH. Self-expanding coil stent with a long delivery system for palliation of unresectable malignant gastric outlet obstruction: a prospective study. *Endoscopy* 2001; **33**: 838-842
- 23 **Lopera JE**, Alvarez O, Castano R, Castaneda-Zuniga W. Initial experience with Song's covered duodenal stent in the treatment of malignant gastroduodenal obstruction. *J Vasc Interv Radiol* 2001; **12**: 1297-1303
- 24 **Profili S**, Meloni GB, Bifulco V, Conti M, Feo CF, Canalis GC. Self-expandable metal stents in the treatment of antropyloric and/or duodenal strictures. *Acta Radiol* 2001; **42**: 176-180
- 25 **Lee JM**, Han YM, Lee SY, Kim CS, Yang DH, Lee SO. Palliation of postoperative gastrointestinal anastomotic malignant strictures with flexible covered metallic stents: preliminary results. *Cardiovasc Intervent Radiol* 2001; **24**: 25-30
- 26 **Espinel J**, Vivas S, Munoz F, Jorquera F, Olcoz JL. Palliative treatment of malignant obstruction of gastric outlet using an endoscopically placed enteral Wallstent. *Dig Dis Sci* 2001; **46**: 2322-2324
- 27 **Jung GS**, Song HY, Seo TS, Park SJ, Koo JY, Huh JD, Cho YD. Malignant gastric outlet obstructions: treatment by means of coaxial placement of uncovered and covered expandable nitinol stents. *J Vasc Interv Radiol* 2002; **13**: 275-283
- 28 **Jeong JY**, Han JK, Kim AY, Lee KH, Lee JY, Kang JW, Kim TJ, Shin SH, Choi BI. Fluoroscopically guided placement of a covered self-expandable metallic stent for malignant antroduodenal obstructions: preliminary results in 18 patients. *AJR Am J Roentgenol* 2002; **178**: 847-852
- 29 **Schieffe I**, Zabel-Langhennig A, Wiedmann M, Huster D, Witzgmann H, Mossner J, Berr F, Caca K. Self-expandable metallic stents for malignant duodenal obstruction caused by biliary tract cancer. *Gastrointest Endosc* 2003; **58**: 213-219
- 30 **Holt AP**, Patel M, Ahmed MM. Palliation of patients with malignant gastroduodenal obstruction with self-expanding metallic stents: the treatment of choice? *Gastrointest Endosc* 2004; **60**: 1010-1017
- 31 **Huang Q**, Dai DK, Qian XJ, Zhai RY. Treatment of gastric outlet and duodenal obstructions with uncovered expandable metal stents. *World J Gastroenterol* 2007; **13**: 5376-5379
- 32 **Kim JH**, Song HY, Shin JH, Choi E, Kim TW, Jung HY, Lee GH, Lee SK, Kim MH, Ryu MH, Kang YK, Kim BS, Yook JH. Metallic stent placement in the palliative treatment of malignant gastroduodenal obstructions: prospective evaluation of results and factors influencing outcome in 213 patients. *Gastrointest Endosc* 2007; **66**: 256-264
- 33 **Lee SM**, Kang DH, Kim GH, Park WI, Kim HW, Park JH. Self-expanding metallic stents for gastric outlet obstruction resulting from stomach cancer: a preliminary study with a newly designed double-layered pyloric stent. *Gastrointest Endosc* 2007; **66**: 1206-1210
- 34 **Lowe AS**, Beckett CG, Jowett S, May J, Stephenson S, Scally A, Tam E, Kay CL. Self-expandable metal stent placement for the palliation of malignant gastroduodenal obstruction: experience in a large, single, UK centre. *Clin Radiol* 2007; **62**: 738-744
- 35 **Maetani I**, Isayama H, Mizumoto Y. Palliation in patients with malignant gastric outlet obstruction with a newly designed enteral stent: a multicenter study. *Gastrointest Endosc* 2007; **66**: 355-360
- 36 **Song HY**, Shin JH, Yoon CJ, Lee GH, Kim TW, Lee SK, Yook JH, Kim BS. A dual expandable nitinol stent: experience in 102 patients with malignant gastroduodenal strictures. *J Vasc Interv Radiol* 2004; **15**: 1443-1449
- 37 **Telford JJ**, Carr-Locke DL, Baron TH, Tringali A, Parsons WG, Gabbrielli A, Costamagna G. Palliation of patients with malignant gastric outlet obstruction with the enteral Wallstent: outcomes from a multicenter study. *Gastrointest Endosc* 2004; **60**: 916-920
- 38 **Bessoud B**, de Baere T, Denys A, Kuoch V, Ducreux M, Precetti S, Roche A, Menu Y. Malignant gastroduodenal obstruction: palliation with self-expanding metallic stents. *J Vasc Interv Radiol* 2005; **16**: 247-253
- 39 **Jeurnink SM**, van Eijck CH, Steyerberg EW, Kuipers EJ, Siersema PD. Stent versus gastrojejunostomy for the palliation of gastric outlet obstruction: a systematic review. *BMC Gastroenterol* 2007; **7**: 18
- 40 **Van Hooft J**, Mutignani M, Repici A, Messmann H, Neuhaus H, Fockens P. First data on the palliative treatment of patients with malignant gastric outlet obstruction using the WallFlex enteral stent: a retrospective multicenter study.

- Endoscopy* 2007; **39**: 434-439
- 41 **Ross AS**, Semrad C, Waxman I, Dye C. Enteral stent placement by double balloon enteroscopy for palliation of malignant small bowel obstruction. *Gastrointest Endosc* 2006; **64**: 835-837
- 42 **Dohmoto M**. New method-endoscopic implantation of rectal stent in palliative treatment of malignant stenosis. *Endoscopia Digestiva* 1991; **3**: 1507-1512
- 43 **Khot UP**, Lang AW, Murali K, Parker MC. Systematic review of the efficacy and safety of colorectal stents. *Br J Surg* 2002; **89**: 1096-1102
- 44 **Choo IW**, Do YS, Suh SW, Chun HK, Choo SW, Park HS, Kang SK, Kim SK. Malignant colorectal obstruction: treatment with a flexible covered stent. *Radiology* 1998; **206**: 415-421
- 45 **Repici A**, Fregonese D, Costamagna G, Dumas R, Kahler G, Meisner S, Giovannini M, Freeman J, Petruziello L, Hervoso C, Comunale S, Faroux R. Ultraflex precision colonic stent placement for palliation of malignant colonic obstruction: a prospective multicenter study. *Gastrointest Endosc* 2007; **66**: 920-927
- 46 **Small AJ**, Baron TH. Comparison of Wallstent and Ultraflex stents for palliation of malignant colonic obstruction: a retrospective, case-matched analysis. *Gastrointest Endosc* 2007; **65**: AB365
- 47 **Deans GT**, Krukowski ZH, Irwin ST. Malignant obstruction of the left colon. *Br J Surg* 1994; **81**: 1270-1276
- 48 **Law WL**, Choi HK, Chu KW. Comparison of stenting with emergency surgery as palliative treatment for obstructing primary left-sided colorectal cancer. *Br J Surg* 2003; **90**: 1429-1433
- 49 **Leitman IM**, Sullivan JD, Brams D, DeCosse JJ. Multivariate analysis of morbidity and mortality from the initial surgical management of obstructing carcinoma of the colon. *Surg Gynecol Obstet* 1992; **174**: 513-518
- 50 **Martinez-Santos C**, Lobato RF, Fradejas JM, Pinto I, Ortega-Deballon P, Moreno-Azcoita M. Self-expandable stent before elective surgery vs. emergency surgery for the treatment of malignant colorectal obstructions: comparison of primary anastomosis and morbidity rates. *Dis Colon Rectum* 2002; **45**: 401-406
- 51 **Saida Y**, Sumiyama Y, Nagao J, Uramatsu M. Long-term prognosis of preoperative "bridge to surgery" expandable metallic stent insertion for obstructive colorectal cancer: comparison with emergency operation. *Dis Colon Rectum* 2003; **46**: S44-S49
- 52 **Smothers L**, Hynan L, Fleming J, Turnage R, Simmang C, Anthony T. Emergency surgery for colon carcinoma. *Dis Colon Rectum* 2003; **46**: 24-30
- 53 **Vandervoort J**, Tham TC. Colonic stents for malignant obstruction--not a bridge too far? *Gastrointest Endosc* 2006; **64**: 921-924
- 54 **Baron TH**. Colonic stenting: technique, technology, and outcomes for malignant and benign disease. *Gastrointest Endosc Clin N Am* 2005; **15**: 757-771
- 55 **Meisner S**, Hensler M, Knop FK, West F, Wille-Jorgensen P. Self-expanding metal stents for colonic obstruction: experiences from 104 procedures in a single center. *Dis Colon Rectum* 2004; **47**: 444-450
- 56 **Small AJ**, Young-Fadok TM, Baron TH. Expandable metal stent placement for benign colorectal obstruction: outcomes for 23 cases. *Surg Endosc* 2008; **22**: 454-462
- 57 **Siddiqui AA**, Loren D, Dudnick R, Kowalski T. Expandable polyester silicon-covered stent for malignant esophageal strictures before neoadjuvant chemoradiation: a pilot study. *Dig Dis Sci* 2007; **52**: 823-829
- 58 **Freeman RK**, Ascoti AJ, Wozniak TC. Postoperative esophageal leak management with the Polyflex esophageal stent. *J Thorac Cardiovasc Surg* 2007; **133**: 333-338
- 59 **Hubmann R**, Bodlaj G, Czompo M, Benko L, Pichler P, Al-Kathib S, Kiblböck P, Shamyieh A, Biesenbach G. The use of self-expanding metal stents to treat acute esophageal variceal bleeding. *Endoscopy* 2006; **38**: 896-901
- 60 **Van Hooft JE**, Bemelman WA, Breumelhof R, Siersema PD, Kruij PM, van der Linde K, Veenendaal RA, Verhulst ML, Marinelli AW, Gerritsen JJ, van Berkel AM, Timmer R, Grubben MJ, Scholten P, Geraedts AA, Oldenburg B, Sprangers MA, Bossuyt PM, Fockens P. Colonic stenting as bridge to surgery versus emergency surgery for management of acute left-sided malignant colonic obstruction: a multicenter randomized trial (Stent-in 2 study). *BMC Surg* 2007; **7**: 12

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## GASTRIC CANCER

# PCR-SSCP-DNA sequencing method in detecting *PTEN* gene mutation and its significance in human gastric cancer

Chuan-Yong Guo, Xuan-Fu Xu, Jian-Ye Wu, Shu-Fang Liu

Chuan-Yong Guo, Xuan-Fu Xu, Jian-Ye Wu, Shu-Fang Liu,  
Department of Gastroenterology, Tenth People's Hospital of  
Tongji University, Shanghai 200072, China

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Foundation of Shanghai, No. grant 200701

Correspondence to: Chuan-Yong Guo, Professor, Depart-  
ment of Gastroenterology, Tenth people's Hospital of Tongji  
University, Shanghai 200072,

China. [guochuanyong@hotmail.com](mailto:guochuanyong@hotmail.com)

Telephone: +86-21-66302535 Fax: +86-21-66303983

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of Hepatology, Postgraduate Institute of Medical Education  
and Research, Chandigarh 160012, India; Toru Ishikawa, MD,  
Department of Gastroenterology, Saiseikai Niigata Second  
Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan

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## Abstract

**AIM:** To discuss the possible effect of *PTEN* gene mutations on occurrence and development of gastric cancer.

**METHODS:** Fifty-three gastric cancer specimens were selected to probe *PTEN* gene mutations in genome of gastric cancer and paracancerous tissues using PCR-SSCP-DNA sequencing method based on microdissection and to observe the protein expression by immunohistochemistry technique.

**RESULTS:** PCR-SSCP-DNA sequencing indicated that 4 kinds of mutation sites were found in 5 of 53 gastric cancer specimens. One kind of mutation was found in exons. AA-TCC mutation was located at 40bp upstream of 3' lateral exon 7 (115946 AA-TCC). Such mutations led to terminator formation in the 297th codon of the *PTEN* gene. The other 3 kinds of mutation were found in introns, including a G-C point mutation at 91 bp upstream of 5' lateral exon 5(90896 G-C), a T-G point mutation at 24 bp upstream of 5' lateral exon 5 (90963 T-G), and a single base A mutation at 7 bp upstream of 5' lateral exon 5 (90980 A del). The *PTEN* protein expression in gastric cancer and paracancerous tissues detected using immunohistochemistry technique indicated that the total positive rate of *PTEN* protein expression was 66% in gastric cancer tissue, which was significantly lower than that (100%) in paracancerous tissues ( $P < 0.005$ ).

**CONCLUSION:** *PTEN* gene mutation and expression may play an important role in the occurrence and development of gastric cancer.

## INTRODUCTION

The occurrence and development of gastric cancer, like other malignant tumors, are a complicated process involving participation of polygene and many factors<sup>[1-4]</sup>. It is generally considered that protein tyrosine phosphatase level plays an important role in the process. Mutation of the *PTEN* gene encoding for protein tyrosine phosphatase and abnormal expression of protein are significantly correlated with the occurrence and development of malignant tumors such as glioblastoma, prostate cancer, malignant melanoma, and breast cancer, etc<sup>[5-11]</sup>. However, only few studies are available on *PTEN* gene mutation and protein expression in gastric cancer<sup>[3,12-15]</sup>. The aim of this study was to detect the *PTEN* gene mutation in gastric cancer and paracancerous tissue from 53 patients by PCR-SSCP-DNA sequencing method and to observe the protein expression by immunohistochemistry technique in order to find the effect of *PTEN* gene on the occurrence and development of gastric cancer.

## MATERIALS AND METHODS

### Objects

Fifty-three gastric cancer and corresponding paracancerous normal tissue samples were obtained at surgery. All the samples were formalin fixed, paraffin embedded, and pathologically confirmed. Of the 53 patients, 41 were males and 12 were females with a mean age of



Table 1 Primer sequence, length and annealing temperature of exons 5-8 in *PTEN* gene

	Primer sequence	Primer length (bp)	Amplification fragment length (bp)	Annealing temperature (°C)
Exon-5F:	ACCTGTTAAGTTTGTATGCAAC	22	379	52
R	TCCAGGAAGAGGAAAGGAAA	20		
Exon-6F:	CATAGCAATTTAGTGAAATAACT	23	274	52
R	GATATGGTTAAGAAAACGTTC	22		
Exon-7F:	TGACAGTTTGACAGTTAAAGG	21	263	58
R	GGATATTTCTCCCAATGAAAG	21		
Exon-8F:	CTCAGATTGCCCTTATAATAGTC	22	558	52
R	TCIGTTACTTGCTACGTAAAC	21		

65.6 years, ranging 39-81 years. The tumor diameter was greater than 3 cm in 37 patients. The tumor was located in gastric antrum of 30 patients, in gastric body of 16 patients, and in gastric cardia of 7 patients, respectively. Invasion was restricted in mucosa and submucosa of 2 patients (I), in muscular layer of 12 patients (II), in chorion and subchorion of 15 patients (III), in neighboring organs of 24 patients through chorion (IV). Lymph node metastasis was found in 32 patients, distant metastasis in 8 patients, embolization in 45 patients. Well-differentiated tumor was found in 1 patient, moderately-differentiated tumor in 35 patients, and poorly-differentiated tumor in 27 patients. pTMN stage I was identified in 13 patients, stage II in 6 patients, stage III in 26 patients, and stage IV in 8 patients.

### Reagents

Tris base, EDTA, 2H<sub>2</sub>O-Na<sub>2</sub>, Taq DNA polymerase and Taq I were purchased from Shanghai Sangon Biological Engineering Technology and Service Co. Ltd. SDS was purchased from AMRESCO Inc. Protein enzyme was purchased from Jingmei Biotechnology Co. Ltd. dNTPs was purchased from Pharmacia Inc. Polyclonal rabbit anti-PTEN antibody and immunohistochemistry staining reagent kit (Rabbit SP Kit) were purchased from Zymed Laboratories Inc. DAAB kit and citrate buffer were purchased from Beijing Zhongshan Biotechnology Co. Ltd. Phosphate-buffered saline (PBS) was purchased from Fuzhou Maixin Biotechnology Co. Ltd.

### Genome DNA extraction from paired gastric cancer and paracancerous tissues

Three paraffin slices (7 µm) were dried in a galvanothermy box at 60°C for 30 min, hydrated in gradient ethanol after deparaffinized in dimethylbenzene, adequately rinsed with tap water and naturally dried. Necrotic tissues were removed under inverted microscope and no carcinoma cells were found in paracancerous tissues. Gastric cancer and paracancerous tissues were put into a 1.5 mL eppendorf tube into which 50 µL digest buffer solution was added. The tube was overturned several times to blend it adequately, bathed in water for 8 h at 65°C and shaken several times. Protein enzyme k was deactivated at 95°C for 8 min and then centrifuged at 10000 r/min for 10-15 min. Transfer supernatant, namely genome DNA, was transferred to another antiseptic tube and stored at 4°C for application.

### PCR amplification of sequence of exons 5-8 in *PTEN* gene

PCR system is composed of 5 µL PCR buffer solution, 5 µL dNTP (2.5 mmol/L), 2 µL primer (F) (10 pmol/µL), 2 µL primer (R) (10 mmol/L), 2 µL DNA template, 1 µL Taq DNA polymerase (5 units/µL), 33 µL ddH<sub>2</sub>O. PCR conditions were at 94°C for 4 min × 1 cycle, at 94°C for 30 s, at 52°C (fifth, sixth and eighth exons) at 58°C (seventh exon) for 30 s, at 72°C for 30 s × 30 cycles, at 72°C for 7 min × 1 cycle. Five µL of the PCR amplified product was put on a 2% agarose gel containing 0.5 g/L EB, 100 bp DNA ladder as a standard reference, electrophoresed for 45 min at 100 V. The results were observed with an ultraviolet transmission reflect analysis instrument and photo was taken with an automatic gel documentation system. Primers used for detecting the mutation of exons 5-8 in the *PTEN* gene are listed in Table 1.

### Enzyme cut reaction with Taq I

Enzyme cut reaction is composed of 3.2 µL ddH<sub>2</sub>O, 1.5 µL buffer Taq I, 10.0 µL PCR, 0.3 µL Taq I (10 unit/µL), and a total volume of 15.0 µL. The mixture was centrifuged for 15 s and heated for 3.5 h at 65°C. Ten µL enzyme cut product was put on a 2% agarose gel containing 0.5 g/L EB, 100 bp DNA ladder as a standard reference, electrophoresed for 45 min at 100 V. The results were observed with an ultraviolet transmission reflect analysis instrument and photo was taken with an automatic gel documentation system to evaluate the enzyme cut reaction.

### SSCP analysis

Eight percent neutral polyacrylamide gel electrophoresis was performed as previously described<sup>[16]</sup>. In brief, 3 mL 40% acrylamide solution, 3 mL 5 × TBE solution, 3 mL 50% glycerin, 6 mL ddH<sub>2</sub>O, 75 µL 10% ammonium persulfate, 7 µL TEMED, were blended adequately and poured into the gel, then concreted for 1 h at room temperature. Four µL PCR product (eighth exon enzyme cut product of exon 8) and 6 µL formamide sample were mixed. The mixture was centrifuged for 15 s, denatured at 95°C for 10 min, bathed in ice for 10 min, put on an 8% neutral polyacrylamide gel, and electrophoresed with 1 × TBE buffer for 8 h at 14°C and 300 V. The fixation

solution was infused into a flat utensil, into which gel was immersed, vibrated for 10 min, and washed 3 times (2 min each time) with ddH<sub>2</sub>O. The gel was immersed into a staining solution, vibrated for 10 min, washed 3 times (20 s each time) with ddH<sub>2</sub>O. The gel was then immersed into a display solution, vibrated until the sample signal became brown and the background became transparent yellow, and rinsed with tap water to stop display. The staining results were observed and photographs were taken.

According to the PCR-SSCP results of genome DNA, the difference in the single strand strip number and electrophoresis transference location, also known as the mobility shift, was considered PCR-SSCP positive.

### DNA sequencing

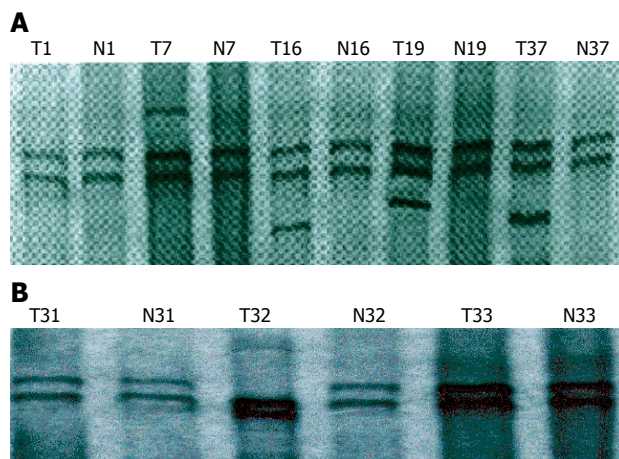
Genome DNA from positive PCR-SSCP samples was amplified again in 80  $\mu$ L reaction system. The product was identified by electrophoresis for bidirectional DNA sequencing.

### Immunohistochemical staining

PBS was used instead of the primary antibody for blank and normal non-immunized rabbit serum was used instead of the primary antibody for negative. Following the specifications provided with the SP staining reagent box, the deparaffinized tissues were cut into 5  $\mu$ m thick sections, washed 3 times (5 min each time) with PBS, incubated at room temperature in 3% H<sub>2</sub>O<sub>2</sub> to eliminate the endogenous peroxidase activity, then wash additional 3 times (3 min each time) with PBS. Antigens were repaired with microwave (citrate buffer pH 6.0), naturally refrigerated to room temperature, washed 3 times (3 min each time) with PBS, incubated at room temperature with normal non-immunized serum solution for 15 min to indicate the non-specific sites, then incubated at room temperature with the primary antibody solution and horse radish peroxidase (HRP) tagged streptavidin for 15 min respectively, washed 3 times (3 min each time) with PBS. DAE stain was rinsed with PBS for 3 min, counter stained with hematoxylin for 1 min, rinsed with tap water for 2 min, differentiated with 1% hydrochloric ethanol, rinsed with tap water for 5 min, dehydrated with gradient alcohol, transparentized with dimethylbenzene. The sections were coated with neutral balata.

### Criteria for positive PTEN protein immunohistochemical staining

Ten high power fields (50-300 cells/HP) were randomly selected for each section to measure histology (H) scores according to the percentage (P) and intensity (I) scores of positive cells (  $H = P \times I$ , P: percentage lower than 10% for score 0, 11%-40% for score 1, 41%-70% for score 2, and higher than 71% for score 3. I: intensity null for score 0, weak (faint yellow) for score 1, moderate (yellow) for score 2, strong (brown) for score 3. H measurement: score 0 or 1 for negative, score 2 or more for positive).



**Figure 1** PCR-SSCP showing exons 5 (A) and 7 (B) in *PTEN* gene. T7, T16, T19 and T37: Positive SSCP; T1: Negative SSCP; T32: Positive SSCP; T31 and T33: Negative SSCP. N: Paracancerous tissue samples; T: Gastric cancer tissue samples.

### Statistical analysis

Fisher's exact probability and chi-square test were used in statistical analysis.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Mutation of exons 5-8 in *PTEN* gene

Detection of the *PTEN* gene exons 5-8 of genome DNA in 53 paired gastric cancer and paracancerous tissue samples indicated that the amplified PCR product had no gene homozygous alteration and no large and/or alteration in the alleles.

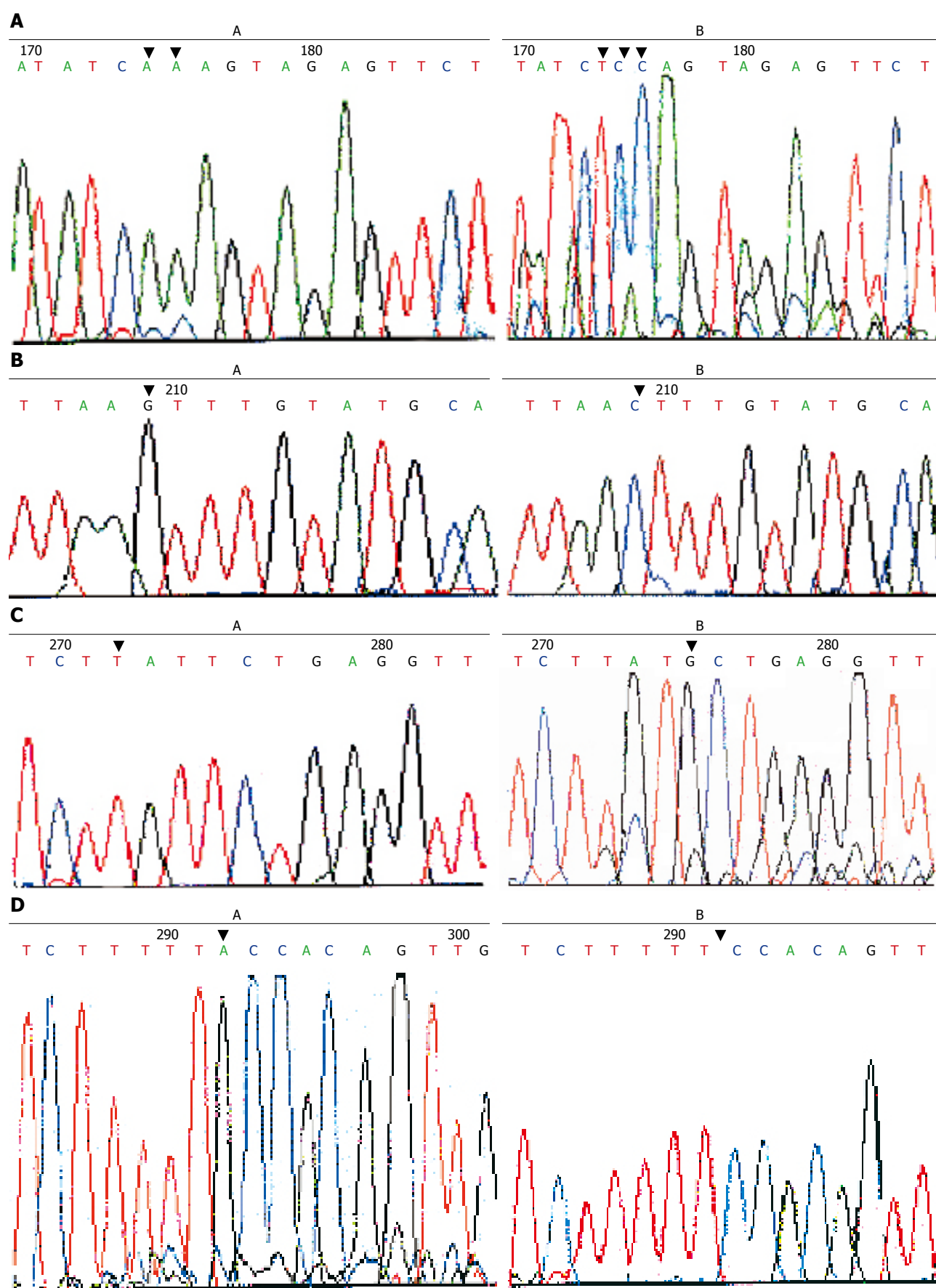
Ten  $\mu$ L reaction product of PCR amplified exon 8 and Taq I enzyme cut reaction on a 2% agarose gel containing 0.5 g/L EB, 100 bp DNA ladder were used as a standard reference. The results indicated that the number and size were in accordance with the theory. The 281 bp, 247 bp, 30 bp segments were relatively justified as the complete enzyme cut reaction.

### SSCP detection

In terms of mutation of exons-5-8 in the *PTEN* gene, positive PCR-SSCP was considered abnormal single strand number and mobility location. Of the 53 gastric cancer tissue samples, mutation occurred in 5 samples, the mutation rate was 9.4%. A surplus shift strip of exon 5 was found in 4 samples, the mutation rate was 7.5% (Figure 1A). Abnormal motility velocity (single strand strip mobility location) was observed in 1 sample at exon 7, the mutation rate was 1.9% (Figure 1B). There was no abnormal SSCP strip in exons 6 and 8.

### DNA sequencing

Genome DNA from positive PCR-SSCP samples was amplified for bidirectional DNA sequencing. The results indicated that only one mutation was found in exons. As in the sample, AA-TCC mutation was located at



**Figure 2** AA-TCC mutation at 40 bp upstream of 3' lateral in exon 7 (A), G-C point mutation at 91 bp upstream of 5' lateral exon 5 (B), T-G point mutation at 24 bp upstream (C) and single base A mutation at 7 bp upstream (D) of 5' lateral exon 5 in paired paracancerous and gastric cancer tissue samples.



Table 2 PTEN protein expression in gastric cancer and paracancerous tissue samples and intensity distribution *n* (%)

Clinicopathological parameters	Cases	PTEN protein expression				<i>P</i>
		-	+	++	+++	
Paracancerous	53	0 (0.0)	10 (18.9)	18 (34.0)	25 (47.2)	< 0.005
Gastric cancer	53	18 (34.0)	17 (32.1)	15 (28.3)	3 (5.7)	
Differentiation extent						< 0.005
Moderate and high differentiation	26	4 (15.4)	6 (23.1)	10 (38.5)	6 (23.1)	
Low differentiation	27	14 (51.9)	8 (29.6)	3 (11.1)	2 (7.4)	

40 bp upstream of 3' lateral in exon 7 (Figure 2A). Such mutations led to terminator formation in codon 297 of the *PTEN* gene. The other 3 kinds of mutation were found in introns, including a G-C point mutation at 91bp upstream of 5' lateral in exon 7 (Figure 2B), a T-G point mutation at 24 bp upstream of 5' lateral in exon 5 (Figure 2C), single base A mutation was deleted at 7 bp upstream of 5' lateral in exon 5 (Figure 2D).

#### PTEN protein expression in gastric cancer and paracancerous tissues

The PTEN protein was expressed in gastric cancer and paracancerous tissue samples. The expression of PTEN protein in gastric cancer samples was 66.0% and 100% in gastric cancer and paracancerous tissue samples, respectively ( $P < 0.005$ , Table 2).

#### Correlation between PTEN protein expression and clinicopathological parameters in gastric cancer patients

PTEN expression was not significantly correlated with the clinicopathological parameters in gastric cancer patients, such as gender and age of the patients, location and size of the carcinoma, distant metastasis, and embolization ( $P > 0.05$ ), but was significantly correlated with infiltrating depth, lymph node metastasis, and pTMN staging ( $P < 0.05$ , Table 3). There was also a significant difference between the moderate and high differentiation groups ( $P < 0.005$ , Table 1).

## DISCUSSION

Protein tyrosine phosphatase level, one of the multi-factors interacting in the period of normal cell growth and division, is determined between protein tyrosine kinase and protein tyrosine phosphatase. The imbalance between the two enzymes affects cell signal transference and cell division, thus leading to malignance of the cells. The occurrence and development of gastric cancer, as other malignant tumors, are an uncontrolled growth and differentiation process of multi-factors involving participation of many genes, including mutation and/or low expression of tumor suppressor gene. At present, researches on structure alteration of tumor suppressor genes in tumor tissues and tumor cell lines, including point mutation, deletion, insertion, cut point, *etc*, indicate that the mutation rate of tumor suppressor genes is 33%-50% in endometrial cancer, 25% in glioblastomas, 21% in ovarian cancer, 13% in prostate cancer, less than 5% in breast and thyroid cancer<sup>[5-11]</sup>.

It was reported that PTEN protein expression is decreased in normal gastric mucosa, intestinal metaplasia, dysplasia and gastric cancer, which is significantly higher in normal gastric mucosa and intestinal metaplasia than in dysplasia and gastric cancer<sup>[17,18]</sup>.

In order to identify the exact role of *PTEN* mutations in occurrence and development of gastric cancer, we used PCR-SSCP-DNA sequencing technique to isolate cancer cells from non-cancer cells to study the sequences of exons 5-8 and certain introns which are frequently mutated. The results indicate that the total mutation rate was 9.4% (5/53), with 3 mutations in introns, including a G-C point mutation at 91 bp upstream, a T-G point mutation of at 24 bp upstream, and a single base A mutation at 7 bp upstream of 5' lateral exon 5. The other AA-TCC mutation was found at 40 bp upstream of 3' lateral exon 7, leading to terminator formation in codon of the *PTEN* gene and pre-termination of the open read frame with the PTEN protein product lacking of the C end that regulates the stability and activity of PTEN protein. Therefore, this mutation may play an important role in the occurrence and development of gastric cancer. The mutation in introns may have effects on the differentiated cut of PTEN transcription product due to the 3 point mutations in introns of the *PTEN* gene.

Furthermore, the study showed that PTEN protein expression in the 53 gastric cancer tissue samples was not significantly correlated with the clinicopathological parameters, such as gender and age of the patients, location and size of the carcinoma, distant metastasis, and embolization, but was significantly correlated with infiltrating depth, lymph node metastasis, and pTMN staging ( $P < 0.05$ ). Along with the increasing infiltrating depth from level I to level IV, the positive expression rate was gradually decreased from 92.9% to 54.2% ( $P < 0.025$ ). There was a significant difference in lymph node metastasis ( $P < 0.05$ ) between negative and positive PTEN expressions (88.9% *vs* 45.7%). The positive PTEN protein expression rate was significantly higher at pTMN stages I and II than at pTMN stages III and IV ( $P < 0.005$ ). These results suggest that PTEN may play an important role in regulation of infiltration and metastasis of gastric cancer cells. Abnormal expressions of PTEN may predict the metastasis and prognosis of gastric cancer<sup>[19-21]</sup>. Furthermore, positive PTEN protein rate was significantly higher in well-and moderately-differentiated gastric cancer than in poorly-differentiated gastric cancer ( $P < 0.025$ ). There was also a significant difference in PTEN protein expression intensity among



**Table 3** Correlation between *PTEN* protein expression and clinicopathological parameters in gastric cancer patients

Clinicopathology parameters	Case ( <i>n</i> )	PTEN protein expression ( <i>n</i> )		Positive rate (%)	<i>P</i>
		Negative	Positive		
Tissues					
Paracancerous	53	0	53	100.0	< 0.005
Gastric cancer	53	18	35	66.0	
Gender					
Male	41	15	26	63.4	< 0.025
Female	12	3	9	66.7	
Age (yr)					
≤ 60	14	5	9	64.3	< 0.01
> 60	39	13	26	66.7	
Size (cm)					
≤ 3	16	6	10	68.8	< 0.025
> 3	37	12	25	64.9	
Location					
Antrum	30	10	20	66.7	< 0.025
Gastric body and cardia	23	8	15	65.2	
Infiltrating depth					
T1, T2	14	1	13	92.9	< 0.005
T3	15	6	9	60.0	
T4	24	11	13	54.2	
Lymph node metastasis					
Without	21	2	19	90.5	< 0.01
With	32	16	16	50.0	
Distant metastasis					
Without	45	15	30	66.7	< 0.025
With	8	3	5	62.5	
Embolization					
Without	8	1	7	87.5	< 0.025
With	45	15	30	66.7	
Differentiation extent					
Moderate and high	26	4	22	84.6	< 0.025
Low	27	14	13	48.1	
PTNM staging					
I, II	19	1	18	94.7	< 0.005
III, IV	34	17	17	50.0	

well, moderately and poorly differentiated gastric cancers ( $P < 0.005$ ), suggesting that *PTEN* protein expression is significantly correlated with histological differentiation of gastric cancer. It is generally accepted that differentiation extent is an indicator for the prognosis of gastric cancer<sup>[22]</sup>. The gene is important in the process of inducing tumor differentiation and *PTEN* protein expression is of certain significance in the prognosis of gastric cancer patients.

In the present study, the mutation rate of gastric cancer was 9.4% (5/53), suggesting that except for gene mutations, other mechanisms are involved in the descending process of *PTEN* protein expression, such as over methylation of nucleotides C and G in promoter or enhancer. Abnormal methylation of CpG islands in promoter is considered one of the important mechanisms underlying gene deactivation and accumulation. The abnormal methylation is considered one of the main pathways promoting occurrence of gastric cancer because over methylation of tumor suppressor genes or other tumor-related genes, such as Rb, APC, p16, p15, hMLH1, E-cadherin, are found in malignant tumors. Another cause might be the abnormal regulation of *PTEN* protein decomposition pathways. Analysis of *PTEN* protein structure revealed that

there were two homologous PESTs and one PSD-95/Dlg/20-1 (PDZ) binding module at the C end of *PTEN* protein. Deletion or structure alteration in the region might result in *PTEN* protein prone to be decomposed. Because the total length of *PTEN* gene DNA is 218 bp including 9 exons and 8 introns, the exact mutation rate of the *PTEN* gene might be higher than 9.4%. The results of this study indicate that expression and mutation of the *PTEN* protein play an important role in the occurrence and development of gastric cancer.

It was reported that inactivation of *PTEN* induces infiltration and metastasis of tumors<sup>[23-28]</sup>. *PTEN* restrains attack and metastasis of tumor cells by regulating matrix metalloproteinases (MMPs) and vascular endothelial growth factor (VEGF)<sup>[29]</sup>. Abnormal expression of *PTEN* protein increases synthesis of MMPs and VEGF, thus leading to attack and metastasis of tumor cells. *PTEN* can also selectively increase dephosphorylation of focal adhesion kinase (FAK) to reduce cell transference by phosphated FAK<sup>[25,30-32]</sup>. Besides, *PTEN* protein and tensin have a homologous sequence<sup>[33]</sup>. Tensin is a cell matrix protein, which participates in adhesion to cells and extracellular matrix (ECM). Our study showed that *PTEN* could restrain cell transference as tensin.

In conclusion, abnormal expression of *PTEN*

protein is usually found in gastric cancer and related to tumor differentiation, infiltrating depth, lymph node metastasis and pTMN staging. PTEN may play an important role in the occurrence and development of gastric cancer. PTEN protein expression phenotype can be considered an indicator for the pathophysiological behavior of gastric cancer.

## COMMENTS

### Background

The occurrence and development of gastric cancer, like other malignant tumors, are a complicated process involving participation of polygene and many factors. It is generally considered that protein tyrosine phosphatase level plays an important role in the process. Mutation of the *PTEN* gene encoding for protein tyrosine phosphatase and abnormal expression of the PTEN protein are significantly correlated with the occurrence and development of malignant tumors, such as glioblastoma, prostate cancer, malignant melanoma, and breast cancer.

### Research frontiers

Discovery of the *PTEN* gene is another important landmark in the field of anti-oncogenes. The relationship between *PTEN* gene and gastric carcinoma was analyzed for the genetic structure, expression and interaction with other genes in this study.

### Innovations and breakthroughs

Few studies on *PTEN* gene mutation and protein expression in gastric cancer are available. However, cancer cells have not been isolated from normal cells that may lead to undetectable *PTEN* gene mutations because of plenty of normal genome DNAs.

### Applications

PTEN protein phenotype can be used as an object index to judge the action of gastric carcinoma based on the cancer cells isolated from normal cells. In this study, we researched the *PTEN* gene mutations using PCR-SSCP-DNA sequencing technology, which can increase the detection rate of *PTEN* gene mutation, suggesting that it can be extended to research other tumor-related genes.

### Peer review

This article describes mutation of the *PTEN* gene in patients with gastric carcinoma. The results indicate that *PTEN* gene plays an important role in the occurrence and development of gastric cancer.

## REFERENCES

- 1 **Cho SH**, Lee CH, Ahn Y, Kim H, Kim H, Ahn CY, Yang KS, Lee SR. Redox regulation of PTEN and protein tyrosine phosphatases in H(2)O(2) mediated cell signaling. *FEBS Lett* 2004; **560**: 7-13
- 2 **Sternberger M**, Schmiedeknecht A, Kretschmer A, Gebhardt F, Leenders F, Czauderna F, Von Carlowitz I, Engle M, Giese K, Beigelman L, Klippel A. GeneBlocs are powerful tools to study and delineate signal transduction processes that regulate cell growth and transformation. *Antisense Nucleic Acid Drug Dev* 2002; **12**: 131-143
- 3 **Sato K**, Tamura G, Tsuchiya T, Endoh Y, Sakata K, Motoyama T, Usuba O, Kimura W, Terashima M, Nishizuka S, Zou T, Meltzer SJ. Analysis of genetic and epigenetic alterations of the *PTEN* gene in gastric cancer. *Virchows Arch* 2002; **440**: 160-165
- 4 **Maehama T**, Taylor GS, Dixon JE. PTEN and myotubularin: novel phosphoinositide phosphatases. *Annu Rev Biochem* 2001; **70**: 247-279
- 5 **Li J**, Yen C, Liaw D, Podsypanina K, Bose S, Wang SI, Puc J, Miliarensis C, Rodgers L, McCombie R, Bigner SH, Giovanella BC, Ittmann M, Tycko B, Hibshoosh H, Wigler MH, Parsons R. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science* 1997; **275**: 1943-1947
- 6 **Jiang YA**, Fan LF, Jiang CQ, Zhang YY, Luo HS, Tang ZJ, Xia D, Wang M. Expression and significance of PTEN, hypoxia-inducible factor-1 alpha in colorectal adenoma and adenocarcinoma. *World J Gastroenterol* 2003; **9**: 491-494
- 7 **Okami K**, Wu L, Riggins G, Cairns P, Goggins M, Evron E, Halachmi N, Ahrendt SA, Reed AL, Hilgers W, Kern SE, Koch WM, Sidransky D, Jen J. Analysis of PTEN/MMAC1 alterations in aerodigestive tract tumors. *Cancer Res* 1998; **58**: 509-511
- 8 **Cohen MM Jr**. Molecular dimensions of gastrointestinal tumors: some thoughts for digestion. *Am J Med Genet A* 2003; **122A**: 303-314
- 9 **Schondorf T**, Ebert MP, Hoffmann J, Becker M, Moser N, Pur S, Gohring UJ, Weisshaar MP. Hypermethylation of the PTEN gene in ovarian cancer cell lines. *Cancer Lett* 2004; **207**: 215-220
- 10 **Mori S**, Ito G, Usami N, Yoshioka H, Ueda Y, Kodama Y, Takahashi M, Fong KM, Shimokata K, Sekido Y. p53 apoptotic pathway molecules are frequently and simultaneously altered in nonsmall cell lung carcinoma. *Cancer* 2004; **100**: 1673-1682
- 11 **Steck PA**, Pershouse MA, Jasser SA, Yung WK, Lin H, Ligon AH, Langford LA, Baumgard ML, Hattier T, Davis T, Frye C, Hu R, Swedlund B, Teng DH, Tavtigian SV. Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers. *Nat Genet* 1997; **15**: 356-362
- 12 **Wang JY**, Huang TJ, Chen FM, Hsieh MC, Lin SR, Hou MF, Hsieh JS. Mutation analysis of the putative tumor suppressor gene PTEN/MMAC1 in advanced gastric carcinomas. *Virchows Arch* 2003; **442**: 437-443
- 13 **Kang YH**, Lee HS, Kim WH. Promoter methylation and silencing of PTEN in gastric carcinoma. *Lab Invest* 2002; **82**: 285-291
- 14 **Byun DS**, Cho K, Ryu BK, Lee MG, Park JI, Chae KS, Kim HJ, Chi SG. Frequent monoallelic deletion of PTEN and its reciprocal association with PIK3CA amplification in gastric carcinoma. *Int J Cancer* 2003; **104**: 318-327
- 15 **Fei G**, Ebert MP, Mawrin C, Leodolter A, Schmidt N, Dietzmann K, Malfertheiner P. Reduced PTEN expression in gastric cancer and in the gastric mucosa of gastric cancer relatives. *Eur J Gastroenterol Hepatol* 2002; **14**: 297-303
- 16 **Zhu X**, Niu N, Liu Y, Du T, Chen D, Wang X, Gu HF, Liu Y. Improvement of the sensitivity and resolution of PCR-SSCP analysis with optimized primer concentrations in PCR products. *J Genet* 2006; **85**: 233-235
- 17 **Yang L**, Kuang LG, Zheng HC, Li JY, Wu DY, Zhang SM, Xin Y. PTEN encoding product: a marker for tumorigenesis and progression of gastric carcinoma. *World J Gastroenterol* 2003; **9**: 35-39
- 18 **Yang XF**, Yang L, Mao XY, Wu DY, Zhang SM, Xin Y. Pathobiological behavior and molecular mechanism of signet ring cell carcinoma and mucinous adenocarcinoma of the stomach: a comparative study. *World J Gastroenterol* 2004; **10**: 750-754
- 19 **Kang GH**, Lee S, Kim WH, Lee HW, Kim JC, Rhyu MG, Ro JY. Epstein-barr virus-positive gastric carcinoma demonstrates frequent aberrant methylation of multiple genes and constitutes CpG island methylator phenotype-positive gastric carcinoma. *Am J Pathol* 2002; **160**: 787-794
- 20 **Lee HS**, Lee HK, Kim HS, Yang HK, Kim WH. Tumour suppressor gene expression correlates with gastric cancer prognosis. *J Pathol* 2003; **200**: 39-46
- 21 **Zheng HC**, Li YL, Sun JM, Yang XF, Li XH, Jiang WG, Zhang YC, Xin Y. Growth, invasion, metastasis, differentiation, angiogenesis and apoptosis of gastric cancer regulated by expression of PTEN encoding products. *World J Gastroenterol* 2003; **9**: 1662-1666
- 22 **Niu WX**, Qin XY, Liu H, Wang CP. Clinicopathological analysis of patients with gastric cancer in 1200 cases. *World J Gastroenterol* 2001; **7**: 281-284

- 23 **Raftopoulou M**, Etienne-Manneville S, Self A, Nicholls S, Hall A. Regulation of cell migration by the C2 domain of the tumor suppressor PTEN. *Science* 2004; **303**: 1179-1181
- 24 **Abe T**, Terada K, Wakimoto H, Inoue R, Tyminski E, Bookstein R, Basilion JP, Chiocca EA. PTEN decreases in vivo vascularization of experimental gliomas in spite of proangiogenic stimuli. *Cancer Res* 2003; **63**: 2300-2305
- 25 **Saito Y**, Gopalan B, Mhashilkar AM, Roth JA, Chada S, Zumstein L, Ramesh R. Adenovirus-mediated PTEN treatment combined with caffeine produces a synergistic therapeutic effect in colorectal cancer cells. *Cancer Gene Ther* 2003; **10**: 803-813
- 26 **Kon H**, Sonoda Y, Kumabe T, Yoshimoto T, Sekiya T, Murakami Y. Structural and functional evidence for the presence of tumor suppressor genes on the short arm of chromosome 10 in human gliomas. *Oncogene* 1998; **16**: 257-263
- 27 **Unoki M**, Nakamura Y. EGR2 induces apoptosis in various cancer cell lines by direct transactivation of BNIP3L and BAK. *Oncogene* 2003; **22**: 2172-2185
- 28 **Stewart AL**, Mhashilkar AM, Yang XH, Ekmekcioglu S, Saito Y, Sieger K, Schrock R, Onishi E, Swanson X, Mumm JB, Zumstein L, Watson GJ, Snary D, Roth JA, Grimm EA, Ramesh R, Chada S. PI3 kinase blockade by Ad-PTEN inhibits invasion and induces apoptosis in RGP and metastatic melanoma cells. *Mol Med* 2002; **8**: 451-461
- 29 **Hwang PH**, Yi HK, Kim DS, Nam SY, Kim JS, Lee DY. Suppression of tumorigenicity and metastasis in B16F10 cells by PTEN/MMAC1/TEP1 gene. *Cancer Lett* 2001; **172**: 83-91
- 30 **Saito Y**, Swanson X, Mhashilkar AM, Oida Y, Schrock R, Branch CD, Chada S, Zumstein L, Ramesh R. Adenovirus-mediated transfer of the PTEN gene inhibits human colorectal cancer growth in vitro and in vivo. *Gene Ther* 2003; **10**: 1961-1969
- 31 **Haier J**, Nicolson GL. PTEN regulates tumor cell adhesion of colon carcinoma cells under dynamic conditions of fluid flow. *Oncogene* 2002; **21**: 1450-1460
- 32 **Garl PJ**, Wenzlau JM, Walker HA, Whitelock JM, Costell M, Weiser-Evans MC. Perlecan-induced suppression of smooth muscle cell proliferation is mediated through increased activity of the tumor suppressor PTEN. *Circ Res* 2004; **94**: 175-183
- 33 **McNeish IA**, Bell SJ, Lemoine NR. Gene therapy progress and prospects: cancer gene therapy using tumour suppressor genes. *Gene Ther* 2004; **11**: 497-503

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## GASTRIC CANCER

# Heparanase expression, degradation of basement membrane and low degree of infiltration by immunocytes correlate with invasion and progression of human gastric cancer

Zun-Jiang Xie, Ying Liu, Li-Min Jia, Ye-Chun He

Zun-Jiang Xie, Ying Liu, Li-Min Jia, Ye-Chun He, Department of Anatomy, Harbin Medical University, Harbin 150081, Heilongjiang Province, China

**Author contributions:** Xie ZJ and Liu Y contributed equally to this work; Xie ZJ and He YC designed research; Xie ZJ, Liu Y and Jia LM performed research; Xie ZJ and Liu Y analyzed the data; Xie ZJ wrote the paper.

**Correspondence to:** Zun-Jiang Xie, Department of Anatomy, Harbin Medical University, Harbin 150081, Heilongjiang Province, China. [xiezj555@hotmail.com](mailto:xiezj555@hotmail.com)

Telephone: +86-451-86690176 Fax: +86-451-86690176

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## Abstract

**AIM:** To disclose the mechanisms that accelerate or limit tumor invasion and metastasis in gastric cancer patients.

**METHODS:** The heparanase expression, continuity of basement, degree of infiltration by dendritic cells and lymphocytes in gastric cancer tissues from 33 the early and late stage patients were examined by immunohistochemistry, *in situ* hybridization and transmission electron microscopy.

**RESULTS:** Heparanase mRNA expression in the late stage patients with gastric cancer was stronger than that in the early stage gastric cancer patients. In the early stage gastric cancer tissues, basement membrane (BM) appeared intact, whereas in the late stage, discontinuous BM was often present. The density of S100 protein positive tumor infiltrating dendritic cells (TIDC) in the early stage gastric cancer tissues was higher than that in the late stage. The infiltrating degree of tumor infiltrating lymphocytes (TIL) in the early stage patients whose tumor tissues contained a high density of TIDC was significantly higher than that in the late stage gastric cancer tissues patients with a low density of TIDC. There were few cancer cells penetrated through the continuous BM of cancer nests in the early stage gastric cancers, but many cancer cells were found outside of the defective BM of cancer nests in the late stage.

**CONCLUSION:** Our results suggest that strong

heparanase expression is related with the degradation of BM which allows or accelerates tumor invasion and metastasis. However, high density of TIDC and degree of infiltration by TIL are associated with tumor progression in human gastric cancers.

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**Key words:** Heparanase; Basement membrane; Tumor infiltrating dendritic cell; Tumor infiltrating lymphocyte; Gastric cancer

**Peer reviewers:** Shingo Tsuji, Professor, Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine(A8), 2-2 Yamadaoka, Suita, Osaka 565-0871, Japan; Toru Ishikawa, MD, Department of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan

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## INTRODUCTION

Gastric cancer is one of the most aggressive malignant tumors, and its incidence is higher than that of any other gastrointestinal malignancy. The prognosis of patients with gastric cancer is often poor, due to tumor invasion and metastasis which are the most common causes of death in gastric cancer<sup>[1,2]</sup>.

Degradation of basement membrane (BM) and extracellular matrix (ECM) around tumor is considered to be associated with invasion and metastasis of gastric cancer<sup>[3]</sup>. Heparanase is an endo- $\beta$ -D-glucuronidase that specifically cleaves carbohydrate chain of heparan sulfate proteoglycans (HSPG)<sup>[4]</sup>. HSPGs are the main component of extracellular matrix and basement membrane which play a barrier to prevent tumor cells from invasion and metastasis<sup>[5]</sup>. Previous studies



have shown that heparanase, produced by malignant tumor cells, mediates degradation of heparan sulfate proteoglycans in the extracellular matrix and basement membrane around tumors, and their expression correlates with the degree of tumor invasion and metastasis in several human malignant tumors<sup>[6-8]</sup>.

Progression of malignant tumors is also restricted by host defense mechanisms<sup>[9,10]</sup>. The tumor infiltrating dendritic cells and lymphocytes are chief immunocytes that inhibit malignant tumor cells from invasion and metastasis. Many authors reported that the infiltration grade of tumor infiltrating dendritic cells are associated with patient survival and prognosis in a large variety of human malignancies<sup>[10-15]</sup>. Recent studies have shown that the number of tumor infiltrating lymphocytes is correlated with the progression of human carcinoma<sup>[16-18]</sup>. However, to our knowledge, the correlation between heparanase expression and infiltration degree of tumor infiltrating dendritic cells and lymphocytes has not been reported so far. The present study was, therefore, undertaken to clarify the relationships between heparanase mRNA expression, degree of degradation of basement membrane, density of tumor infiltrating dendritic cells, infiltrating degree of tumor infiltrating lymphocytes, and tumor invasion and progression in human gastric cancer patients.

## MATERIALS AND METHODS

### Tumor samples

Tissue samples were obtained from 33 patients with primary gastric cancer who underwent curative surgery in the Second Clinical Hospital of Harbin Medical University (Harbin, China). Ten patients had stage I, 8 stage II, 7 stage III, 8 stage IV cancer according to the TNM classification (UICC, TNM classification, 5th Edition, 1997)<sup>[19]</sup>. Histological stage grouping was evaluated. Stages I and II ( $n = 18$ ) were referred to the early stage, stages III and IV ( $n = 15$ ) were referred to the late stage. All fresh tumor tissues were divided into two parts, one part was fixed in 0.1 mol/L phosphate buffer (pH 7.4) containing 4% paraformaldehyde for immunohistochemistry and *in situ* hybridization, the other part was immersed in 0.1 mol/L phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde for transmission electron microscopy.

### In situ hybridization

Paraffin-embedded tissue sections were prepared for heparanase staining. Following reagents were purchased from Maxim Biotech (South San Francisco, CA, USA). Tissue sections (4  $\mu$ m) were deparaffinized, dehydrated and incubated in 0.2 mol/L HCl for 20 min. After washed with  $2 \times$  SSC, the sections were incubated with proteinase K for 10 min at 37°C, fixed with PBS containing 4% paraformaldehyde for 5 min, washed with  $2 \times$  SSC, and then prehybridized for 2 h at 63°C in a buffer containing 50% deionized formamide,  $4 \times$  SSC,  $2 \times$  Denhardt's solution and 250  $\mu$ g/mL RNA. Hybridization was performed in 50% deionized formamide,  $4 \times$  SSC,  $2 \times$  Denhardt's

solution, 10% dextran sulfate and 500  $\mu$ g/mL RNA. The final concentration of DIG-labeled heparanase probe was about 500 ng/mL. The probe was placed on the section, covered with parafilm and incubated at 63°C overnight in a moisture chamber. After hybridization, excess probes were removed by washing in  $2 \times$  SSC followed by RNase treatment with 100 U/mL RNase T1 at 37°C for 30 min. The sections were washed at 65°C in  $2 \times$  SSC for 10 min, washed three times in  $0.2 \times$  SSC and 50% deionized formamide (10 min each time), and incubated with an anti-DIG antibody conjugated with alkaline phosphatase. For the following color reaction, 5-bromo-4-chloro-3-indolyl phosphatase was used. Finally, the sections were counterstained with Mayer's hematoxylin.

### Immunohistochemistry

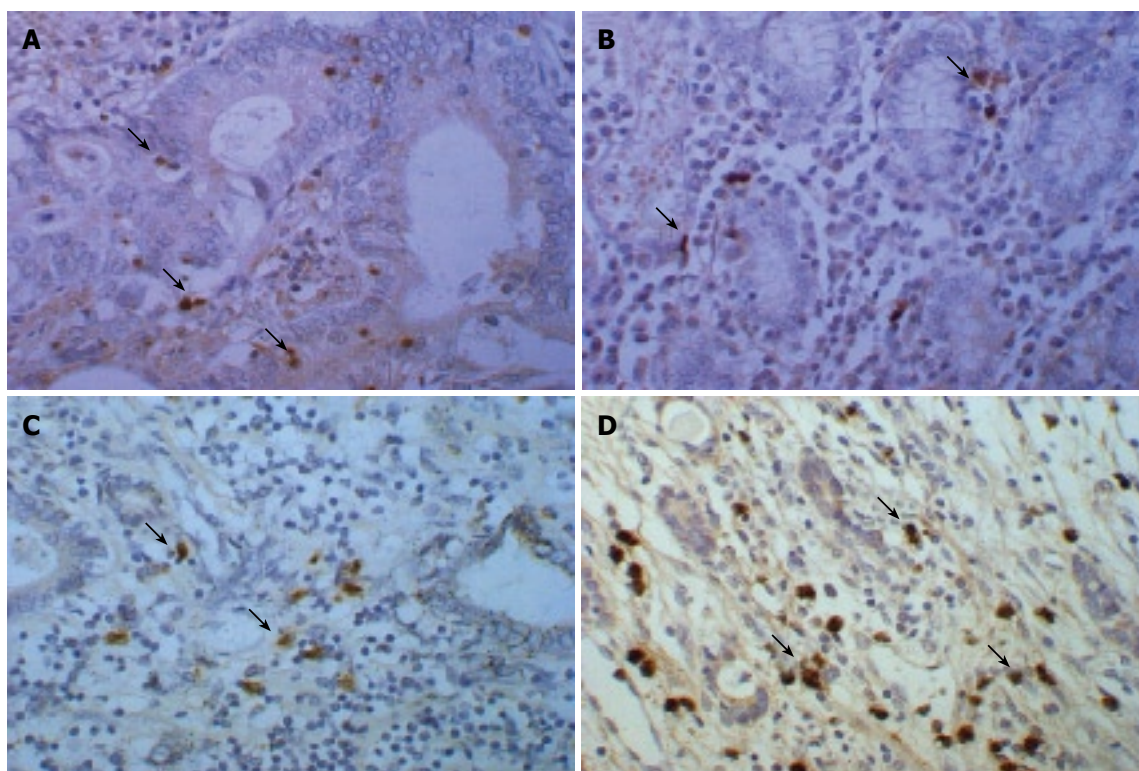
Paraffin-embedded specimens were prepared for S100 protein immunohistochemical staining. The specimens were cut into 5- $\mu$ m thick sections and mounted on glass slides. The sections were then deparaffinized in xylene for 20 min and dehydrated in ascending concentrations of ethanol. Endogenous peroxidase was blocked by incubating the sections with 3.0% H<sub>2</sub>O<sub>2</sub> in methanol. After incubated in normal bovine serum for 10 min, the sections were incubated with anti-S100 protein antibody (Sigma, St. Louis, MO, USA) for 2 h at 37°C. After washed with PBS, the sections were incubated with biotinylated immunoglobulin and streptavidin conjugated to horseradish peroxidase (ABC kit, Sigma, St. Louis, MO, USA). Immunostaining was developed using DAB/ H<sub>2</sub>O<sub>2</sub> solution. Finally, the sections were lightly counterstained with hematoxylin.

### Transmission electron microscopy

Specimens fixed in 0.1 mol/L phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde were rinsed with the phosphate buffer and postfixed in 0.1 mol/L phosphate buffer containing 1% OsO<sub>4</sub> for 2 h, dehydrated through ascending concentrations of ethanol, and embedded in Epon 812 using aclar film (Nisshin EM, Tokyo, Japan). Semi-thin sections were stained with toluidine blue and observed under a light microscope. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under a H-600 transmission electron microscope (Hitachi, Tokyo, Japan).

### Statistical analysis

Under the light microscope, S100 protein immunohistochemistry and heparanase mRNA stained sections were examined using the image analysis system computer software (BeiHan Image Centre, Beijing, China). Twenty sections from each kind of staining were analyzed, two high-power fields ( $\times 400$ ) (each field is 0.255 mm<sup>2</sup>) were randomly selected from each section. The number and area density of positive cells in each section were automatically calculated by the computer. The results were expressed as mean  $\pm$  SD. Student's *t*-test was used to compare the S100 protein positive cells and heparanase expressing cells in the early stage gastric



**Figure 1** Expression and distribution of S100 protein and heparanase mRNA in gastric cancer tissues. Immunohistochemical staining of S100 protein ( $\times 400$ ) with a high density of tumor infiltrating dendritic cells positively stained for S100 protein in the early stage gastric cancer tissues (A) and a low density of such cells in the late stage gastric cancer tissues (B), heparanase mRNA expression by in situ hybridization ( $\times 400$ ) with a low heparanase mRNA expression level in the early stage gastric cancer tissues (C) and a high heparanase mRNA expression level in the late stage gastric cancer tissues (D) (Arrows: Positively expressed cells).

**Table 1** Densities of S100 positive TIDC and heparanase expression in early and late gastric cancer tissues (mean  $\pm$  SD)

	Densities of S100 positive TIDC			Heparanase expression		
	Early stage	Late stage	P-value	Early stage	Late stage	P-value
Cases (n)	18	15		18	15	
Number density	0.25 $\pm$ 0.19	0.03 $\pm$ 0.02	< 0.01	0.09 $\pm$ 0.08	0.33 $\pm$ 0.25	< 0.01
Area density	1.47 $\pm$ 1.15	0.21 $\pm$ 0.18	< 0.01	0.76 $\pm$ 0.64	3.47 $\pm$ 3.17	< 0.01

cancer tissues with those in the late stage gastric cancer tissues.  $P < 0.05$  was considered statistically significant.

## RESULTS

### **Distribution and density of tumor infiltrating dendritic cells in gastric cancer tissue**

S100 protein positive cells showing typical morphology of dendritic cells and distinct cytoplasmic processes or veils, were detected in tissues from patients with gastric cancer at the early or late stage (Figure 1A and B). S100 protein positive cells were found mainly in stroma around the nests of cancer cells and connective tissue surrounding the tumor. In addition, S100 protein positive tumor infiltrating dendritic cells were also scattered among the cancer cells. Patients with gastric cancer at the early stage showed a high density of S100 protein positive tumor infiltrating dendritic cells (Figure 1A), while those at the late stage had a low density of S100 protein positive tumor infiltrating dendritic cells (Figure 1B). There was a significant difference in the density of S100

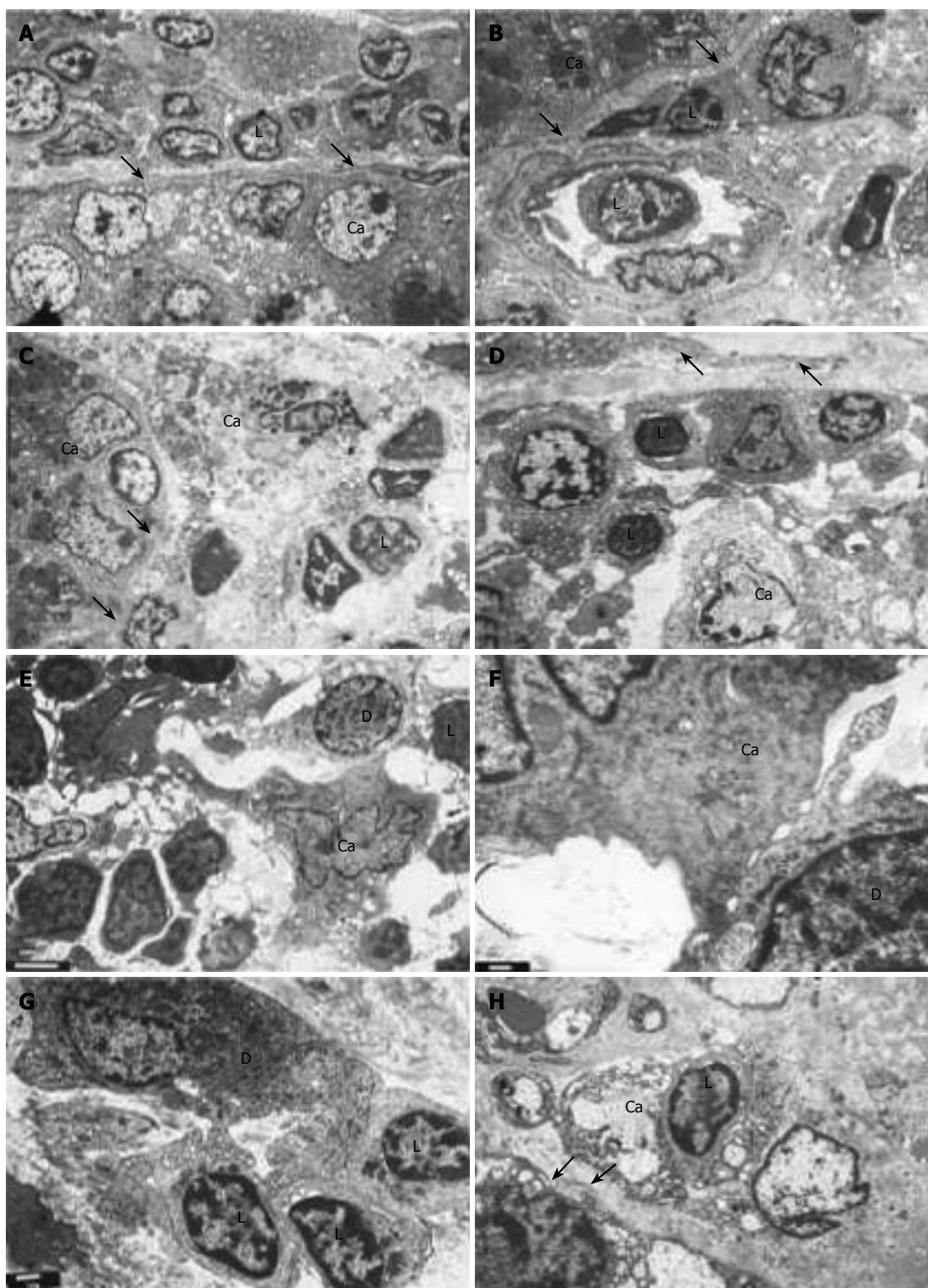
protein positive tumor infiltrating dendritic cells between gastric cancer patients at the early and late stage ( $P < 0.01$ , Table 1). The density of tumor infiltrating dendritic cells was significantly correlated with tumor invasion and clinical stage.

### **Heparanase mRNA expression in gastric cancer tissue**

Heparanase mRNA positive labeling occurred mainly in cytoplasm and nuclei of gastric cancer cells. Heparanase mRNA was weakly expressed in the early stage gastric cancer tissues (Figure 1C) and strongly expressed in the late stage gastric cancer tissues (Figure 1D). The density of heparanase mRNA positive cells was significantly higher in the late stage gastric cancer tissues than in the early stage gastric cancer tissues ( $P < 0.01$ , Table 1). Heparanase mRNA expression was significantly correlated with invasion and TNM stage of gastric cancer.

### **Transmission electron microscopy (TEM)**

The basement membrane was intact in the early stage



**Figure 2** Transmission electron microscopy micrographs of the human gastric cancer tissues. (A)-(D) showing the early stage cancer tissues (Arrows indicate basement membrane). **A:** The continuous basement membrane which consisted of the electron-dense outer layer and the electron-lucent inner layer was observed. The numerous tumor infiltrating lymphocytes (L) were located in one side of the basement membrane ( $\times 2500$ ); **B and C:** The intact basement membrane was found on the margin of cancer nests. The tumor infiltrating lymphocytes (L) appeared around the cancer cell (Ca) (B  $\times 4000$ ; C  $\times 3000$ ); **D:** The cancer cell (Ca) was surrounded by the tumor infiltrating lymphocytes (L), and the basement membrane is clearly visualized ( $\times 2500$ ); (E)-(H) showing the late stage cancer tissues. **E:** The relationships were displayed between cancer cells (Ca) or tumor infiltrating lymphocytes (L) and tumor infiltrating dendritic cells (D). Note the tumor with absent basement membrane ( $\times 4000$ ); **F:** A higher magnification of E exhibited the contact relationship between the cancer cell (Ca) and tumor infiltrating dendritic cell (D) ( $\times 20000$ ); **G:** The tumor infiltrating dendritic cell (D) was surrounded by several tumor infiltrating lymphocytes (L), and formed the dendritic cell-lymphocyte cluster ( $\times 5000$ ); **H:** The tumor infiltrating lymphocyte (L) appeared near the cancer cell (Ca), and the discontinuous or defective basement membrane of cancer nest can also be seen (double arrow) ( $\times 5000$ ).

gastric cancer tissue (Figure 2A-D). The continuous and well-formed basement membrane was found at the margin of



cancer nests in the early stage gastric cancer tissue (Figure 2B and C). The basement membrane was consisted of an electron-dense outer layer and an electron-lucent inner layer (Figure 2A and D). In contrast, the basement membrane at the rim of cancer nests was discontinuous and defective or absent in the late stage gastric cancer tissue (Figure 2E and H). Numerous tumor infiltrating lymphocytes were observed in the surrounding tissues of cancer nests and cells of the early stage gastric cancer (Figure 2B, C and D). In addition, many tumor infiltrating lymphocytes were arranged along one side of the basement membrane (Figure 2A). Lymphocytes in blood vessels were found near the cancer nests (Figure 2B), and invasion of cancer cells was noted outside of cancer nests (Figure 2C). The infiltrating degree of tumor infiltrating lymphocytes was high in the early stage gastric cancer tissues with intact basement membrane and few lymphocytes infiltrated into the tumor-surrounding tissue of the late stage gastric cancer with discontinuous basement membrane. Few cancer cells penetrated through the intact basement membrane of cancer nests in the early stage gastric cancer tissues, but many cancer cells were observed outside of the discontinuous basement membrane of cancer nests in the late stage gastric cancer tissues. There was a close contact between cancer cells or tumor infiltrating lymphocytes and tumor infiltrating dendritic cells (Figure 2E), and the contact between cancer cells and tumor infiltrating dendritic cells was also observed (Figure 2F). Many tumor infiltrating lymphocytes were distributed around the tumor infiltrating dendritic cells, forming a dendritic cell-lymphocyte cluster. Tumor infiltrating dendritic cells were closely contacted with tumor infiltrating lymphocytes (Figure 2G). Tumor infiltrating lymphocytes were also found near the cancer cells (Figure 2H).

## DISCUSSION

It is generally accepted that one principal reason for the poor prognosis of patients with malignant tumors is the invasion and metastasis of cancer cells. The basement membrane plays an important role as a barrier in preventing cancer cells from invasion and metastasis<sup>[9]</sup>. The previous studies have demonstrated that heparanase which can degrade the basement membrane is one of the key enzymes involved in the tumor invasion and metastasis *in vivo*, such as pancreatic cancer, head and neck cancers, esophageal cancer, gastric cancer and colon cancer<sup>[7,8,20-22]</sup>. The heparanase can also shows *in vitro* human squamous cell carcinoma cell lines<sup>[23]</sup>. In the present study, we examined heparanase mRNA expression in the early and late stages of human gastric cancer by *in situ* hybridization. Our results show that heparanase mRNA expression was significantly higher in the late stage than in the early stage gastric cancer tissues ( $P < 0.01$ ), and that heparanase mRNA expression was correlated significantly with tumor invasion and TNM stages of cancer.

Whether high expression of heparanase can promote the invasion of cancer cells by degrading the basement membrane remains unclear. Our TEM study has showed

that intact basement membrane of cancer nests appeared in regions where heparanase expression was low in the early stage gastric cancer tissues, whereas discontinuous basement membrane of cancer nests was often present in places where heparanase expression was high in the late stage gastric cancer tissues. Our morphological observation directly proved that heparanase activity was associated with degradation of basement membrane. Lipponen<sup>[9]</sup> reported that invasion of superficial bladder cancer is related to the loss of continuous basement membrane, which is in agreement with our present TEM study.

In the present study, few cancer cells were observed to penetrate through the continuous basement membrane of cancer nests in the early stage gastric cancer tissue, but many cancer cells could be found outside of discontinuous basement membrane of cancer nests in the late stage gastric cancer tissue. These results suggest that the state of basement membrane, which is determined by heparanase mRNA levels, correlates with invasion of cancer cells. The present study also suggested that the discontinuity of basement membrane facilitate the invasion of cancer cells<sup>[23]</sup>. The discontinuity of basement membrane could probably results from the degradation of basement membrane by proteolytic enzymes, such as heparanase, which are presumably actively secreted by cancer cells<sup>[24]</sup>.

The present study also showed that the state of basement membrane as a mechanical barrier and host immune defense system were interrelated. The immune defense system plays a critical role in preventing and limiting the development of malignant tumors<sup>[24]</sup>. The tumor infiltrating immunocytes situated around the tumor were considered a key factor for maintaining the status of local antitumor immunity<sup>[25]</sup>. The tumor infiltrating dendritic cells and lymphocytes are the main components of immunocytes in the tumor-surrounding tissues. Reportedly, the quantity of tumor infiltrating dendritic cells correlates with the clinical outcome of different tumor types<sup>[11,12,14,15]</sup>. Zeid and Muller demonstrated that the density of S100 positive dendritic cells in lung tumors is related to tumor subtype and differentiation, and a high dendritic cell density is associated with enhanced patient survival<sup>[11]</sup>. S100 protein has been widely used as a marker for identification of dendritic cells<sup>[26-28]</sup>. Tsujitani *et al* showed that the infiltration of dendritic cells is related to tumor invasion and lymph node metastasis in human gastric cancer<sup>[12]</sup>. A high number of dendritic cells in tumor tissue correlate with a good prognosis<sup>[29]</sup>. The present study showed that the density of S100 protein positive cells was higher in the early stage than in the late stage gastric cancer tissue ( $P < 0.01$ ), suggesting that the density of tumor infiltrating dendritic cells correlates significantly with tumor invasion and clinical stages.

It was reported that invasion and metastasis of malignant tumor are related with the infiltrating degree of tumor infiltrating lymphocytes in tumor tissues<sup>[30]</sup>. Ropponen *et al* have shown the relationship between the number of tumor infiltrating lymphocytes and the prognosis of patients with colorectal cancer<sup>[31]</sup>. Aaltomaa *et al* demonstrated that a high number of



tumor infiltrating lymphocytes in tumor tissue correlate with a good prognosis of patients with breast cancer<sup>[32]</sup>. In the present study, the infiltrating degree of tumor infiltrating lymphocytes in the early stage gastric cancer patients whose tumor tissues contained a high density of tumor infiltrating dendritic cells was significantly higher than that in the late stage gastric cancer patients with a low density of these cells, indicating that the infiltrating degree of tumor infiltrating lymphocytes is associated with the progression of gastric cancer. These results suggest that there exists a certain relation between tumor infiltrating dendritic cells and lymphocytes. When the tumor becomes large, dendritic cells and lymphocytes in the whole body are overwhelmed by a large number of tumor cells. In addition, these increased tumor cells will also prevent and limit infiltration by dendritic cells and lymphocytes. Thus, the decreases in tumor-infiltrating DCs and TILs may not be due to tumor development, but due to tumor growth.

Suzuki *et al* showed that dendritic cells are attached to groups of lymphocytes and form dendritic cell-lymphocyte clusters to promote T-cell activation for the generation of tumor-specific immunity in the invasive margin of the colorectal cancer stroma<sup>[33]</sup>. Dendritic cells present antigen to lymphocytes, stimulate naïve T lymphocyte proliferation and activation to kill tumor cells<sup>[34]</sup>. Bell *et al*<sup>[35]</sup> suggested that the peritumoral clustering of mature dendritic cells reflects a state in which they interact directly with clusters of tumor infiltrating lymphocytes to generate an antitumor immune response. In the present study, tumor infiltrating lymphocytes were distributed around tumor infiltrating dendritic cells and formed dendritic cell-lymphocyte cluster, and the close contacts were found between tumor infiltrating dendritic cell and tumor infiltrating lymphocytes. The contact between tumor infiltrating dendritic cells and lymphocytes may indicate the process that tumor infiltrating dendritic cells present antigen to lymphocytes to activate them for antitumor immunity<sup>[36,37]</sup>.

Loss of integrity in basement membrane results from high heparanase expression. In the present study, few cancer cells were observed to penetrate through the continuous basement membrane of cancer nests in the early stage gastric cancer tissue, but many cancer cells could be found outside of the discontinuous basement membrane of cancer nests in the late stage gastric cancer tissue. These results suggest that the state of basement membrane, which is determined by heparanase, correlates with invasion of cancer cells. In the late stage gastric cancer tissue, increased cancer cells outside of discontinuous basement membrane of cancer nests could prevent and limit infiltration by dendritic cells and lymphocytes. Thus, heparanase expression or loss of integrity in basement membrane is associated with the infiltrating degree of tumor infiltrating dendritic cells and lymphocytes.

In summary, heparanase expression, degradation of basement membrane, density of tumor infiltrating dendritic cells and infiltrating degree of tumor infiltrating lymphocytes are associated with tumor

invasion, TNM stages and progression in human gastric cancer. When the tumor has reached an advanced stage, discontinuous basement membrane, degraded by high expression heparanase, allows cancer cells to penetrate and is favorable to tumor invasion and metastasis, and can predict a poor prognosis of patients with gastric cancer. Moreover, at the late stage, a low degree of infiltration by dendritic cells and lymphocytes reflecting the presence of a weak local antitumor immune response in gastric cancer tissues also indicates that patients with less infiltrating immunocytes gastric cancer would have a poor prognosis, whereas the result is contrary in the early gastric cancer tissue. These factors including interactions between heparanase and basement membrane as well as between tumor infiltrating dendritic cells and lymphocytes may play a crucial role in tumor invasion and metastasis. Further study is required to understand the precise mechanism of interactions between both of them in the process of tumor invasion and metastasis.

## COMMENTS

### Background

The prognosis of patients with gastric cancer is often poor, due to tumor invasion and metastasis which are the most common causes for death of gastric cancer patients. It is crucially important to disclose the mechanisms underlying tumor invasion and metastasis. Heparanase, tumor infiltrating dendritic cells (TIDC) and tumor infiltrating lymphocytes (TIL) play a critical role in preventing and limiting the development of malignant tumors.

### Research frontiers

Recent investigations have shown that heparanase expression which can degrade the basement membrane is one of the key enzymes involved in tumor invasion and metastasis, but few studies are available on the relationship between heparanase expression, basement membrane degradation, density of dendritic cells, infiltrating degree of lymphocytes, and tumor invasion and progression in gastric cancer patients.

### Innovations and breakthroughs

High heparanase expression levels are related with the degradation of basement membrane which allows or accelerates tumor invasion and metastasis. However, high TIDC density and TIL infiltration degree are associated with progression of gastric cancer.

### Applications

The study defined the mechanisms underlying tumor invasion and metastasis. Heparanase expression, basement membrane degradation, and TIDC and TIL infiltration degree, can be used as prognostic biomarkers for gastric cancer.

### Terminology

Heparanase is an endo- $\beta$ -D-glucuronidase that specifically cleaves the carbohydrate chain of heparan sulfate proteoglycan (HSPG). HSPG is the main component of extracellular matrix and basement membrane, which as a barrier can protect tumor cells from invasion and metastasis.

### Peer review

This is a histopathological analysis of heparanase expression, dendritic cells and lymphocytes infiltrating to tumor in patients gastric cancer at the early or late stage. In addition, the authors showed the electron microscopic pictures of gastric cancer tissue infiltrated by dendritic cells and lymphocytes. This is an interesting report on heparanase expression in human gastric cancer.

## REFERENCES

- 1 Poste G, Fidler IJ. The pathogenesis of cancer metastasis. *Nature* 1980; **283**: 139-146
- 2 Ajani JA, Mansfield PF, Ota DM. Potentially resectable gastric carcinoma: current approaches to staging and preoperative therapy. *World J Surg* 1995; **19**: 216-220

- 3 **Grigioni WF**, D'Errico A, Fortunato C, Fiorentino M, Mancini AM, Stetler-Stevenson WG, Sobel ME, Liotta LA, Onisto M, Garbisa S. Prognosis of gastric carcinoma revealed by interactions between tumor cells and basement membrane. *Mod Pathol* 1994; **7**: 220-225
- 4 **Nakajima M**, Irimura T, Nicolson GL. Heparanases and tumor metastasis. *J Cell Biochem* 1988; **36**: 157-167
- 5 **Bernfield M**, Gotte M, Park PW, Reizes O, Fitzgerald ML, Lincecum J, Zako M. Functions of cell surface heparan sulfate proteoglycans. *Annu Rev Biochem* 1999; **68**: 729-777
- 6 **Tang W**, Nakamura Y, Tsujimoto M, Sato M, Wang X, Kurozumi K, Nakahara M, Nakao K, Nakamura M, Mori I, Kakudo K. Heparanase: a key enzyme in invasion and metastasis of gastric carcinoma. *Mod Pathol* 2002; **15**: 593-598
- 7 **Ohkawa T**, Naomoto Y, Takaoka M, Nobuhisa T, Noma K, Motoki T, Murata T, Uetsuka H, Kobayashi M, Shirakawa Y, Yamatsuji T, Matsubara N, Matsuoka J, Haisa M, Gunduz M, Tsujigiwa H, Nagatsuka H, Hosokawa M, Nakajima M, Tanaka N. Localization of heparanase in esophageal cancer cells: respective roles in prognosis and differentiation. *Lab Invest* 2004; **84**: 1289-1304
- 8 **Nobuhisa T**, Naomoto Y, Ohkawa T, Takaoka M, Ono R, Murata T, Gunduz M, Shirakawa Y, Yamatsuji T, Haisa M, Matsuoka J, Tsujigiwa H, Nagatsuka H, Nakajima M, Tanaka N. Heparanase expression correlates with malignant potential in human colon cancer. *J Cancer Res Clin Oncol* 2005; **131**: 229-237
- 9 **Lipponen PK**. The prognostic value of basement membrane morphology, tumour histology and morphometry in superficial bladder cancer. *J Cancer Res Clin Oncol* 1993; **119**: 295-300
- 10 **Xie ZJ**, Jia LM, He YC, Gao JT. Morphological observation of tumor infiltrating immunocytes in human rectal cancer. *World J Gastroenterol* 2006; **12**: 1757-1760
- 11 **Zeid NA**, Muller HK. S100 positive dendritic cells in human lung tumors associated with cell differentiation and enhanced survival. *Pathology* 1993; **25**: 338-343
- 12 **Tsujitani S**, Kakeji Y, Watanabe A, Kohnoe S, Maehara Y, Sugimachi K. Infiltration of dendritic cells in relation to tumor invasion and lymph node metastasis in human gastric cancer. *Cancer* 1990; **66**: 2012-2016
- 13 **Lespagnard L**, Gancberg D, Rouas G, Leclercq G, de Saint-Aubain Somerhausen N, Di Leo A, Piccart M, Verhest A, Larsimont D. Tumor-infiltrating dendritic cells in adenocarcinomas of the breast: a study of 143 neoplasms with a correlation to usual prognostic factors and to clinical outcome. *Int J Cancer* 1999; **84**: 309-314
- 14 **Iwamoto M**, Shinohara H, Miyamoto A, Okuzawa M, Mabuchi H, Nohara T, Gon G, Toyoda M, Tanigawa N. Prognostic value of tumor-infiltrating dendritic cells expressing CD83 in human breast carcinomas. *Int J Cancer* 2003; **104**: 92-97
- 15 **Sandel MH**, Dadabayev AR, Menon AG, Morreau H, Melief CJ, Offringa R, van der Burg SH, Janssen-van Rhijn CM, Ensink NG, Tollenaar RA, van de Velde CJ, Kuppen PJ. Prognostic value of tumor-infiltrating dendritic cells in colorectal cancer: role of maturation status and intratumoral localization. *Clin Cancer Res* 2005; **11**: 2576-2582
- 16 **Tsujihashi H**, Uejima S, Akiyama T, Kurita T. Immunohistochemical detection of tissue-infiltrating lymphocytes in bladder tumors. *Urol Int* 1989; **44**: 5-9
- 17 **Eerola AK**, Soini Y, Paakko P. A high number of tumor-infiltrating lymphocytes are associated with a small tumor size, low tumor stage, and a favorable prognosis in operated small cell lung carcinoma. *Clin Cancer Res* 2000; **6**: 1875-1881
- 18 **Tormanen-Napankangas U**, Soini Y, Paakko P. High number of tumour-infiltrating lymphocytes is associated with apoptosis in non-small cell lung carcinoma. *APMIS* 2001; **109**: 525-532
- 19 **Sobin LH**, Fleming ID. TNM Classification of Malignant Tumors, fifth edition (1997). Union Internationale Contre le Cancer and the American Joint Committee on Cancer. *Cancer* 1997; **80**: 1803-1804
- 20 **Koliopanos A**, Friess H, Kleeff J, Shi X, Liao Q, Pecker I, Vlodavsky I, Zimmermann A, Buchler MW. Heparanase expression in primary and metastatic pancreatic cancer. *Cancer Res* 2001; **61**: 4655-4659
- 21 **Beckhove P**, Helmke BM, Ziouta Y, Bucur M, Dorner W, Mogler C, Dyckhoff G, Herold-Mende C. Heparanase expression at the invasion front of human head and neck cancers and correlation with poor prognosis. *Clin Cancer Res* 2005; **11**: 2899-2906
- 22 **Takaoka M**, Naomoto Y, Ohkawa T, Uetsuka H, Shirakawa Y, Uno F, Fujiwara T, Gunduz M, Nagatsuka H, Nakajima M, Tanaka N, Haisa M. Heparanase expression correlates with invasion and poor prognosis in gastric cancers. *Lab Invest* 2003; **83**: 613-622
- 23 **Kurokawa H**, Katsube K, Podyma KA, Ikuta M, Iseki H, Nakajima M, Akashi T, Omura K, Takagi M, Yanagishita M. Heparanase and tumor invasion patterns in human oral squamous cell carcinoma xenografts. *Cancer Sci* 2003; **94**: 277-285
- 24 **Chauhan SS**, Goldstein LJ, Gottesman MM. Expression of cathepsin L in human tumors. *Cancer Res* 1991; **51**: 1478-1481
- 25 **Perrot I**, Blanchard D, Freymond N, Isaac S, Guibert B, Pacheco Y, Lebecque S. Dendritic cells infiltrating human non-small cell lung cancer are blocked at immature stage. *J Immunol* 2007; **178**: 2763-2769
- 26 **Matsuda H**, Mori M, Tsujitani S, Ohno S, Kuwano H, Sugimachi K. Immunohistochemical evaluation of squamous cell carcinoma antigen and S-100 protein-positive cells in human malignant esophageal tissues. *Cancer* 1990; **65**: 2261-2265
- 27 **Tsujitani S**, Kakeji Y, Watanabe A, Kohnoe S, Maehara Y, Sugimachi K. Infiltration of S-100 protein positive dendritic cells and peritoneal recurrence in advanced gastric cancer. *Int Surg* 1992; **77**: 238-241
- 28 **Ambe K**, Mori M, Enjoji M. S-100 protein-positive dendritic cells in colorectal adenocarcinomas. Distribution and relation to the clinical prognosis. *Cancer* 1989; **63**: 496-503
- 29 **Inoue K**, Furihata M, Ohtsuki Y, Fujita Y. Distribution of S-100 protein-positive dendritic cells and expression of HLA-DR antigen in transitional cell carcinoma of the urinary bladder in relation to tumour progression and prognosis. *Virchows Arch A Pathol Anat Histopathol* 1993; **422**: 351-355
- 30 **Lipponen PK**, Eskelinen MJ, Jauhiainen K, Harju E, Terho R. Tumour infiltrating lymphocytes as an independent prognostic factor in transitional cell bladder cancer. *Eur J Cancer* 1992; **29A**: 69-75
- 31 **Ropponen KM**, Eskelinen MJ, Lipponen PK, Alhava E, Kosma VM. Prognostic value of tumour-infiltrating lymphocytes (TILs) in colorectal cancer. *J Pathol* 1997; **182**: 318-324
- 32 **Aaltomaa S**, Lipponen P, Eskelinen M, Kosma VM, Marin S, Alhava E, Syrjanen K. Lymphocyte infiltrates as a prognostic variable in female breast cancer. *Eur J Cancer* 1992; **28A**: 859-864
- 33 **Suzuki A**, Masuda A, Nagata H, Kameoka S, Kikawada Y, Yamakawa M, Kasajima T. Mature dendritic cells make clusters with T cells in the invasive margin of colorectal carcinoma. *J Pathol* 2002; **196**: 37-43
- 34 **Schuler G**, Steinman RM. Dendritic cells as adjuvants for immune-mediated resistance to tumors. *J Exp Med* 1997; **186**: 1183-1187
- 35 **Bell D**, Chomarat P, Broyles D, Netto G, Harb GM, Lebecque S, Valladeau J, Davoust J, Palucka KA, Banchereau J. In breast carcinoma tissue, immature dendritic cells reside within the tumor, whereas mature dendritic cells are located in peritumoral areas. *J Exp Med* 1999; **190**: 1417-1426
- 36 **Flechner ER**, Freudenthal PS, Kaplan G, Steinman RM. Antigen-specific T lymphocytes efficiently cluster with dendritic cells in the human primary mixed-leukocyte reaction. *Cell Immunol* 1988; **111**: 183-195
- 37 **Austyn JM**, Weinstein DE, Steinman RM. Clustering with dendritic cells precedes and is essential for T-cell proliferation in a mitogenesis model. *Immunology* 1988; **63**: 691-696



# Killing of p53-deficient hepatoma cells by parvovirus H-1 and chemotherapeutics requires promyelocytic leukemia protein

Maike Sieben, Kerstin Herzer, Maja Zeidler, Vera Heinrichs, Barbara Leuchs, Martin Schuler, Jan J Cornelis, Peter R Galle, Jean Rommelaere, Markus Moehler

Maike Sieben, Kerstin Herzer, Maja Zeidler, Vera Heinrichs, Peter R Galle, Markus Moehler, First Department of Internal Medicine, Johannes Gutenberg University of Mainz, Mainz 55101, Germany

Barbara Leuchs, Jan J Cornelis, Jean Rommelaere, German Cancer Research Center, Infection and Cancer Program, Dept. F010 and Institut National de la Santé et de la Recherche Médicale Unité 701, Heidelberg 69120, Germany

Martin Schuler, Department of Medicine (Cancer Research), West German Cancer Center, University Hospital Essen, Essen 45122, Germany

**Author contributions:** Sieben M and Herzer K contributed equally to this work; Moehler M corresponds the paper; Sieben M, Herzer K, Zeidler M, Cornelis JJ, Rommelaere J, Moehler M designed research; Herzer K, Zeidler M, Heinrichs V, Leuchs B performed research; Schuler M, Cornelis JJ contributed new reagents/analytic tools; Sieben M, Herzer K, Cornelis JJ, Galle PR, Rommelaere J, Moehler M analyzed data; and Sieben M and Moehler M wrote the paper.

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**Correspondence to:** Markus Moehler, First Department of Internal Medicine of Johannes Gutenberg University of Mainz, Langenbeckstrasse 1, Mainz 55101, Germany. [moehler@mail.uni-mainz.de](mailto:moehler@mail.uni-mainz.de)

Telephone: +49-6131-176839 Fax: +49-6131-176621

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## Abstract

**AIM:** To evaluate the synergistic targeting and killing of human hepatocellular carcinoma (HCC) cells lacking p53 by the oncolytic autonomous parvovirus (PV) H-1 and chemotherapeutic agents and its dependence on functional promyelocytic leukemia protein (PML).

**METHODS:** The role of p53 and PML in regulating cytotoxicity and gene transfer mediated by wild-type (wt) PV H-1 were explored in two pairs of isogenic human hepatoma cell lines with different p53 status. Furthermore, H-1 PV infection was combined with cytostatic drug treatment.

**RESULTS:** While the HCC cells with different p53 status studied were all susceptible to H-1 PV-induced apoptosis, the cytotoxicity of H-1 PV was more

pronounced in p53-negative than in p53-positive cells. Apoptosis rates in p53-negative cell lines treated by genotoxic drugs were further enhanced by a treatment with H-1 PV. In flow cytometric analyses, H-1 PV infection resulted in a reduction of the mitochondrial transmembrane potential. In addition, H-1 PV cells showed a significant increase in PML expression. Knocking down PML expression resulted in a striking reduction of the level of H-1 PV infected tumor cell death.

**CONCLUSION:** H-1 PV is a suitable agent to circumvent the resistance of p53-negative HCC cells to genotoxic agents, and it enhances the apoptotic process which is dependent on functional PML. Thus, H-1 PV and its oncolytic vector derivatives may be considered as therapeutic options for HCC, particularly for p53-negative tumors.

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**Key words:** Autonomous parvovirus; Apoptosis; p53; Promyelocytic leukemia protein; Human hepatocellular carcinoma; Hepatocytes

**Peer reviewer:** Dr. Toru Ikegami, Department of Surgery and Science, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan

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## INTRODUCTION

Abrogation of function of tumor suppressors such as p53 or the promyelocytic leukemia protein (PML) are common events in human tumors and lead to more aggressive cancer phenotypes<sup>[1,2]</sup>. At early stages during the process of carcinogenesis, activated oncogenes sensitize primary cells towards the p53-dependent stress response, in which the nuclear phosphoprotein p53 serves as a ge-

nostic stabilizer, inhibitor of cell cycle progression and angiogenesis, and facilitator of apoptosis<sup>[3-5]</sup>. In order to overcome this endogenous defense mechanism, cancer cell variants are strongly selected for p53 mutations, with p53 gene alterations identified in approximately half of all human tumors<sup>[6-8]</sup>. Thus, loss of p53 function usually results in a more aggressive cancer phenotype and worse clinical outcome. Studies using *in vitro* cell cultures and *in vivo* animal models demonstrated that the deficiency of p53 correlates with enhanced tumorigenesis, tumor-induced angiogenesis, and an increased resistance towards intracellular stresses which is mainly due to abrogation of an effective apoptotic response to chemotherapy or radiation<sup>[9]</sup>.

The tumor suppressor PML is predominantly localized in distinct nuclear domains that are termed PML-nuclear bodies (PML-NBs), and consist of multiprotein complexes implicated in apoptosis regulation, cellular senescence, and antiviral response<sup>[10,11]</sup>. PML expression results in potent growth-suppressive<sup>[12]</sup> and apoptosis-inducing effects<sup>[13,14]</sup>, and PML-deficient mice and cells exhibit defects in multiple apoptosis pathways<sup>[15]</sup>. One major goal of therapeutic oncology is to identify ways to kill the tumor cells that became resistant to conventional treatments due to their lack of functional p53 or PML<sup>[16]</sup>. The rapid expansion of the field of gene transfer technologies led to the development of retroviral or adenoviral p53 expression vectors and their use to restore sensitivity to genotoxic agents or to directly induce apoptosis in preclinical tumor models<sup>[17-20]</sup>.

Promising new approaches to tumor-directed therapy include oncolytic parvoviruses (PVs), which are of particular interest, since they are endowed with oncolytic properties and also increase the host immune response by priming effector immune cells against the tumors<sup>[21-24]</sup>. The autonomous PV of the rat (H-1 PV) and its close relatives, such as the minute virus of mice (MVM) and in addition the most commonly used herpes simplex virus and adenovirus are emerging as promising candidates because of a number of their properties<sup>[25]</sup>.

Notably, these viruses preferentially replicate in and kill transformed and tumor-derived cells in culture<sup>[21,22,26,27]</sup>. In addition, recombinant PVs have recently been produced with the aim to increase the anti-tumor effect of the natural viruses<sup>[28]</sup>. In particular, PVs may be suitable to target and kill tumor cells and simultaneously deliver appropriate transgenes, e.g. genes coding for immuno-stimulatory factors<sup>[25]</sup>. As H-1 PV is seldom pathogenic to its natural adult hosts<sup>[29]</sup> and infects humans without any apparent consequences<sup>[21,28]</sup>, the prospects for the clinical use of PVs are intriguing. *In vivo*, these viruses may combat tumor development or repress established tumors, what makes them promising tools in cancer therapy<sup>[25]</sup>.

The factors controlling the sensitivity of target (in particular human) cells to PV-induced killing are still largely unknown. Cells transformed with oncogenes display both an enhanced capacity for accumulating the viral cytotoxic nonstructural (NS) protein<sup>[22]</sup> and a greater intrinsic responsiveness to NS1-mediated killing<sup>[30]</sup>. On

the other hand, our previous investigation of the cytotoxicity of H-1 PV in hepatoma cell cultures failed to go into a requirement for functional p53. So far, the inactivation of p53 was only found in human leukemia cells and transformed rat fibroblasts and correlates with a greater susceptibility to H-1 PV-induced cell killing<sup>[31]</sup>.

Thus, in order to better understand the role of the cell genetic background and effector pathways in the H-1 PV-induced cytotoxicity, we compared two isogenic pairs of p53-positive *versus* negative human tumor cell lines of hepatocellular carcinoma (HCC) origin. This system allowed us to assess the impact of p53 on the susceptibility of host cells to both H-1 PV gene expression and killing activity, and H-1 PV vector-reduced reporter gene transduction. To further understand the molecular mechanism underlying H-1 PV-induced cell killing, another tumor suppressor, the PML, was investigated for its influence on H-1 PV infection. We used RNA interference to knock down PML expression in the described cellular systems, and determined the consequences for the outcome of H-1 PV infection with regard to the host cell p53 status.

The present study shows that H-1 PV triggers an apoptotic type of death in human HCC cells, and that p53 is dispensable for this process. In contrast, PML, which is induced by H-1 PV infection, helps PV killing carcinoma cells, irrespective of their p53 status. Given the known dependence of apoptosis induction by radio-chemotherapeutic agents on the target cell p53 status<sup>[32]</sup>, PVs appear to be suitable adjuvants to eliminate tumor cell populations resistant against these agents by means of combined treatments.

## MATERIALS AND METHODS

### Tumor cells

The Hep3B cells were derived from a HCC<sup>[33]</sup> and HepG2 cells from a human hepatoblastoma<sup>[32]</sup>. HepG2 cells were propagated in Dulbecco's modified Eagle medium (DMEM; Life Technologies GmbH, Karlsruhe, Germany), and Hep3B in Eagle minimal essential medium (Eurobio GmbH, Raunheim, Germany). Both media were supplemented with 10% fetal calf serum (FCS), 5 mol/L glutamine, 100 µg/mL penicillin, and 5 mol/L Hepes<sup>[21]</sup>. The Hep3B4P line is a Hep3B derivative stably transfected with tamoxifen-regulated wt p53-estrogen receptor chimera (p53-mERTm-pBabepuro)<sup>[34]</sup>. p53-mERTm-pBpuro contains the *Bam*HI fragment of human cDNA p53 cloned in frame with and N-terminally to a modified estrogen receptor containing a gly to arg mutation at residue 525<sup>[35]</sup>. This mutation renders the hormone binding domain insensitive to estradiol but responsive to the synthetic estrogen 4-OH-tamoxifen<sup>[35]</sup>. To induce p53 in the experiments, tamoxifen was added at a concentration of 750 nmol/L 1 d before H-1 PV infection.

HepG2 303 is a HepG2 derivative stably transfected with a dominant-negative p53 mutant (dn-p53) kindly provided by A. Levine (ΔV143A) as described by Schuler *et al*<sup>[36]</sup>. Since the dn-p53 transfection plasmid contained the puromycin resistance gene, HepG2 303 cells were



selected with puromycin (0.5 µg/mL) for 4 wk (4 consecutive days each week).

### **Virus infection**

For infection, H-1 PV was produced in NB-E cells and purified over cesium chloride gradients as described earlier. Wild-type (wt) H-1 PV titration by plaque assays was performed according to published methods<sup>[26]</sup>. The multiplicity of infection (MOI) is given by the number of plaque-forming units (pfu) inoculated per cell. For experimental infections, exponentially growing cell cultures were incubated for 1 h with H-1 PV at indicated MOIs. Cells were cultured for up to 8 d post infection (p.i.).

### **Cell treatments**

For combined treatment with H-1 PV and chemotherapeutics, cells were first infected with H-1 PV (MOI = 20 pfu/cell) in complete medium. One hour after infection, the chemotherapeutic agents Irinotecan (100 µg/mL), 5-Fluorouracil (5-FU) (5 µg/mL), or Cisplatin (0.25 µg/mL) were added, and cells were further incubated for 3 d at 37°C. Apoptosis rates were then quantitatively determined by FACS. Herein, the treated cells were harvested *via* trypsinization, washed with PBS, stained with propidium iodide and annexin V, and apoptosis levels were assessed, using the FACScan flow cytometry with CellQuest software (Becton Dickinson, San Jose, CA). Anticancer agents were purchased from Pfizer (Irinotecan), Hexal AG (5-FU), and Gry Pharma GmbH (Cisplatin).

### **Analysis of virus protein expression**

Cultures were infected with H-1 PV at a MOI of 20 pfu/cell. After washing with PBS, cells were lysed in RIPA buffer (10 mol/L Tris-HCl, 150 mol/L NaCl, 1 mol/L EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 5% SDS) containing protease inhibitors. Protein concentrations were determined using the Bio-Rad protein assay (Bio-Rad, Munich, Germany). Total proteins (50 µg) were diluted into an equal volume, subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a nitrocellulose membrane (Amersham Pharmacia Biotech, Freiburg, Germany). Non-specific binding sites were blocked by incubating the membrane for 2 h in PBS containing 10% low-fat milk powder and 0.2% Tween-20 (Sigma, Deisenhofen, Germany). The blot was further incubated with the rabbit polyclonal antibody SP8 directed against carboxy-terminal peptides of NS1<sup>[37]</sup>, then with an anti-rabbit peroxidase-conjugated antibody, and processed for enhanced chemoluminescence detection (Amersham Pharmacia Biotech, Freiburg, Germany).

### **Characterisation of the p53 and PML status of human tumor cells**

Cultures grown for 48 h were processed for Western blotting as described above for viral proteins, p53 was detected using the monoclonal DO-7 antibody<sup>[38]</sup>. Actin was used as an internal loading control.

### **RNA interference**

To knock down PML expression by RNA interference, the targeting oligonucleotide 5'-GAGCTCAAG TGC-GACATCA-3' (PML sense) was inserted into the pSUPER vector. This target region is present in all PML isoforms and was verified by BLAST searches to confirm specificity. For control experiments, empty pSUPER vectors were used.

### **Measurement of PV-induced cell lysis**

Hep3B and HepG2 cells were infected with H-1 PV at a MOI of 20 pfu/cell and further grown for 1 to 3 d. Cell permeabilization was then measured by using a standard toxicity assay (Toxilight, Cambrex Bio Science, Rockland Inc., USA) assessing the concentration of cellular adenylate kinase (AK) in culture supernatants according to the manufacturer's recommendations<sup>[39]</sup>.

### **FACScan analysis of apoptosis**

For quantification of the percentage of apoptotic cells in H-1 PV-infected cultures (MOI = 20 pfu/cell), adherent cells were dissociated with 0.25% trypsin and collected, together with cells floating in the medium, by centrifugation at 800 g. Cells were washed twice with PBS and stained with propidium iodide and annexin V (Becton Dickinson, Heidelberg, Germany). Fluorescence was measured with a minimum of 10 000 events per sample in a FACScan according to the manufacturer's instructions (Becton Dickinson). Data analysis was performed using the software Cell Quest (Becton Dickinson)<sup>[21]</sup>.

### **Analysis of mitochondrial membrane potential**

To measure the mitochondrial transmembrane potential, the cationic lipophilic fluorochrome JC-1 (5, 5, 6, 6-tetrachloro-1, 1, 3, 3-tetraethylbenzimidazolyl-carbocyanine iodide) (Molecular Probes, Inc., Eugene, OR) was used. JC-1 exists as a monomer in solution, emitting green fluorescence. In a reaction driven by the mitochondrial transmembrane potential, JC-1 can adopt a dimeric configuration and emit red fluorescence<sup>[40,41]</sup>. Mock- or H-1 PV-infected cultures ( $5 \times 10^4$  cells/mL) were incubated with JC-1 (5 µg/mL) for 20 min at room temperature in the dark, washed once in PBS and immediately analyzed by flow cytometry (FACScan, Becton Dickinson, Heidelberg, Germany) using Cellquest software. The red fluorescence of JC-1 indicates intact mitochondria, whereas green fluorescence shows monomeric JC-1 that remained unprocessed due to breakdown of the mitochondrial membrane potential<sup>[42]</sup>. After gating out small sized (i.e., non-cellular) debris, 10 000 events were collected for each analysis. The emitted green fluorescence signals were used as a measure for the loss of mitochondrial membrane potential<sup>[43]</sup>.

### **Statistical analysis**

Protein and (real time) gene expression values were analyzed for differences, using the one-sided Student's *t*-test. A *P*-value lower than 0.05 was considered as statistically significant.

## RESULTS

### Characterization of the p53 status in the hepatoma cell line pairs

In order to validate the system chosen to analyze the role of p53 and its effector pathways in H-1 PV-induced cellular cytotoxicity, we first confirmed the differential activity and expression of p53 in the two pairs of isogenic p53-positive and p53-negative HCC cell lines. The cell line HepG2 expresses functional wt p53 while Hep3B is a p53-null (p53<sup>-/-</sup>) cell line<sup>[44]</sup>. Hep3B4P cells transfected with a tamoxifen-regulated p53-estrogen receptor chimera<sup>[34]</sup> were cultured with different concentrations of 4-OH-tamoxifen to induce p53. Upon transfection of a p53-transactivated luciferase construct (pConluc/pgu-pluc), 4-OH-tamoxifen induced dose-dependent the p53 activity, up to 25 times at 750 nmol/L of 4-OH-tamoxifen (data not shown). At this concentration, the induction of p53 expression was determined to have no detectable effects on the growth of non-infected cells in accordance to previous reports<sup>[38,45]</sup>. The cell lines studied differ in expression of p53 as expected (Figure 1A). HepG2 and HepG2 303 show p53 bands in both cases, because HepG2 303 is a HepG2 derivative stably transfected with a dominant-negative p53 mutant (dn-p53).

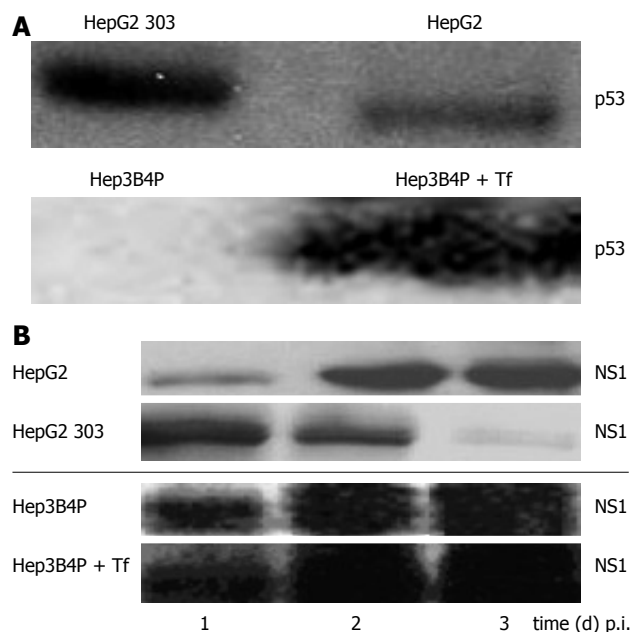
### Production of parvoviral non-structural proteins in virus-infected p53 different tumor cells

In order to assess the effect of p53 on H-1 PV replication, expression of the replicative cytotoxic NS1 protein was analyzed in infected tumor cell line pairs. As illustrated in Figure 1B, all cultures were proficient in NS1 accumulation within a few days p.i., as detected by Western blot analysis. It was noteworthy; however the p53 expression somehow impaired the capacity for NS1 production, as apparent from the delayed appearance (HepG2 system) or reduced steady-state level (Hep3B4P system) of NS1 in the p53-positive cells. As previously reported<sup>[21]</sup>, NS1 expression levels were in accordance with the respective amounts of viral DNA intermediates as well as luciferase activities of parvoviral vectors.

### H-1 PV toxicity for human tumor cell pairs differing in their p53 status

We previously observed that a p53-negative hepatoma cell line was lightly susceptible to H-1 PV-induced cytotoxicity<sup>[21]</sup>. This prompted us to compare the p53-deficient cells with their p53-positive counterparts in terms of their relative sensitivities to H-1 PV-induced killing. The levels of cytotoxicity in H-1 PV-inoculated Hep3B4P and HepG2 cultures were first monitored by measuring cell permeabilization through the release of adenylate kinase (AK) in the medium for up to 3 d p.i. (Figure 2A). Compared to their mock-treated controls, increased cell death was observed for all hepatoma cell cultures.

In keeping with their above-mentioned greater efficiency in cytotoxic NS1 production, the p53-negative cell cultures moved to be significantly more sensitive to the toxic effect of H-1 PV than their p53-positive de-



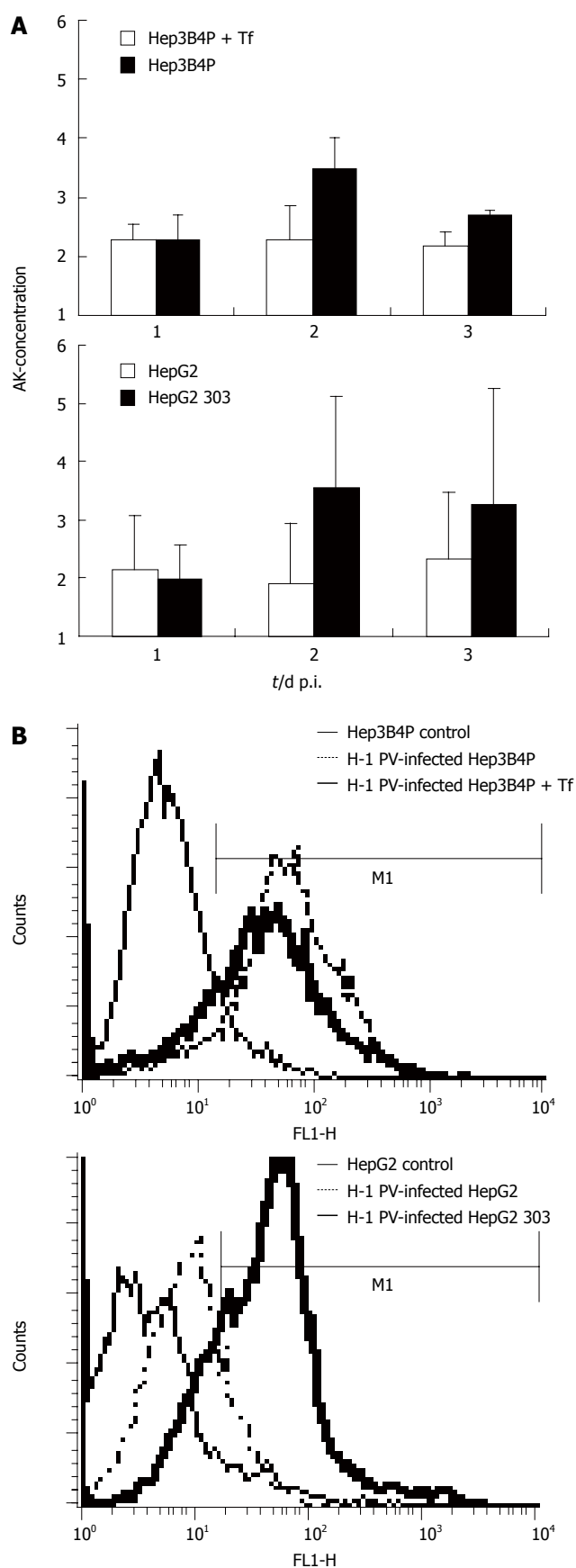
**Figure 1** A: Characterisation of the p53 status and analysis of parvoviral proteins in different human tumor cells by Western blot. Cells were cultured for 2 d and lysed with RIPA buffer, and 50 µg of total protein was subjected to SDS-PAGE. For p53 protein detection, blots were incubated with the monoclonal DO-7 antibody; B: Production of parvoviral proteins in H-1 PV-infected p53 different tumor cells. Hep3B4P and HepG2 cells were H-1 PV-infected (MOI = 20 pfu/cell) and grown for 1 to 3 d. After lysis with RIPA buffer, 50 µg of total proteins were equally diluted and separated on SDS-PAGE. For parvoviral protein detection, blots were incubated with the NS1-specific antibody<sup>[37]</sup>.

rivative. Indeed, supernatants from HepG2 303 cultures contained about twice the amounts of AK than those from the HepG2 parent after H-1 PV infection. Likewise, tamoxifen induction of p53 in the Hep3B4P cells correlated with an increase in their resistance to H-1 PV cytotoxicity.

This difference was confirmed by FACS analysis quantifying the expression of annexin V, a known marker of apoptosis<sup>[30]</sup> (Figure 2B). In agreement with the above viability assay, H-1 PV infection induced annexin V in all hepatoma cells tested, yet to a higher level in p53-negative compared to p53-positive lines. Thus, p53 status correlated with sensitivity of hepatoma cells to the induction of H-1 PV-mediated apoptosis.

### H-1 PV infection enhances depolarization of the inner mitochondrial membrane

The depolarization of the inner mitochondrial membrane has been associated with apoptosis in tumor cells exposed to different cytostatic agents or viruses<sup>[46]</sup>. This prompted us to investigate the effect of H-1 PV infection on this parameter in hepatoma cells. To this end, the profiles of JC-1 fluorescence were compared between mock- and H-1 PV-infected Hep3B4P cells. As illustrated in Figure 3, H-1 PV infection correlated with a striking increase in the fraction of hepatoma cells displaying depolarized mitochondrial membranes. In keeping with above data, this change occurred as soon as 1 d p.i. (Figure 3A) in p53-negative (Hep3B4P) in contrast to positive (Hep3B4P + Tf) cells. The p53-positive cells show an increase



**Figure 2** **A:** Cytotoxicity and induction of apoptosis in H-1 PV-infected p53 different tumor cell line pairs. The early damage of tumor cells upon H-1 PV infection was measured with a standardized toxicity test via supernatant adenylate kinase (AK) concentration; **B:** Induction of apoptosis in H-1 PV-infected tumor cells is shown in histograms for annexin V (FL1-H) of Hep3B4P and HepG2 cells. Data are given as mean values of triplicates.

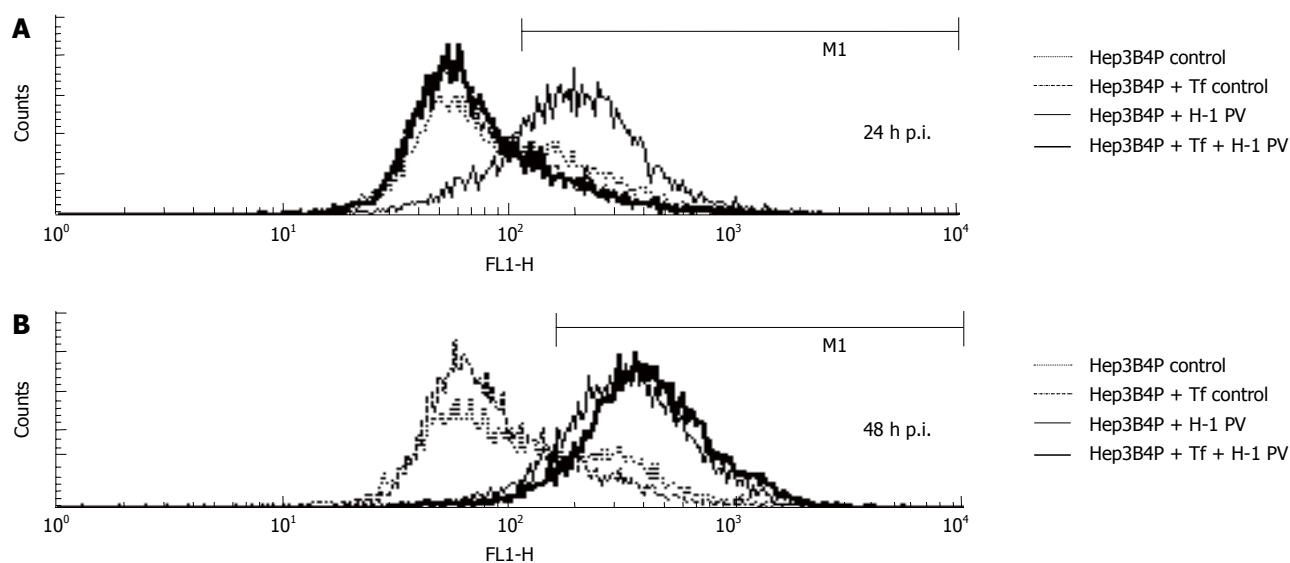
of the depolarization of membran potential not before 2 d p.i. (Figure 3B).

### Interplay of the tumor suppressor PML and H-1 PV infection

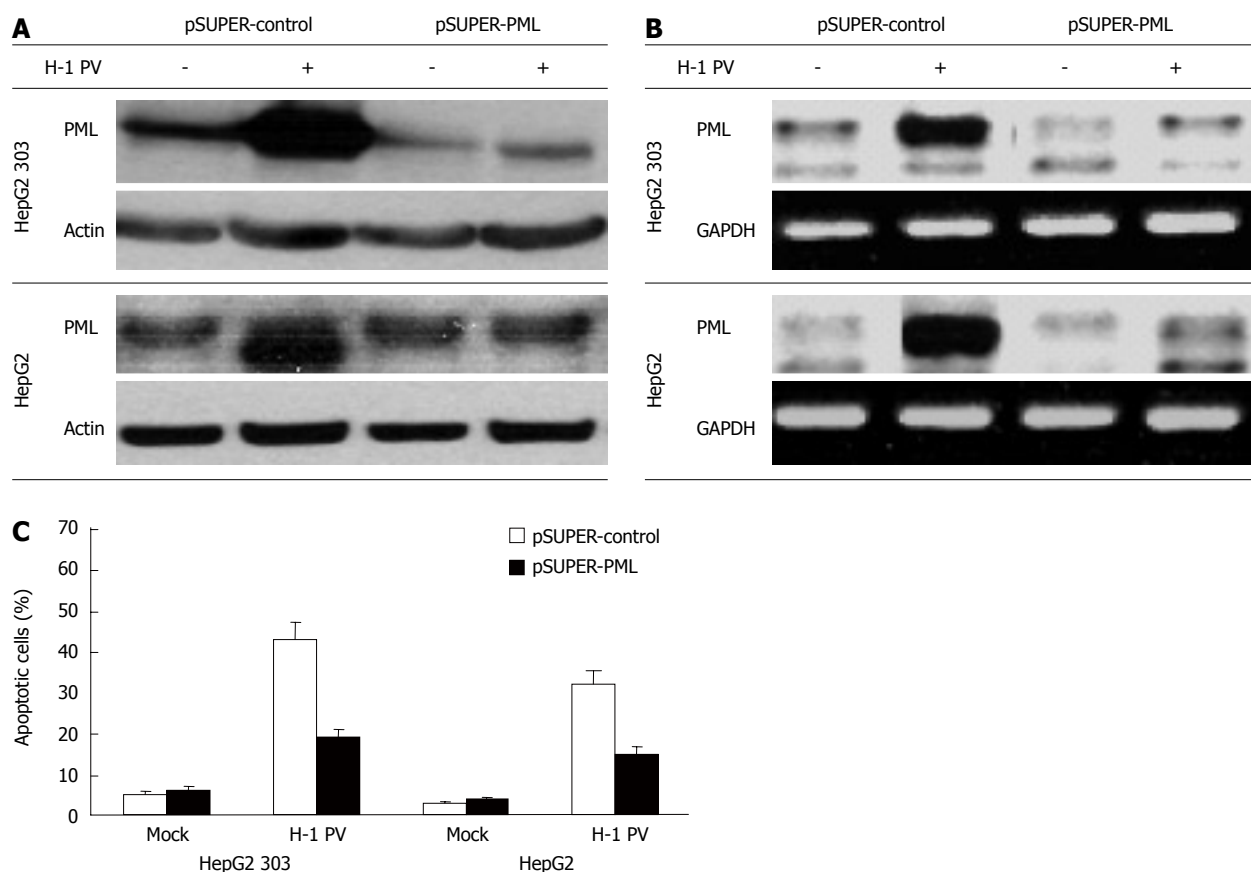
With the intention to identify factors influencing the sensitivity of hepatoma cells for H-1 PV, we determined whether the expression of the tumor suppressor protein PML was affected by H-1 PV-inoculation, and conversely, whether it had an impact on the outcome of infection. As shown in Figure 4, H-1 PV infection caused a strong increase in PML expression both on the protein (Figure 4A) and on the RNA (Figure 4B) level. To assess a possible role of PML in the control of H-1 PV-induced apoptosis in hepatoma cells, PML expression was reduced by RNA interference through expression of a short hairpin (sh) RNA (pSUPER-PML) which specifically targets PML. In contrast to the control pSUPER vector, the shPML construct strongly inhibited expression of endogenous PML in transfected HepG2 cells, irrespective of their p53 status (Figure 4B). Interestingly, knocking down of PML expression was found to correlate with a marked reduction of the efficiency of H-1 PV in inducing apoptosis in both HepG2 cells and their p53-negative HepG2 303 derivatives (Figure 4C). This result reveals PML to be centrally involved in regulation of apoptosis of hepatoma cells upon H-1 PV infection.

### Treatment with chemotherapeutic agents combined with H-1 PV infection

The genetic drift of cancer cells leads to the appearance of variants resisting conventional genotoxic anticancer treatments. This prompted us to test whether hepatoma cells escaping chemotherapy may still be killed by H-1 PV, i.e. whether the combination of chemotherapeutics with H-1 PV meant an advantage. In a first step, this possibility was explored *in vitro* by determining whether H-1 PV infection enhanced the fraction of apoptotic hepatoma cells in cultures treated with cisplatin (Cis), irinotecan (Iri), or 5-FU. As illustrated in Figure 5B, in p53-deficient HepG2 303 cultures, H-1 PV was found to cooperate with all three agents in enhancing the overall fraction of treated cells undergoing apoptosis. Interestingly, the beneficial effect of the combined treatment or either of its individual components was not (cisplatin, irinotecan) or hardly (5-FU) significant in the p53-positive parental line HepG2 (Figure 5A). Therefore, p53 appeared to impair the cooperation of H-1 PV with genotoxic agents, possibly due to the fact that the chemotherapeutic agents enhanced the above-mentioned negative impact of p53 on the parvoviral life cycle. It is noteworthy that the p53-deficient HepG2 303 cells were less (irinotecan, 5-FU) or even more (cisplatin) sensitive to the chemotherapeutic tested, compared with the p53-positive HepG2 parent (Figure 5). This was surprising, given the usually lower susceptibility of p53-negative cells to the induction of apoptosis by genotoxic agents, but is not without precedent as p53 can be functionally replaced by re-



**Figure 3** Analysis of mitochondrial membrane potential. Kinetics of reduction of the mitochondrial membrane potential. Hepatoma cells were left untreated (control) or infected (H-1 PV) for the indicated periods of time and analyzed by flow cytometry using the fluorochrome JC-1. The percentage of cells with decreased mitochondrial membrane potential is shown. After one (A) and two (B) days incubation, the percentage of cells with decreased mitochondrial membrane potential were determined by flow cytometry.



**Figure 4** H-1 PV-induced apoptosis is mediated by PML. HepG2 (p53<sup>+</sup>) and HepG2 303 (p53<sup>-</sup>) cells were transfected with pSUPER or pSUPER-PML as indicated, and infected with H-1 PV for 48 h. Cells were harvested and subjected to Western blot (A) or PCR analysis (B). Hepatoma cells were treated as described, harvested, and subjected to cytotoxicity assay. Apoptosis was determined as described in Material and Methods (C).

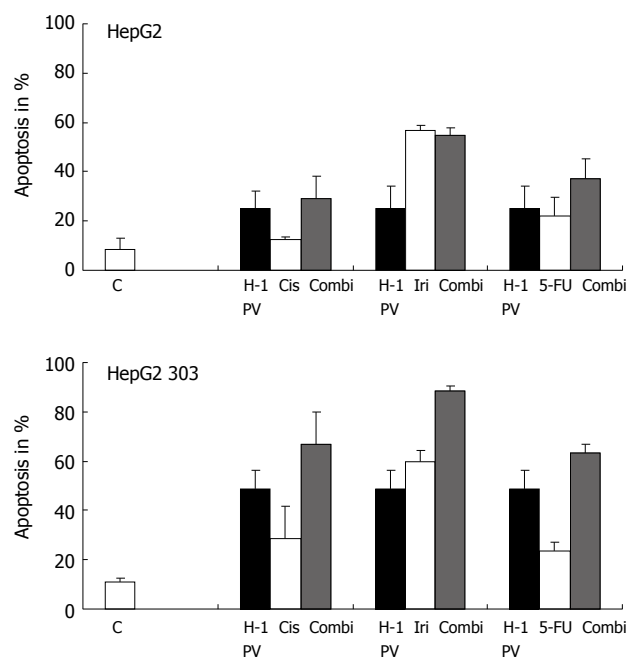
lated products in drug-induced killing of some tumor cells<sup>[47]</sup>.

## DISCUSSION

The development of gene transfer techniques for tumor suppressor protein negative cancers is a rapidly expanding field: For HCC, different viruses have been assessed to specifically target p53-negative tumors or to transfer the wt p53 gene to reconstitute apoptotic pathways in tumor cells<sup>[17-20,44,48-51]</sup>. However, several of the viral delivery systems are limited by their immuno-

suppressor protein negative cancers is a rapidly expanding field: For HCC, different viruses have been assessed to specifically target p53-negative tumors or to transfer the wt p53 gene to reconstitute apoptotic pathways in tumor cells<sup>[17-20,44,48-51]</sup>. However, several of the viral delivery systems are limited by their immuno-





**Figure 5** Treatment of p53 different tumor cells with chemotherapeutic agents. The p53 different HepG2 cells were treated with various chemotherapeutic agents alone or combined with H-1 PV infection (MOI = 20 pfu/cell). H-1 PV- or mock-infected cells were seeded into 6-well plates, and 1 h after infection cultures were treated with chemotherapeutic agents as indicated. Apoptosis was measured on day 3 by FACScan analysis. Data given represent mean values of triplicates.

genic or pathogenic side effects<sup>[52,53]</sup>. We have shown in this paper that oncolytic PV H-1 may be effective in the treatment of HCC. Even more, these oncolytic viruses directly targeting p53-negative carcinomas may be attractive alternative vectors as well as ideal tools for combination with classical chemotherapeutic agents. As the rat PV H-1 is seldom pathogenic to its natural adult hosts and infects humans without any apparent clinical consequences<sup>[28,29]</sup>, we considered the PV H-1 for tumor cell-targeted therapy, in particular in p53-negative tumors. In addition, immune reaction to PVs, such as AAV or H-1 PV might not induce any severe side effects<sup>[21-24]</sup>.

Thus, we first characterized the susceptibility to H-1 PV infection and cell killing of pairs of human tumor cells which differ in their wt p53 levels. In concordance with earlier data<sup>[21]</sup>, H-1 PV-induced killing of tested tumor cells was dependent on MOI and correlated with NS1 expression levels. Similarly, amenability to gene transfer after recombinant PV infection was higher in p53 lacking cells<sup>[21]</sup>. Despite both isogenic HepG2 cells were susceptible to H-1 PV-induced apoptosis, cell death was more pronounced in HepG2 303. As well in other human and rodent cell systems studied so far, susceptibility to H-1 PV-induced cell killing correlated with the capacity of the host cells to sustain both, parvoviral DNA amplification and NS1 protein expression<sup>[20,28,54-56]</sup>. With this regard, earlier data revealed cellular processes underlying the PV-induced tumor cell killing. Further on p53 displayed a key role in the G<sub>1</sub>/S checkpoint in response to DNA damage<sup>[57]</sup> as a regulator of cell cycle

progression and a mediator of apoptosis in many cell lines<sup>[58]</sup>. Thus, p53 could prevent cell progression to S-Phase. According to this some S-phase factors such as p53 have been involved in the regulation of PV DNA replication<sup>[31,35]</sup>. Indeed, the rare H-1 PV-resistant variant clones named KS cells, isolated from the H-1 PV-susceptible human p53-negative erythroleukemia cell line K562, differed from the parental wt p53-positive cells by a reduced oncogenic potential in immunocompromised mice. Similarly, rat fibroblasts overexpressing mutant p53 protein were more sensitive to H-1 PV infection than parental cells<sup>[31]</sup>.

Our data further demonstrate that H-1 PV induces significantly expression of PML on the RNA and protein level and, thus, increases the susceptibility to cell death in H-1 PV-infected tumor cells. The significance of this effect was clearly demonstrated by the knock down of PML in H-1 PV-infected cells with the consequence of impaired apoptosis upon H-1 PV infection. Recently, we showed that the hepatotropic hepatitis C virus (HCV) was able to impair apoptosis in hepatoma cells by inhibition of p53 function *via* interaction with PML<sup>[59]</sup>. Polypeptides from other viruses were also shown to interact with PML, and to disable its biological function in apoptosis regulation, growth suppression and cellular senescence. For example, adenoviral E1A protein abrogates oncogenic Ras- and PML-IV-induced cellular senescence by overriding PML function<sup>[60]</sup>.

Furthermore PODs/PML bodies have been associated with transcription, cell growth, and antiviral responses<sup>[61,62]</sup>. DNA and RNA viruses also frequently target PODs, presumably to facilitate the early stages of transcription and replication<sup>[63-67]</sup>. PODs can also be targeted for reorganization following viral infection<sup>[66]</sup>. For example, adenovirus protein E4-ORF3 localizes to PODs/PML bodies, thereby causing a physical restructuring of the bodies from spherical to extended fibril-like structures termed nuclear tracks<sup>[66]</sup>. Additionally, many viruses induce interferon expression, what increases the size and number of PODs<sup>[62,68]</sup>. Although PV infection did not induce an interferon response, a dramatic relocalization of PODs has been seen late in MVM infection. However, Cziepluch *et al.*<sup>[69]</sup> suggested that H-1 virus does not target known nuclear bodies for DNA replication but rather induced the formation of a novel structure in the nucleus of infected cells. Within that study, PML-expression was not directly investigated.

In addition, PVs were recently reported to replicate in association with distinct nuclear bodies<sup>[69]</sup>, which appear to lately merge with PML and PML-NBs<sup>[70]</sup>. PML may interact with viral products and participate in the regulation of virus replication. PML proteins may thus modulate viral cytopathic effects, in keeping with the above-mentioned involvement of PML in growth inhibition and death processes. As PV H-1 infection enhances PML expression and function, this might be a central molecular mechanism for an effective treatment of HCC.

We furthermore examined the effects of various chemotherapeutic agents in combination with H-1 PV

infection on growth inhibition using isogenic cancer cells with different p53 status. H-1 PV infection enhanced the cytotoxicity of chemotherapeutic agents in the treatment of two HCC. In p53-deficient HepG2 303 cultures, H-1 PV was found to cooperate with all three agents in enhancing the overall fraction of treated cells undergoing apoptosis. The treatment with Irinotecan, 5-Fluorouracil and Cisplatin combined with H-1 PV infection more strongly inhibited the growth of the p53-negative HepG2 303 cells than treatment with chemotherapeutics alone. Therefore H-1 PV infection enhances cytostatic drug therapy in p53-negative tumors. Furthermore, irrespective of the p53 status, H-1 PV was able to induce programmed cell death in these human tumor cells, as it was also shown for other PVs<sup>[71]</sup>. Chemotherapeutic treatment alone did not induce such a high apoptosis rate compared to combined treatment with H-1 PV. Comparable data have also been published for other oncolytic viruses<sup>[72-75]</sup>. Thus, our data show a beneficial interaction between chemotherapy and oncolytic viral therapy and suggest that H-1 PV infection may enhance the effectiveness of chemotherapeutic agents in the treatment of HCC.

In summary, our results strongly suggest that p53-impaired tumors-which have a poor prognosis-may be particularly suitable to PV H-1-induced therapy<sup>[39,43,45]</sup>. Though p53 deficiency in tumors may induce resistance to chemotherapeutic agents, this will not affect the tumor cell susceptibility to H-1 PV-induced oncolytic infections<sup>[51]</sup>. As recombinant H-1 PV had a high capacity to transduce transgenes in these cells, the therapeutic potential of H-1 PV-based recombinant vectors carrying suicide genes or cytokines should be further assessed in p53-negative tumors. The PV H-1 may then also overcome other tumor resistance mechanisms against autocrine and paracrine apoptotic triggers developed in these tumor entities<sup>[50,51]</sup>. We conclude that our strategy using H-1 PV infection in combination with chemotherapeutic treatment can enhance the cytotoxic effect of anti-cancer agents. Furthermore, H-1 PV induced the expression of PML, thus increased the susceptibility to cell death in H-1 PV-infected tumor cells. So PML may operate as a positive element which controls in a direct or indirect way the susceptibility of hepatoma cells to apoptosis-activity of H-1 PV.

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## COMMENTS

### Background

Oncolytic parvoviruses (PVs) are endowed with oncolytic properties and also increase the host immune response against the tumor by priming effector cells.

### Research frontiers

Authors evaluated the synergistic targeting and killing of human hepatocellular carcinoma (HCC) cells lacking p53 by PV H-1 and chemotherapeutic agents.

### Innovations and breakthroughs

Analysing the regulating the cell killing pathways and gene transfer mediated by PV H-1 in pairs of human hepatocellular cell lines with different p53 status, H-1 PV is quite a suitable agent to circumvent the resistance of p53-negative HCC to genotoxic agents, and enhances the apoptotic process which is dependent on functional PML.

### Applications

Especially for p53-negative human tumors authors consider PV H-1 as therapeutic option for human HCC.

### Peer review

This manuscript described that H-1 PV is a novel agent for treating p53 negative HCC via the induction of PML and apoptosis. This study is a well designed, well exerted, and well written manuscript.

## REFERENCES

- Gurrieri C, Nafa K, Merghoub T, Bernardi R, Capodici P, Biondi A, Nimer S, Douer D, Cordon-Cardo C, Gallagher R, Pandolfi PP. Mutations of the PML tumor suppressor gene in acute promyelocytic leukemia. *Blood* 2004; **103**: 2358-2362
- Alves VA, Nita ME, Carrilho FJ, Ono-Nita SK, Wakamatsu A, Lehrbach DM, de Carvalho MF, de Mello ES, Gayotto LC, da Silva LC. p53 immunostaining pattern in Brazilian patients with hepatocellular carcinoma. *Rev Inst Med Trop Sao Paulo* 2004; **46**: 25-31
- Clarke AR, Purdie CA, Harrison DJ, Morris RG, Bird CC, Hooper ML, Wyllie AH. Thymocyte apoptosis induced by p53-dependent and independent pathways. *Nature* 1993; **362**: 849-852
- Lane DP. Cancer. p53, guardian of the genome. *Nature* 1992; **358**: 15-16
- Vogelstein B, Lane D, Levine AJ. Surfing the p53 network. *Nature* 2000; **408**: 307-310
- Baker SJ, Fearon ER, Nigro JM, Hamilton SR, Preisinger AC, Jessup JM, vanTuinen P, Ledbetter DH, Barker DF, Nakamura Y, White R, Vogelstein B. Chromosome 17 deletions and p53 gene mutations in colorectal carcinomas. *Science* 1989; **244**: 217-221
- Nigro JM, Baker SJ, Preisinger AC, Jessup JM, Hostetter R, Cleary K, Bigner SH, Davidson N, Baylin S, Devilee P. Mutations in the p53 gene occur in diverse human tumour types. *Nature* 1989; **342**: 705-708
- Chang F, Syrjanen S, Syrjanen K. Implications of the p53 tumor-suppressor gene in clinical oncology. *J Clin Oncol* 1995; **13**: 1009-1022
- Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell* 1993; **74**: 957-967
- Sternsdorf T, Jensen K, Will H. Evidence for covalent modification of the nuclear dot-associated proteins PML and Sp100 by PIC1/SUMO-1. *J Cell Biol* 1997; **139**: 1621-1634
- Salomoni P, Pandolfi PP. The role of PML in tumor suppression. *Cell* 2002; **108**: 165-170
- Le XF, Yang P, Chang KS. Analysis of the growth and transformation suppressor domains of promyelocytic leukemia gene, PML. *J Biol Chem* 1996; **271**: 130-135
- Fogal V, Gostissa M, Sandy P, Zacchi P, Sternsdorf T, Jensen K, Pandolfi PP, Will H, Schneider C, Del Sal G. Regulation of p53 activity in nuclear bodies by a specific PML isoform. *EMBO J* 2000; **19**: 6185-6195
- Guo A, Salomoni P, Luo J, Shih A, Zhong S, Gu W, Pandolfi PP. The function of PML in p53-dependent apoptosis. *Nat Cell Biol* 2000; **2**: 730-736
- Wang Z, Seliger B, Mike N, Momburg F, Knuth A, Ferrone S. Molecular analysis of the HLA-A2 antigen loss by melanoma cells SK-MEL-29.1.22 and SK-MEL-29.1.29. *Cancer Res* 1998; **58**: 2149-2157

- 16 **El-Deiry WS.** Insights into cancer therapeutic design based on p53 and TRAIL receptor signaling. *Cell Death Differ* 2001; **8**: 1066-1075
- 17 **Fujiwara T,** Cai DW, Georges RN, Mukhopadhyay T, Grimm EA, Roth JA. Therapeutic effect of a retroviral wild-type p53 expression vector in an orthotopic lung cancer model. *J Natl Cancer Inst* 1994; **86**: 1458-1462
- 18 **Wills KN,** Maneval DC, Menzel P, Harris MP, Sutjipto S, Vaillancourt MT, Huang WM, Johnson DE, Anderson SC, Wen SF. Development and characterization of recombinant adenoviruses encoding human p53 for gene therapy of cancer. *Hum Gene Ther* 1994; **5**: 1079-1088
- 19 **Sandig V,** Brand K, Herwig S, Lukas J, Bartek J, Strauss M. Adenovirally transferred p16INK4/CDKN2 and p53 genes cooperate to induce apoptotic tumor cell death. *Nat Med* 1997; **3**: 313-319
- 20 **Kirn D,** Martuza RL, Zwiebel J. Replication-selective virotherapy for cancer: Biological principles, risk management and future directions. *Nat Med* 2001; **7**: 781-787
- 21 **Moehler M,** Blechacz B, Weiskopf N, Zeidler M, Stremmel W, Rommelaere J, Galle PR, Cornelis JJ. Effective infection, apoptotic cell killing and gene transfer of human hepatoma cells but not primary hepatocytes by parvovirus H1 and derived vectors. *Cancer Gene Ther* 2001; **8**: 158-167
- 22 **Rommelaere J,** Cornelis JJ. Autonomous Parvoviruses[A]. In: Hernáiz Driever P, Rabkin SD, editors. Replication-Competent Viruses for Cancer Therapy. Monographs in Virology. Basel: Karger, 2001: 100-129
- 23 **Moehler M,** Zeidler M, Schede J, Rommelaere J, Galle PR, Cornelis JJ, Heike M. Oncolytic parvovirus H1 induces release of heat-shock protein HSP72 in susceptible human tumor cells but may not affect primary immune cells. *Cancer Gene Ther* 2003; **10**: 477-480
- 24 **Moehler MH,** Zeidler M, Wilsberg V, Cornelis JJ, Woelfel T, Rommelaere J, Galle PR, Heike M. Parvovirus H-1-induced tumor cell death enhances human immune response in vitro via increased phagocytosis, maturation, and cross-presentation by dendritic cells. *Hum Gene Ther* 2005; **16**: 996-1005
- 25 **Cornelis JJ,** Salome N, Dinsart C, Rommelaere J. Vectors based on autonomous parvoviruses: novel tools to treat cancer? *J Gene Med* 2004; **6** Suppl 1: S193-S202
- 26 **Chen YQ,** de Foresta F, Hertoghs J, Avalosse BL, Cornelis JJ, Rommelaere J. Selective killing of simian virus 40-transformed human fibroblasts by parvovirus H-1. *Cancer Res* 1986; **46**: 3574-3579
- 27 **Cornelis JJ,** Becquart P, Duponchel N, Salome N, Avalosse BL, Namba M, Rommelaere J. Transformation of human fibroblasts by ionizing radiation, a chemical carcinogen, or simian virus 40 correlates with an increase in susceptibility to the autonomous parvoviruses H-1 virus and minute virus of mice. *J Virol* 1988; **62**: 1679-1686
- 28 **Rommelaere J,** Cornelis JJ. Antineoplastic activity of parvoviruses. *J Virol Methods* 1991; **33**: 233-251
- 29 **Jacoby RO,** Ball-Goodrich LJ, Besselsen DG, McKisic MD, Riley LK, Smith AL. Rodent parvovirus infections. *Lab Anim Sci* 1996; **46**: 370-380
- 30 **Mousset S,** Ouadrhiri Y, Caillet-Fauquet P, Rommelaere J. The cytotoxicity of the autonomous parvovirus minute virus of mice nonstructural proteins in FR3T3 rat cells depends on oncogene expression. *J Virol* 1994; **68**: 6446-6453
- 31 **Telerman A,** Tynnder M, Dupressoir T, Robaye B, Sigaux F, Shaulian E, Oren M, Rommelaere J, Amson R. A model for tumor suppression using H-1 parvovirus. *Proc Natl Acad Sci USA* 1993; **90**: 8702-8706
- 32 **Muller M,** Strand S, Hug H, Heinemann EM, Walczak H, Hofmann WJ, Stremmel W, Krammer PH, Galle PR. Drug-induced apoptosis in hepatoma cells is mediated by the CD95 (APO-1/Fas) receptor/ligand system and involves activation of wild-type p53. *J Clin Invest* 1997; **99**: 403-413
- 33 **Ponchel F,** Puisieux A, Tabone E, Michot JP, Froschl G, Morel AP, Frebourg T, Fontaniere B, Oberhammer F, Ozturk M. Hepatocarcinoma-specific mutant p53-249ser induces mitotic activity but has no effect on transforming growth factor beta 1-mediated apoptosis. *Cancer Res* 1994; **54**: 2064-2068
- 34 **Friedman SL,** Shaulian E, Littlewood T, Resnitzky D, Oren M. Resistance to p53-mediated growth arrest and apoptosis in Hep 3B hepatoma cells. *Oncogene* 1997; **15**: 63-70
- 35 **Vater CA,** Bartle LM, Dionne CA, Littlewood TD, Goldmacher VS. Induction of apoptosis by tamoxifen-activation of a p53-estrogen receptor fusion protein expressed in E1A and T24 H-ras transformed p53-/- mouse embryo fibroblasts. *Oncogene* 1996; **13**: 739-748
- 36 **Schuler M,** Maurer U, Goldstein JC, Breitenbucher F, Hoffarth S, Waterhouse NJ, Green DR. p53 triggers apoptosis in oncogene-expressing fibroblasts by the induction of Noxa and mitochondrial Bax translocation. *Cell Death Differ* 2003; **10**: 451-460
- 37 **Faisst S,** Faisst SR, Dupressoir T, Plaza S, Pujol A, Jauniaux JC, Rhode SL, Rommelaere J. Isolation of a fully infectious variant of parvovirus H-1 supplanting the standard strain in human cells. *J Virol* 1995; **69**: 4538-4543
- 38 **Galmarini CM,** Falette N, Tabone E, Levrat C, Britten R, Voorzanger-Rousselot N, Roesch-Gateau O, Vanier-Viorner A, Puisieux A, Dumontet C. Inactivation of wild-type p53 by a dominant negative mutant renders MCF-7 cells resistant to tubulin-binding agent cytotoxicity. *Br J Cancer* 2001; **85**: 902-908
- 39 **Olsson T,** Gulliksson H, Palmeborn M, Bergstrom K, Thore A. Leakage of adenylate kinase from stored blood cells. *J Appl Biochem* 1983; **5**: 437-445
- 40 **Lawrence JW,** Darkin-Rattray S, Xie F, Neims AH, Rowe TC. 4-Quinolones cause a selective loss of mitochondrial DNA from mouse L1210 leukemia cells. *J Cell Biochem* 1993; **51**: 165-174
- 41 **Loeffler M,** Kroemer G. The mitochondrion in cell death control: certainties and incognita. *Exp Cell Res* 2000; **256**: 19-26
- 42 **Reers M,** Smith TW, Chen LB. J-aggregate formation of a carbocyanine as a quantitative fluorescent indicator of membrane potential. *Biochemistry* 1991; **30**: 4480-4486
- 43 **Scaffidi C,** Schmitz I, Zha J, Korsmeyer SJ, Krammer PH, Peter ME. Differential modulation of apoptosis sensitivity in CD95 type I and type II cells. *J Biol Chem* 1999; **274**: 22532-22538
- 44 **Vollmer CM,** Ribas A, Butterfield LH, Disette VB, Andrews KJ, Eilber FC, Montejo LD, Chen AY, Hu B, Glaspy JA, McBride WH, Economou JS. p53 selective and nonselective replication of an E1B-deleted adenovirus in hepatocellular carcinoma. *Cancer Res* 1999; **59**: 4369-4374
- 45 **Ran Z,** Rayet B, Rommelaere J, Faisst S. Parvovirus H-1-induced cell death: influence of intracellular NAD consumption on the regulation of necrosis and apoptosis. *Virus Res* 1999; **65**: 161-174
- 46 **Duverger V,** Sartorius U, Klein-Bauernschmitt P, Krammer PH, Schlehofer JR. Enhancement of cisplatin-induced apoptosis by infection with adeno-associated virus type 2. *Int J Cancer* 2002; **97**: 706-712
- 47 **Vayssade M,** Haddada H, Faridoni-Laurens L, Tourpin S, Valent A, Benard J, Ahomadegbe JC. P73 functionally replaces p53 in Adriamycin-treated, p53-deficient breast cancer cells. *Int J Cancer* 2005; **116**: 860-869
- 48 **Anderson SC,** Johnson DE, Harris MP, Engler H, Hancock W, Huang WM, Wills KN, Gregory RJ, Sutjipto S, Wen SF, Lofgren S, Shepard HM, Maneval DC. p53 gene therapy in a rat model of hepatocellular carcinoma: intra-arterial delivery of a recombinant adenovirus. *Clin Cancer Res* 1998; **4**: 1649-1659
- 49 **Bookstein R,** Demers W, Gregory R, Maneval D, Park J, Wills K. p53 gene therapy in vivo of herpatocellular and liver metastatic colorectal cancer. *Semin Oncol* 1996; **23**: 66-77
- 50 **Borresen-Dale AL.** TP53 and breast cancer. *Hum Mutat* 2003; **21**: 292-300
- 51 **Picksley SM,** Spicer JF, Barnes DM, Lane DP. The p53-

- MDM2 interaction in a cancer-prone family, and the identification of a novel therapeutic target. *Acta Oncol* 1996; **35**: 429-434
- 52 **Alt M**, Caselmann WH. Liver-directed gene therapy: molecular tools and current preclinical and clinical studies. *J Hepatol* 1995; **23**: 746-758
- 53 **Bischoff JR**, Kirn DH, Williams A, Heise C, Horn S, Muna M, Ng L, Nye JA, Sampson-Johannes A, Fattaey A, McCormick F. An adenovirus mutant that replicates selectively in p53-deficient human tumor cells. *Science* 1996; **274**: 373-376
- 54 **Dupressoir T**, Vanacker JM, Cornelis JJ, Duponchel N, Rommelaere J. Inhibition by parvovirus H-1 of the formation of tumors in nude mice and colonies in vitro by transformed human mammary epithelial cells. *Cancer Res* 1989; **49**: 3203-3208
- 55 **Ries SJ**, Brandts CH, Chung AS, Biederer CH, Hann BC, Lipner EM, McCormick F, Korn WM. Loss of p14ARF in tumor cells facilitates replication of the adenovirus mutant dl1520 (ONYX-015). *Nat Med* 2000; **6**: 1128-1133
- 56 **St George JA**. Gene therapy progress and prospects: adenoviral vectors. *Gene Ther* 2003; **10**: 1135-1141
- 57 **Ciciarello M**, Mangiacasale R, Casenghi M, Zaira Limongi M, D'Angelo M, Soddu S, Lavia P, Cundari E. p53 displacement from centrosomes and p53-mediated G1 arrest following transient inhibition of the mitotic spindle. *J Biol Chem* 2001; **276**: 19205-19213
- 58 **Cui Q**, Yu JH, Wu JN, Tashiro S, Onodera S, Minami M, Ikejima T. P53-mediated cell cycle arrest and apoptosis through a caspase-3- independent, but caspase-9-dependent pathway in oridonin-treated MCF-7 human breast cancer cells. *Acta Pharmacol Sin* 2007; **28**: 1057-1066
- 59 **Herzer K**, Weyer S, Krammer PH, Galle PR, Hofmann TG. Hepatitis C virus core protein inhibits tumor suppressor protein promyelocytic leukemia function in human hepatoma cells. *Cancer Res* 2005; **65**: 10830-10837
- 60 **Ferbeyre G**, de Stanchina E, Lin AW, Querido E, McCurrach ME, Hannon GJ, Lowe SW. Oncogenic ras and p53 cooperate to induce cellular senescence. *Mol Cell Biol* 2002; **22**: 3497-3508
- 61 **Doucas V**. The promyelocytic (PML) nuclear compartment and transcription control. *Biochem Pharmacol* 2000; **60**: 1197-1201
- 62 **Lavau C**, Marchio A, Fagioli M, Jansen J, Falini B, Lebon P, Grosveld F, Pandolfi PP, Pelicci PG, Dejean A. The acute promyelocytic leukaemia-associated PML gene is induced by interferon. *Oncogene* 1995; **11**: 871-876
- 63 **Ahn JH**, Hayward GS. Disruption of PML-associated nuclear bodies by IE1 correlates with efficient early stages of viral gene expression and DNA replication in human cytomegalovirus infection. *Virology* 2000; **274**: 39-55
- 64 **Day PM**, Roden RB, Lowy DR, Schiller JT. The papillomavirus minor capsid protein, L2, induces localization of the major capsid protein, L1, and the viral transcription/replication protein, E2, to PML oncogenic domains. *J Virol* 1998; **72**: 142-150
- 65 **Doucas V**, Ishov AM, Romo A, Juguilon H, Weitzman MD, Evans RM, Maul GG. Adenovirus replication is coupled with the dynamic properties of the PML nuclear structure. *Genes Dev* 1996; **10**: 196-207
- 66 **Everett RD**, Maul GG. HSV-1 IE protein Vmw110 causes redistribution of PML. *EMBO J* 1994; **13**: 5062-5069
- 67 **Wu FY**, Ahn JH, Alcendor DJ, Jang WJ, Xiao J, Hayward SD, Hayward GS. Origin-independent assembly of Kaposi's sarcoma-associated herpesvirus DNA replication compartments in transient cotransfection assays and association with the ORF-K8 protein and cellular PML. *J Virol* 2001; **75**: 1487-1506
- 68 **Fabunmi RP**, Wigley WC, Thomas PJ, DeMartino GN. Interferon gamma regulates accumulation of the proteasome activator PA28 and immunoproteasomes at nuclear PML bodies. *J Cell Sci* 2001; **114**: 29-36
- 69 **Cziepluch C**, Lampel S, Grewenig A, Grund C, Lichter P, Rommelaere J. H-1 parvovirus-associated replication bodies: a distinct virus-induced nuclear structure. *J Virol* 2000; **74**: 4807-4815
- 70 **Young PJ**, Jensen KT, Burger LR, Pintel DJ, Lorson CL. Minute virus of mice NS1 interacts with the SMN protein, and they colocalize in novel nuclear bodies induced by parvovirus infection. *J Virol* 2002; **76**: 3892-3904
- 71 **Poole BD**, Karetnyi YV, Naides SJ. Parvovirus B19-induced apoptosis of hepatocytes. *J Virol* 2004; **78**: 7775-7783
- 72 **Eisenberg DP**, Adusumilli PS, Hendershott KJ, Yu Z, Mullerad M, Chan MK, Chou TC, Fong Y. 5-fluorouracil and gemcitabine potentiate the efficacy of oncolytic herpes viral gene therapy in the treatment of pancreatic cancer. *J Gastrointest Surg* 2005; **9**: 1068-1077; discussion 1077-1079
- 73 **Mullerad M**, Bochner BH, Adusumilli PS, Bhargava A, Kikuchi E, Hui-Ni C, Kattan MW, Chou TC, Fong Y. Herpes simplex virus based gene therapy enhances the efficacy of mitomycin C for the treatment of human bladder transitional cell carcinoma. *J Urol* 2005; **174**: 741-746
- 74 **Raykov Z**, Grekova S, Galabov AS, Balboni G, Koch U, Aprahamian M, Rommelaere J. Combined oncolytic and vaccination activities of parvovirus H-1 in a metastatic tumor model. *Oncol Rep* 2007; **17**: 1493-1499
- 75 **Toyoizumi T**, Mick R, Abbas AE, Kang EH, Kaiser LR, Molnar-Kimber KL. Combined therapy with chemotherapeutic agents and herpes simplex virus type 1 ICP34.5 mutant (HSV-1716) in human non-small cell lung cancer. *Hum Gene Ther* 1999; **10**: 3013-3029

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## Bcl-x<sub>L</sub> and Myeloid cell leukaemia-1 contribute to apoptosis resistance of colorectal cancer cells

Henning Schulze-Bergkamen, Roland Ehrenberg, Lothar Hickmann, Binje Vick, Toni Urbanik, Christoph C Schimanski, Martin R Berger, Arno Schad, Achim Weber, Steffen Heeger, Peter R Galle, Markus Moehler

Henning Schulze-Bergkamen, Roland Ehrenberg, Lothar Hickmann, Binje Vick, Toni Urbanik, Christoph C Schimanski, Peter R Galle, Markus Moehler, First Department of Medicine, Johannes-Gutenberg-University Mainz, Mainz 55101, Germany

Martin R Berger, German Cancer Research Center, Heidelberg 69120, Germany

Arno Schad, Institute of Pathology, University of Mainz, Mainz 55101, Germany

Achim Weber, Department of Pathology, Institute of Surgical Pathology, University Hospital, Zürich 8091, Switzerland

Steffen Heeger, Merck Pharma GmbH, Darmstadt 64293, Germany

**Author contributions:** Schulze-Bergkamen H, Ehrenberg R contributed equally to this work; Schulze-Bergkamen H, Ehrenberg R, Hickmann L, Urbanik T and Vick B performed the experiments of the study and made substantial contributions to conception and design of the study, interpretation of the data and statistical analysis; Schulze-Bergkamen H drafted the manuscript; Moehler M, Heeger S and Galle PR made substantial contributions to conception, design and interpretation of data; Schimanski CC and Berger MR participated in the design of the study and in the analysis of colorectal carcinoma tissue samples; Schad A and Weber A performed the immunohistochemical analysis of colorectal carcinoma tissues; all authors read and approved the final manuscript; this study contains essential parts of the medical thesis work of Ehrenberg R and Hickmann L.

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**Correspondence to:** Henning Schulze-Bergkamen, MD, PhD, First Department of Medicine, Johannes-Gutenberg-University Mainz, Langenbeckstrasse 1, Mainz 55101, Germany. [bergkam@uni-mainz.de](mailto:bergkam@uni-mainz.de)

Telephone: +49-6131-172462 Fax: +49-6131-175529

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and immunohistochemistry. Bcl-x<sub>L</sub> and Mcl-1 protein expression was knocked down or increased in CRC cell lines by applying specific siRNAs or expression plasmids, respectively. After modulation of protein expression, CRC cells were treated with chemotherapeutic agents, an antagonistic epidermal growth factor receptor (EGFR1) antibody, an EGFR1 tyrosine kinase inhibitor, or with the death receptor ligand TRAIL. Apoptosis induction and cell viability were analyzed.

**RESULTS:** Here we show that in human CRC tissue and various CRC cell lines both Bcl-x<sub>L</sub> and Mcl-1 are expressed. Bcl-x<sub>L</sub> expression was higher in CRC tissue than in surrounding non-malignant tissue, both on protein and mRNA level. *Mcl-1* mRNA expression was significantly lower in malignant tissues. However, protein expression was slightly higher. Viability rates of CRC cells were significantly decreased after knock down of Bcl-x<sub>L</sub> expression, and, to a lower extent, after knock down of Mcl-1 expression. Furthermore, cells with reduced Bcl-x<sub>L</sub> or Mcl-1 expression was more sensitive towards oxaliplatin- and irinotecan-induced apoptosis, and in the case of Bcl-x<sub>L</sub> also towards 5-FU-induced apoptosis. On the other hand, upregulation of Bcl-x<sub>L</sub> by transfection of an expression plasmid decreased chemotherapeutic drug-induced apoptosis. EGF treatment clearly induced Bcl-x<sub>L</sub> and Mcl-1 expression in CRC cells. Apoptosis induction upon EGFR1 blockage by cetuximab or PD168393 was increased by inhibiting Mcl-1 and Bcl-x<sub>L</sub> expression. More strikingly, CD95- and TRAIL-induced apoptosis was increased by Bcl-x<sub>L</sub> knock down.

**CONCLUSION:** Our data suggest that Bcl-x<sub>L</sub> and, to a lower extent, Mcl-1, are important anti-apoptotic factors in CRC. Specific downregulation of Bcl-x<sub>L</sub> is a promising approach to sensitize CRC cells towards chemotherapy and targeted therapy.

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### Abstract

**AIM:** To explore the role of Bcl-x<sub>L</sub> and Myeloid cell leukaemia (Mcl)-1 for the apoptosis resistance of colorectal carcinoma (CRC) cells towards current treatment modalities.

**METHODS:** Bcl-x<sub>L</sub> and Mcl-1 mRNA and protein expression were analyzed in CRC cell lines as well as human CRC tissue by Western blot, quantitative PCR

**Key words:** Colorectal carcinoma; Bcl-x<sub>L</sub>; Myeloid cell leukaemia-1; Epidermal growth factor receptor 1; Apoptosis; 5-fluorouracil; Irinotecan; Oxaliplatin

**Peer reviewers:** Shu Zheng, Professor, Scientific Director of Cancer Institute, Zhejiang University, Secondary Affiliated Hospital, Zhejiang University, 88# Jiefang Road, Hangzhou 310009, Zhejiang Province, China; Dr. John M Carethers, GI

Section, 111D, VA San Diego Healthcare System, 3350 La Jolla Village Drive, San Diego CA 92161, United States; Wei Tang, MD, EngD, Assistant Professor, H-B-P Surgery Division, Artificial Organ and Transplantation Division, Department of surgery, Graduate School of Medicine, the University of Tokyo, Tokyo 113-8655, Japan

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## INTRODUCTION

Colorectal carcinoma (CRC) is one of the most common malignancies in the Western world. In palliative care, novel treatment approaches including combination of chemotherapy and targeted therapies, such as epidermal growth factor receptor (EGFR) 1 blockage, have improved survival of cancer patients<sup>[1]</sup>. However, 5-year survival of patients with metastatic CRC remains < 5%. Current established systemic therapy options include 5-fluorouracil (5-FU), oxaliplatin, irinotecan, the EGFR1 antibody cetuximab and the vascular endothelial growth factor (VEGF)-A antibody bevacizumab.

Apoptosis is a genetically programmed process of controlled suicide, which is critical for multicellular organisms during development and for tissue homeostasis. However, in cancer, tumor cells acquire resistance to apoptosis. Thus, the ratio of apoptosis and cell division is altered, resulting in a net gain of malignant tissue. Additionally, defects in apoptosis signalling in cancer cells impair response to therapy and contribute to the limited efficacy of different therapy regimens in metastatic disease<sup>[2]</sup>.

Stabilization of mitochondrial integrity is a key mechanism for the survival of a malignant cell and its resistance to therapy<sup>[3]</sup>. Mitochondrial integrity is regulated by pro- and anti-apoptotic members of the Bcl-2 family, such as Bcl-x<sub>L</sub> and Mcl-1 (Myeloid cell leukaemia-1, anti-apoptotic) and Bid, Bad and Bax (pro-apoptotic). Mcl-1 is essential for development, differentiation and survival in a variety of cell types<sup>[4,5]</sup>. It is involved in important interactions of Bcl-2 family members and thereby regulates mitochondrial activation<sup>[6]</sup>. Mcl-1 protein levels are elevated in various human tumors, such as hepatocellular carcinoma<sup>[7]</sup> and non-small cell lung cancer<sup>[8]</sup>. Importantly, it contributes to the resistance of cancer cells towards apoptosis induction<sup>[6,9]</sup>. Downregulation of Mcl-1 has been shown to sensitize cancer cells towards apoptosis induction, e.g. after treatment with the death receptor ligand TRAIL [tumor necrosis factor (TNF)-related apoptosis-inducing ligand]<sup>[10,11]</sup>. In addition, Mcl-1 degradation is necessary for mitochondrial activation after genotoxic stress<sup>[12]</sup>. Like Mcl-1, Bcl-x<sub>L</sub> is known to promote cell survival by

counteracting pro-apoptotic Bcl-2 family members, such as Bim, Bax and Bid. In cancer, overexpression of Bcl-x<sub>L</sub> is associated with tumor progression, poor prognosis and resistance to chemotherapy. In CRC, Bcl-x<sub>L</sub> expression is correlated with an advanced disease stage<sup>[13]</sup>. A role of Bcl-x<sub>L</sub> in cancer was first suggested when it was found that expression of Her-2/Neu in breast cancer cells increased Bcl-x<sub>L</sub> expression and rendered cells resistant to tamoxifen-induced apoptosis<sup>[14]</sup>. Ectopic expression of Bcl-x<sub>L</sub> in CRC blocks curcumin-induced apoptosis<sup>[15]</sup>. On the other hand, downregulation of Bcl-x<sub>L</sub> by antisense technique induces cell death, e.g. after treatment with chemotherapeutic drugs<sup>[16,17]</sup>. An important trigger for Bcl-x<sub>L</sub> expression in CRC is NF-κB<sup>[18]</sup>. CRC cells frequently harbor genetic aberrations that promote NF-κB-mediated induction of Bcl-x<sub>L</sub>.

The last decade has ushered in new advances for the treatment of patients with CRC. The older cytotoxic chemotherapy drug 5-FU underwent new formulation, and two new drugs, oxaliplatin and irinotecan, were investigated as adjunctive therapies. Finally, targeted therapies, including monoclonal antibodies against VEGF-A (bevacizumab) and EGFR1 (cetuximab), are now standard treatment for metastatic CRC. For patients with metastatic disease, the survival rate has doubled. Among others, a promising approach to overcome apoptosis resistance of CRC cells is the engagement of the death receptors belonging to the tumor necrosis factor receptor gene superfamily with the death ligand TRAIL (Apo2L)<sup>[19]</sup>.

In our study, we investigated the role of the anti-apoptotic Bcl-2 family members Bcl-x<sub>L</sub> and Mcl-1 for the apoptosis sensitivity of CRC. Both Bcl-2 family proteins were specifically modulated in CRC cells by RNA interference and overexpression, respectively, and the impact on apoptosis sensitivity towards chemotherapy and targeted therapy including TRAIL and EGFR1 blockage was explored.

## MATERIALS AND METHODS

### Reagents and cell lines

SW480, HT29, Caco-2 (all isolated from primary tumor tissue) and SW620 (derived from lymph node metastasis), all human CRC cell lines (adenocarcinomas), were purchased from ATCC. Cell lines were cultured in RPMI 1640 (Invitrogen, Karlsruhe, Germany), supplemented with 10% fetal calf serum (FCS, Biochrom, Berlin, Germany), Pen/Strep (1%) (PAA Laboratories, Pasching, Austria), HEPES (1%) (Cambrex, Verviers, Belgium) and L-Glutamin (1%) (Cambrex). Cells were cultivated in reduced medium (FCS concentration decreased to 0.5%) in all experiments. Reagents were purchased from the following suppliers: chemotherapeutic agents from Sigma (Deisenhofen, Germany); TRAIL (with enhancer, applied in a concentration of 1 ng/mL) from Alexis Biochemicals (San Diego, CA, USA); PD168393 from Calbiochem (Schwalbach, Germany); Protein A (for co-treatment with anti-APO-1, in a concentration of 10 ng/mL) and EGF

from Sigma. Cetuximab was supplied by Merck Pharma (Darmstadt, Germany). Anti-APO-1 was kindly provided by Peter H. Krammer (German Cancer Research Center).

### Tissue samples

CRC tissue samples as well as non-neoplastic colorectal tissues were obtained from patients undergoing elective surgery for colorectal cancer at the University of Mainz. Analysis of CRC samples was approved by the local ethics committee. The morphological classification of the carcinomas was conducted according to WHO specifications. Tissues samples were used for immunohistochemical staining as well as for mRNA extraction.

### Immunohistochemical staining

Paraffin-embedded tissue sections (which all included carcinoma as well as normal epithelial cells in one section) were subjected to immunostaining, using a biotin/streptavidin-peroxidase technique (Vector Laboratories Inc., Burlingame, CA). They were deparaffinized in xylene and dehydrated in ethanol, and dried in a steamer with 10 mmol Na-citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked by incubating the slices for 5 min with 3.0% hydrogen peroxide at room temperature, followed by washing in TPBS (0.5% TWEEN in PBS). The sections were then incubated for 30 min at room temperature with TNB (1% BSA, 0.5% protein-blocking reagent in TBS) prior to an overnight incubation at 4°C with polyclonal rabbit Bcl-xS/L (clone S-18, Santa Cruz Biotechnology Inc., Santa Cruz, California) or polyclonal rabbit Mcl-1 (S-19, Santa Cruz). Both were diluted 1:160 in TNB. Bound antibody was detected using biotinylated anti-rabbit IgG secondary antibody (Vector) and streptavidin-peroxidase complex (Vector), followed by incubating with diaminobenzidine as substrate. Sections were counterstained with Mayer's haematoxylin. As negative controls, sections were incubated in the presence of nonimmunized rabbit IgG as first antibody.

### Viability test

Cell viability was determined by a colorimetric 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. CRC cell lines were seeded onto 12-well plates. On day 1 after seeding, cells were treated as indicated. 100 µL MTT (5 mg/mL) was added to each well. After 4 h incubation at 37°C the supernatant was discarded and cells were washed with PBS. For cell lysis 0.5 mL 1-propanol was added for another 20 min. After transfer of 100 µL of each sample to a flat-bottomed 96-well microtiter plate; the optical density was determined at 550 nm. The viability of "1" was defined as the absorbance obtained from mock transfected cells or untreated cells, respectively.

### Detection of apoptosis

CRC cell lines were seeded onto 12-well plates. On day 1 after seeding, cells were treated as indicated. Cells were then collected, washed, and resuspended in lysis buffer containing 0.1% (w/v) sodium citrate, 0.1% (v/v) Triton

X-100 and 50 µg/mL propidium iodide (Sigma). After overnight incubation at 4°C, nuclei from apoptotic cells were quantified by flow cytometry according to the method of Nicoletti *et al.*<sup>[20]</sup>.

### Cell lysis and Western blotting

Cell lysis and Western blotting were performed as described before<sup>[7]</sup>. Immunodetection was performed using the indicated primary antibodies: anti-Mcl-1 (S19) (Santa Cruz Biotechnology, Heidelberg, Germany), anti-Bcl-x<sub>L</sub> (Labvision/NeoMarkers, Warm Springs Blvd. Fremont, Canada), and mouse anti-α-Tubulin clone B-5-1-2 (Sigma).

### RNAi and transfection

For small interfering RNA (siRNA)-mediated knock down of *Mcl-1* and *Bcl-x<sub>L</sub>*, the following siRNA sequences were applied (MWG Biotech, Ebersberg, Germany): *Mcl-1*, 5'-aagaucaacagacguucucTT-3' (sense) and 5'-gagaacgucugugauacuuTT-3' (antisense). *Bcl-x<sub>L</sub>*, 5'-gcu ug-ggaaagaaugcaaTT-3' (sense) and 5'-uugcauuuuau cccaag-cAG-3' (antisense). As a non-silencing control, siRNA specific for green fluorescent protein (GFP) was used: 5'-ggcuacguccaggagcgaccTT-3' (sense) and 5'-ggg ggcuc-cuggacguagccTT-3' (antisense), where capitals represent deoxyribonucleotides and lower case letters represent ribonucleotides. SW480 cells were transiently transfected with Lipofectamin RNAiMAX (Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol and analyzed 24 h after transfection. For Mcl-1 and Bcl-x<sub>L</sub> expression, we applied specific expression vectors (pEF4<sub>Mcl-1</sub> or pcDNA3<sub>Bcl-xL</sub>) or the corresponding empty vectors (pEF4<sub>empty</sub> or pcDNA3<sub>empty</sub>, respectively), all kindly provided by Peter H. Krammer, German Cancer Research Center (Heidelberg, Germany). SW480 cells were transfected with plasmids using Transfectin (Biorad, München, Germany) according to the manufacturer's protocol.

### Real-time quantitative polymerase chain reaction (RT-QPCR)

To analyze RNAi efficiency, total RNA from CRC cells was extracted using RNeasy Mini Kit (Qiagen) 24 h after transfection of siRNA. One µg of total RNA was reverse transcribed using an oligo-dT primer with the Omniscript RT kit (Qiagen) and afterwards analyzed for specific mRNA expression by RT-QPCR using the QuantiTect SYBR Green PCR Kit (Qiagen) and the following primers: *Actin* forward: 5'-GGACTTCGAGCAAGAGAT GG-3', *Actin* reverse: 5'-AGCACTGTGTTGGCGTAC AG-3', *Mcl-1* forward: 5'-TAAGGACAAAACGGGACT GG-3', *Mcl-1* reverse: 5'-ACCAGCTCCTACTCCAGC AA-3'. *Bcl-x<sub>L</sub>* forward: 5'-GTAAACTGGGGTTCGC ATTGT-3', *Bcl-x<sub>L</sub>* reverse: 5'-TGCTGCATTGTTCCC ATAGA-3'. The relative increase in reporter fluorescent dye emission was monitored. The level of *Mcl-1* or *Bcl-x<sub>L</sub>* (gene of interest, GOI) mRNA, respectively, relative to actin, was calculated using the formula: Relative GOI mRNA expression =  $2 [C_t (GOI_{control}) - C_t (GOI_{treated}) + C_t (Actin_{treated}) - C_t (Actin_{control})]$ , where  $C_t$

is defined as the number of the cycle in which emission exceeds an arbitrarily defined threshold. For evaluation of *Bcl-x<sub>L</sub>* and *Mcl-1* mRNA expression in tumor as well as non-neoplastic colon tissues, *RP II* instead of actin was measured as housekeeping gene: *RP II* forward: 5'-GCACCACGTCCAATGACAT-3', *RP II* reverse: 5'-GTGCGGCTGCTTCATAA-3'.

### Statistical analysis

All results are expressed as mean  $\pm$  SD. Data were analyzed by Student's *t*-test (paired, two sided). *P* < 0.05 was considered significant.

## RESULTS

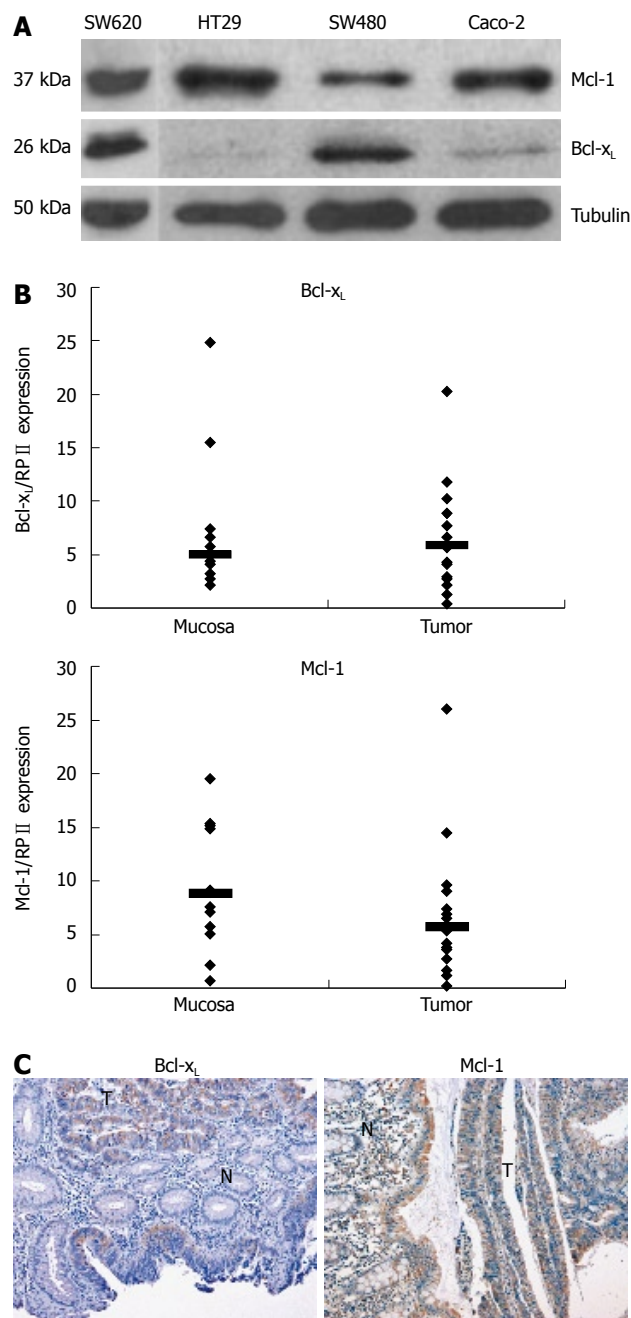
### Expression of the anti-apoptotic Bcl-2 family members Bcl-x<sub>L</sub> and Mcl-1 in CRC

Apoptosis resistance is a well-known phenomenon which counteracts chemotherapeutic drug-induced cell death of CRC cells. Anti-apoptotic Bcl-2 family members such as Bcl-x<sub>L</sub> and Mcl-1 contribute to the apoptosis resistance in different tumor entities. First, we analyzed expression of Bcl-x<sub>L</sub> and Mcl-1 in various CRC cell lines. All cell lines tested showed a profound expression of Mcl-1 on protein level (Figure 1A). Bcl-x<sub>L</sub> expression was rather low in HT29 and Caco-2 and high in SW480 cells (Figure 1A).

Next, we analyzed expression of *Bcl-x<sub>L</sub>* and *Mcl-1* mRNA in human CRC tissues by quantitative PCR. Bcl-x<sub>L</sub> levels were higher in CRC tissues compared to non-malignant, adjacent tissue (Median of relative expression: 1.2, *n* = 9, *P* < 0.2, not significant, Figure 1B). Six of 9 patients showed a higher *Bcl-x<sub>L</sub>* expression, 2 patients showed a lower expression, and in 1 patient, expression was virtually equal. *Mcl-1* mRNA expression was significantly lower in carcinoma tissue compared to non-malignant tissue (Median of relative expression: 0.41, *n* = 9, *P* < 0.01). In addition, we performed immunohistochemical analysis of Bcl-x<sub>L</sub> and Mcl-1 in CRC. In all tissues tested (*n* = 6), expression of Bcl-x<sub>L</sub> was profoundly higher in carcinoma cells compared to surrounding epithelial cells (Figure 1C). Furthermore, Mcl-1 expression was also (slightly) higher compared to surrounding epithelial cells in all probes tested (*n* = 4).

### Sensitivity of Bcl-x<sub>L</sub> and Mcl-1 expressing CRC cells towards chemotherapeutic drug-induced apoptosis and EGFR1 inhibition

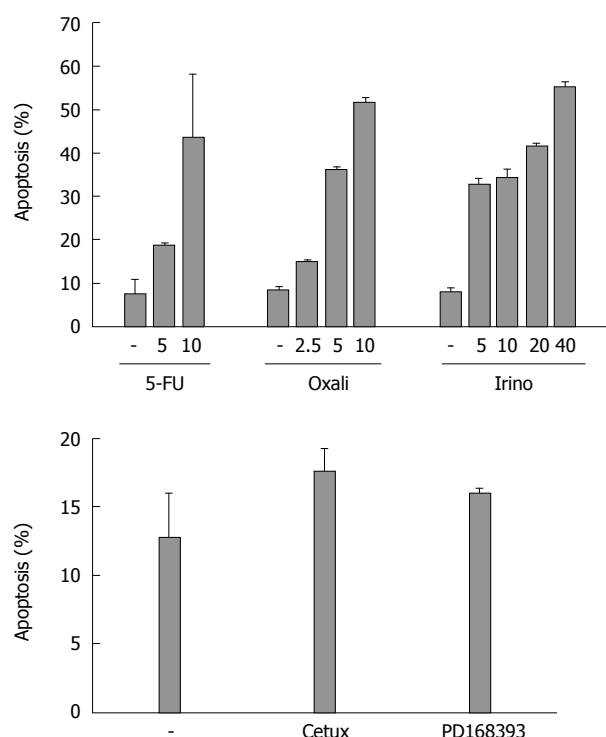
Subsequently, we tested the sensitivity of CRC cell lines towards chemotherapeutic drug-induced apoptosis. We treated SW480 cells with different agents frequently applied for the treatment of patients with CRC: the chemotherapeutic agents irinotecan, oxaliplatin and 5-FU (Figure 2). After 48 h, oxaliplatin (10  $\mu$ g/mL) and irinotecan (40  $\mu$ g/mL) induced apoptosis in more than 50% of the cells. 5-FU (10  $\mu$ g/mL) induced apoptosis in nearly 45% of CRC cells. Treatment with the antagonistic EGFR1 antibody cetuximab induced apoptosis in 18% of cells after 48 h (compared to 13% of apoptosis



**Figure 1** Mcl-1 and Bcl-x<sub>L</sub> expression in CRC. **A:** The CRC cell lines, HT29, SW620, SW480, and Caco-2, were analyzed for the basal expression of the Bcl-2 family members Bcl-x<sub>L</sub> and Mcl-1. Whole cell lysates were prepared, separated, and immunoblotted with antibodies against Bcl-x<sub>L</sub>, Mcl-1 and  $\alpha$ -tubulin; **B:** CRC tissues and normal colorectal tissues were tested for mRNA expression (*n* = 9 patients). mRNA expression levels of *Bcl-x<sub>L</sub>*, *Mcl-1* and *RP II* were measured in all tissue samples by quantitative real-time PCR. mRNA expression levels of *Bcl-x<sub>L</sub>* or *Mcl-1* were normalized to *RP II* in each sample. Each PCR reaction was run in triplicates. Median is added; **C:** Immunohistochemical analysis of human CRC tissues was performed as described in the Methods section. All sections included carcinoma as well as normal epithelial tissues to directly compare Bcl-x<sub>L</sub> as well as Mcl-1 expression in neoplastic and non-malignant tissues. Representative analysis of immunoperoxidase detection of Bcl-x<sub>L</sub> and Mcl-1 in paraffin embedded carcinoma tissue (T) and adjacent non-tumor tissue (N) is presented.

in control cells, *P* = 0.07, Figure 2). Apoptosis induction in cells treated with the EGFR1 tyrosine kinase inhibitor PD168393 was not significant (16% vs 13%, *P* = 0.15).





**Figure 2** Drug-induced apoptosis in CRC cells. SW480 cells were treated with the chemotherapeutic agents 5-FU, oxaliplatin ("oxali") and irinotecan ("irino") for 48 h (concentrations as indicated, upper panel). In addition, cells were treated with the EGFR1 antibody cetuximab ("cetux", 20 µg/mL) or the EGFR1 tyrosine kinase inhibitor PD168393 (7 µmol/L, lower panel) in FCS reduced medium. Cells were then harvested and analyzed for apoptosis induction by flow cytometry. Assays were performed in triplicates. Values are mean  $\pm$  SD.

### Modulation of Bcl-x<sub>L</sub> and Mcl-1 expression and its impact on chemotherapeutic drug-induced apoptosis

In order to analyze the functional contribution of Bcl-x<sub>L</sub> and Mcl-1 on apoptosis sensitivity of CRC cells, we specifically modulated their expression. Transfection of specific siRNA sequences effectively knocked down expression of Bcl-x<sub>L</sub> and Mcl-1 mRNA and protein in SW480 cells (Figures 3A and 4A). For example, 24 h after transfecting 20 nmol/L of specific siRNAs, mRNA expressions were reduced by 75% or 74%, respectively. For 10, 20 and 40 nmol/L of siRNA concentration, an efficient knock down of Bcl-x<sub>L</sub> and Mcl-1 was observed after 24 h (data not shown). As could be detected in Western blot assays, Bcl-x<sub>L</sub> and Mcl-1 protein expression were also drastically reduced 24 h after siRNA transfection. On the other hand, transfection of expression plasmids for Bcl-x<sub>L</sub> increased protein expression (Figure 3C). Next, we tested the effect of specific knock down of Mcl-1 *vs* Bcl-x<sub>L</sub> on apoptosis sensitivity of SW480 cells. Knock down of Bcl-x<sub>L</sub> significantly enhanced apoptosis induction in untreated cells compared to control transfected cells (21% to 38%,  $P < 0.05$ , Figure 3B). These results were confirmed in viability assays: Viability was decreased by 33% (Figure 3B). In contrast, Mcl-1 knock down only moderately enhanced spontaneous apoptosis rates (20% to 25%,  $P < 0.05$ , Figure 4B). Correspondingly, viability was not decreased in CRC cells after Mcl-1 knock down (Figure 4B).

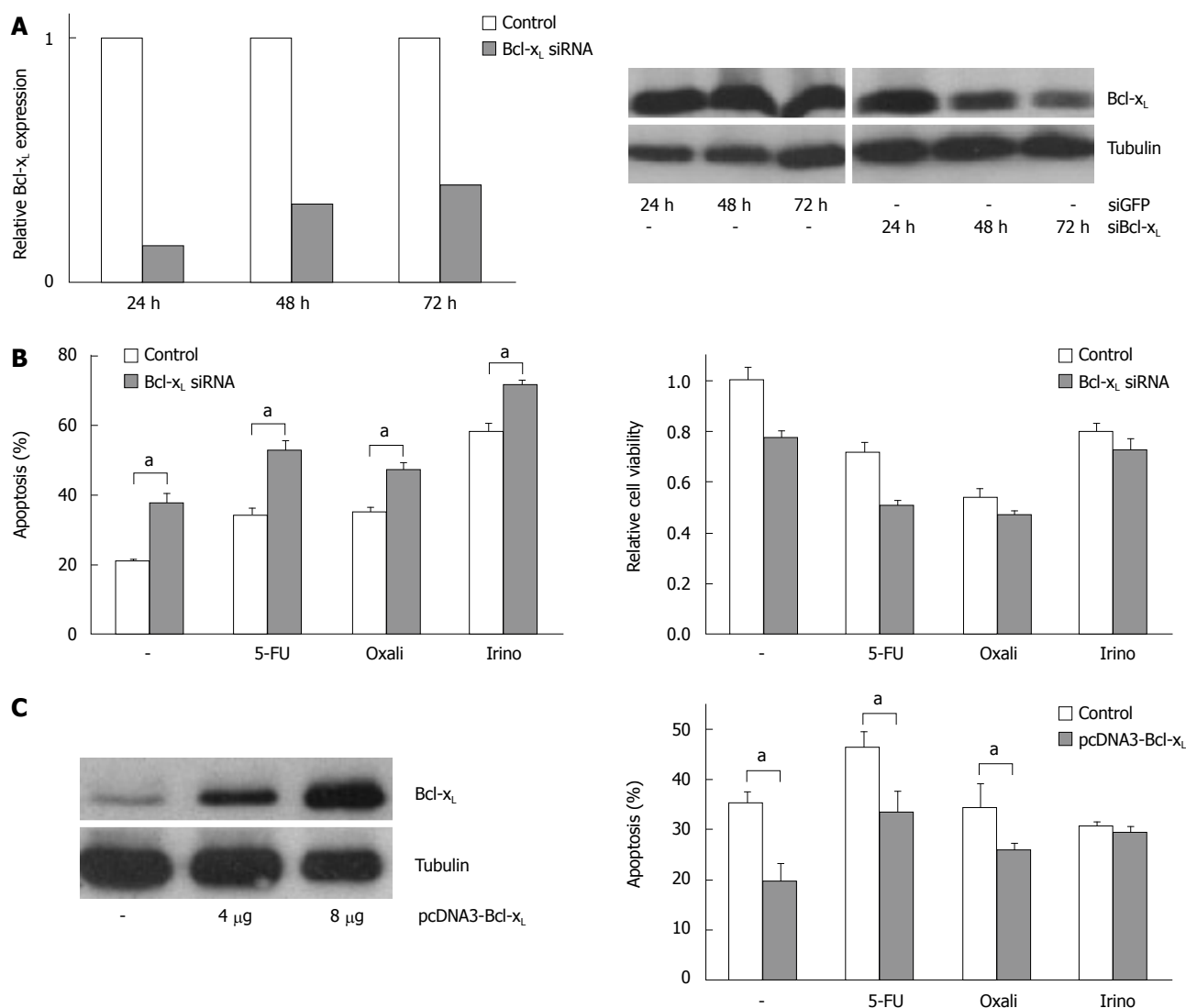
Subsequently, we treated CRC cells with the chemotherapeutic agents 5-FU, oxaliplatin and irinotecan. Knock down of Bcl-x<sub>L</sub> in combination with chemotherapy resulted in an increase of apoptosis induction (5-FU: 34% (chemotherapy alone) to 53% (chemotherapy plus Bcl-x<sub>L</sub> knock down); oxaliplatin: 35% to 47% and irinotecan: 58% to 72%,  $P < 0.05$ , Figure 3B). Silencing of Mcl-1 expression led to a slight enhancement of irinotecan-induced apoptosis (33% to 35%, not significant, Figure 4B) and to a moderate, but significant increase of oxaliplatin-induced apoptosis (27% to 33%,  $P < 0.05$ ). Surprisingly, 5-FU-induced apoptosis was decreased in cells with lower Mcl-1 expression (32% to 28%, not significant, Figure 4B). Transfection of the Bcl-x<sub>L</sub> expression plasmid enhanced viability of CRC cells: spontaneous apoptosis rates were 20% instead of 36% in control transfected cells ( $P < 0.05$ , Figure 3C). Moreover, 5-FU (33% *vs* 46%,  $P < 0.05$ ), and irinotecan (26% *vs* 34%,  $P < 0.05$ )-induced apoptosis, but not oxaliplatin-induced apoptosis (29% *vs* 30%, n.s.), was reduced by transfecting Bcl-x<sub>L</sub> (Figure 3C). After transfection of Mcl-1 expression plasmids, no significant impact on chemotherapeutic-drug-induced apoptosis was observed (data not shown).

### Modulation of Bcl-x<sub>L</sub> and Mcl-1 expression and its impact on EGFR1 blockage

We next analyzed apoptosis induction in CRC cells after targeted therapy approaches. Inhibition of EGFR1 signalling has already entered clinical routine in the treatment of patients with CRC. Cetuximab, a monoclonal antibody against EGFR1, demonstrates anti-tumor efficacy both as a single agent and in combination with irinotecan- and oxaliplatin-based chemotherapy. EGF is known to contribute to an apoptosis resistant phenotype of carcinoma cells. First, we tested the influence of EGF treatment on Bcl-x<sub>L</sub> and Mcl-1 expression *in vitro*. EGF treatment of CRC cells induced expression of Bcl-x<sub>L</sub> and Mcl-1 after 1.5 and 2.5 h, respectively (Figure 5A). We next analyzed the role of Mcl-1 and Bcl-x<sub>L</sub> for the resistance towards targeted therapy. First, we treated CRC cells with cetuximab. Treatment with cetuximab alone (100 µg/mL) did not induce apoptosis SW480 cells after 24 h (Figure 5B). However, in cells with reduced Bcl-x<sub>L</sub>, a significant increase in apoptosis induction after treatment with cetuximab (44% *vs* 37%,  $P < 0.05$ , Figure 5B) or the EGFR1 tyrosine kinase inhibitor PD168393 (0.7 µmol/L; 41% *vs* 29%,  $P < 0.01$ , Figure 5B) was observed. Moreover, knock down of Mcl-1 also resulted in a moderate, but significant sensitization towards cetuximab (28% *vs* 23%; Figure 5B,  $P < 0.01$ ) and PD168393 (32% *vs* 24%, Figure 5B,  $P < 0.05$ ).

### Modulation of Bcl-x<sub>L</sub> and Mcl-1 expression and its impact on TRAIL- and CD95-mediated apoptosis

The death receptor ligand TRAIL is a promising anti-cancer agent (for recent review<sup>[21]</sup>) and already has been tested in clinical studies in CRC patients. Thus, we analyzed the impact of Bcl-x<sub>L</sub> and Mcl-1 modulation



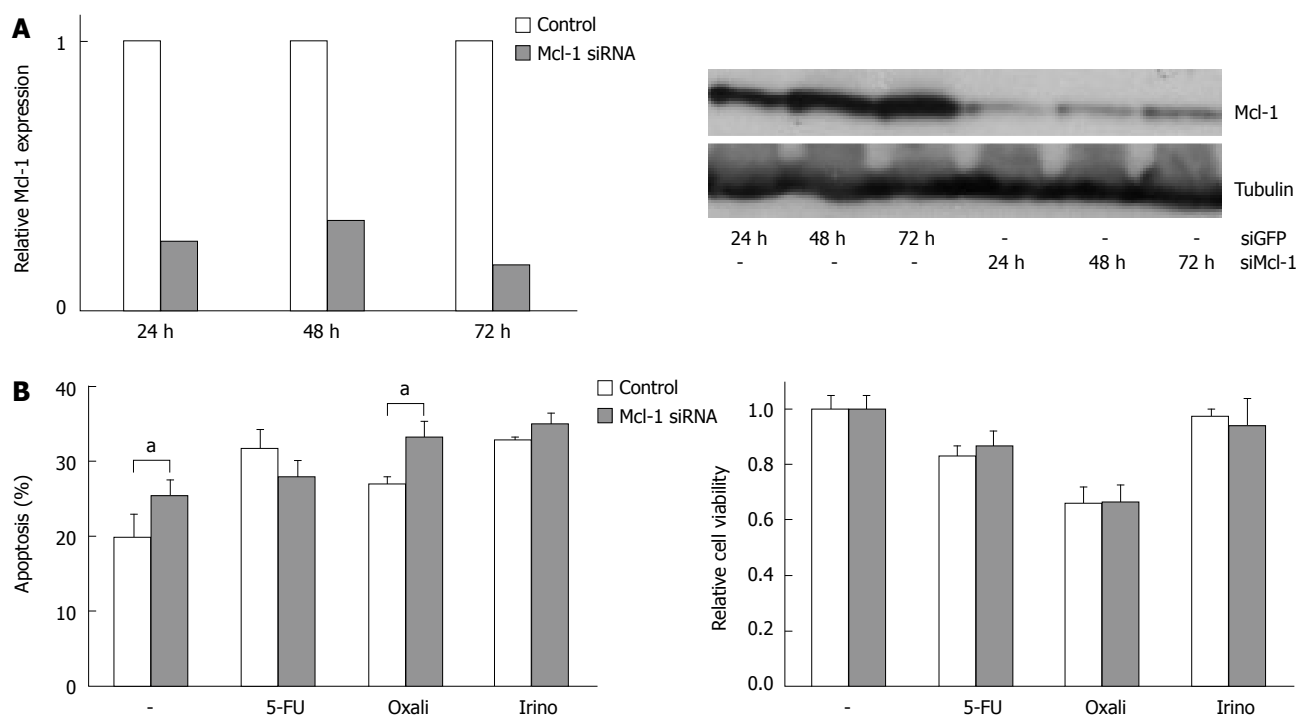
**Figure 3** Modulation of Bcl-x<sub>L</sub> expression alters chemotherapeutic drug-induced apoptosis. **A:** SW480 cells were transfected with siRNA specific for Bcl-x<sub>L</sub> or transfected with siRNA specific for GFP as control (20 nmol/L). After the indicated time post transfection, total RNA was extracted and analyzed for Bcl-x<sub>L</sub> expression by quantitative real-time PCR (left panel). Relative expression was calculated as described in the materials and methods section. In addition, after the indicated time post transfection, cells were lysed and analyzed for Bcl-x<sub>L</sub> expression by Western blot (right panel).  $\alpha$ -Tubulin expression was used to control equal loading; **B:** SW480 cells were transfected with siRNA specific for Bcl-x<sub>L</sub> or transfected with siRNA specific for GFP as control. 24 h post transfection, cells were treated with 5-FU (10  $\mu$ g/mL), oxaliplatin (16  $\mu$ g/mL) and irinotecan (60  $\mu$ g/mL) for further 24 h. Cells were then harvested and analyzed for apoptosis induction (left panel). In addition, cell viability was measured by MTT assay and is shown relative to mock treated controls (right panel); **C:** SW480 cells were transfected with pcDNA3 Bcl-x<sub>L</sub> or pcDNA3 empty vector as control. 24 h post transfection, cells were analyzed for Bcl-x<sub>L</sub> expression using Western blotting (left panel). In addition, 24 h post transfection, cells were treated with 5-FU (15  $\mu$ g/mL), oxaliplatin (10  $\mu$ g/mL) and irinotecan (40  $\mu$ g/mL) for further 24 h. Cells were then harvested and analyzed for apoptosis induction by flow cytometry. (B) and (C) assays were performed in triplicates. Values are means  $\pm$  SD, \* $P$  < 0.05.

on TRAIL-induced apoptosis of CRC cells. TRAIL efficiently induced apoptosis in SW480 cells (22% *vs* 3% apoptosis in control cells,  $P$  < 0.05, Figure 6A). Importantly, Bcl-x<sub>L</sub> downregulation enhanced TRAIL-induced apoptosis more than two-fold (46% *vs* 22%,  $P$  < 0.001, Figure 6A). To further evaluate the sensitizing effect of Bcl-x<sub>L</sub> knock down for TRAIL efficacy, we included another CRC cell line, SW620, in our study. After Bcl-x<sub>L</sub> knock down, TRAIL-induced apoptosis was increased from 16% to 27% ( $P$  < 0.05, Figure 6A). Mcl-1 knock down sensitized SW480 cells to TRAIL (17% *vs* 11%,  $P$  < 0.05, Figure 6B). Upregulation of Bcl-x<sub>L</sub> by transfection of expression plasmids decreased TRAIL-induced cell death ( $P$  < 0.05, Figure 6C). Subsequently, we tested the effect of Bcl-x<sub>L</sub> and Mcl-1 knock down on CD95-

induced apoptosis of CRC cells (Figure 6D). SW480 cells were highly susceptible to CD95 stimulation by the agonistic CD95 antibody anti-APO-1 (65% *vs* 3% after 24 h of anti-APO-1 treatment, Figure 6D). Bcl-x<sub>L</sub> knock down further increased anti-APO-1 induced apoptosis from 65% to 80% ( $P$  < 0.001). Mcl-1 knock down did not further enhance CD95-triggered apoptosis of CRC cells (Figure 6D).

## DISCUSSION

In the present study we demonstrate an important role of the anti-apoptotic Bcl-2 family members Bcl-x<sub>L</sub> and, to a lower extent, Mcl-1, for the apoptosis sensitivity of CRC cells. Bcl-x<sub>L</sub> expression is considerably enhanced in



**Figure 4** Modulation of Mcl-1 expression alters chemotherapeutic drug-induced apoptosis. **A:** SW480 cells were transfected with siRNA specific for Mcl-1, or transfected with siRNA specific for GFP as control (20 nmol/L). After the indicated time post transfection, total RNA was extracted and analyzed for Mcl-1 expression by quantitative real-time PCR (left panel). Relative expression was calculated as described in the materials and methods section. In addition, after the indicated time post transfection, cells were lysed and analyzed for Mcl-1 expression by Western blot (right panel).  $\alpha$ -Tubulin expression was used to control equal loading; **B:** SW480 cells were transfected with siRNA specific for Mcl-1 or transfected with control siRNA. 24 h post transfection cells were treated with 5-FU (10  $\mu$ g/mL), oxaliplatin (16  $\mu$ g/mL) and irinotecan (60  $\mu$ g/mL) for further 24 h. Apoptosis induction was measured by flow cytometry (left panel) and MTT assay (right panel). Values are mean  $\pm$  SD,  $^aP < 0.05$ .

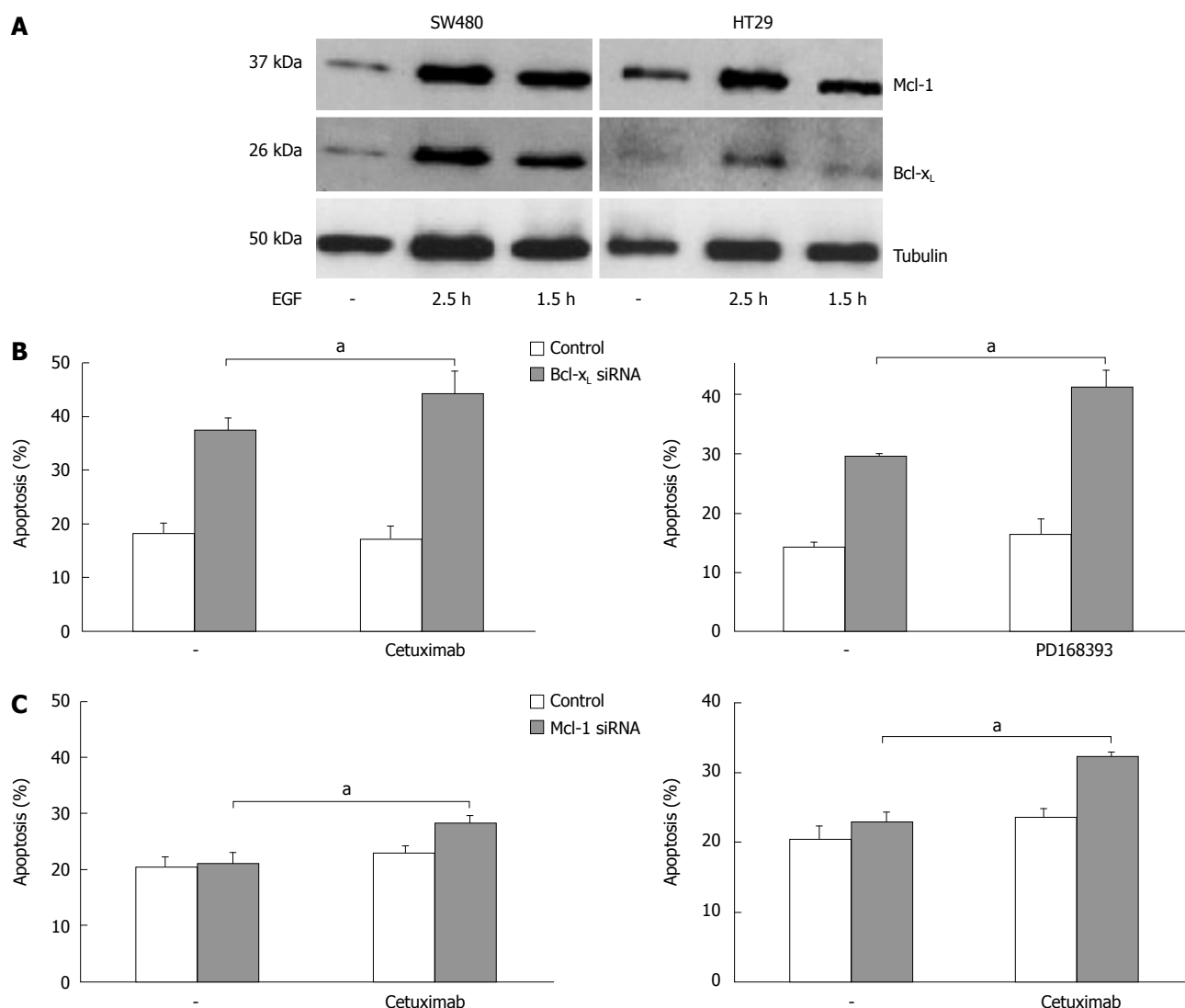
CRC tissue compared to adjacent non-malignant tissue. After knock down of Bcl-x<sub>L</sub> by RNA interference, CRC cells prove to be more sensitive towards chemotherapy, EGFR1 blockage, CD95 triggering and treatment with the death receptor ligand TRAIL. The sensitizing effect of Mcl-1 knock down is comparatively moderate. Our data suggest, that Bcl-2 family members, such as Bcl-x<sub>L</sub>, are promising targets to improve treatment of patients with CRC. Since strategies to inhibit Bcl-x<sub>L</sub> activity have already been applied in preclinical studies, our data are of particular interest<sup>[22]</sup>.

In the past two decades, substantial progress has been made in the treatment of colon cancer, the second most common cancer in western countries. However, therapy resistance of CRC remains a common clinical problem, so that recurrence and metastasis of CRC remain major obstacles in oncology. Thus, new strategies to overcome resistance to current treatment options are needed.

Numerous defects in apoptosis signalling have been described in CRC. These defects appear to be involved in colorectal tumorigenesis, by facilitating tumor cell progression<sup>[23]</sup>. In addition, defects in apoptosis signalling represent principle mechanisms through which cancer cells are enabled to survive therapy, since chemotherapy and irradiation induce cell death mainly by apoptosis induction<sup>[24]</sup>. These defects include, among others, stabilization of mitochondria, inactivation of death receptor signalling and overexpression of EGFR1<sup>[2]</sup>.

Anti-apoptotic proteins of the Bcl-2 family, such as Bcl-2, Bcl-x<sub>L</sub> and Mcl-1 critically regulate mitochondrial integrity. Increased expression of anti-apoptotic Bcl-2 proteins counteracts chemotherapeutic drug-induced apoptosis in cancer cells. In our study, human CRC tissues revealed enhanced Bcl-x<sub>L</sub> expression both on mRNA and protein levels. In line with our study, previous studies also observed enhanced Bcl-x<sub>L</sub> expression in CRC tissues<sup>[13,25]</sup>. In contrary, Northern blot as well as immunohistochemical analysis failed to detect Bcl-2 expression in CRC<sup>[25]</sup>. The reason for increased Bcl-x<sub>L</sub> expression in CRC remains elusive. However, a consistent finding is that oncogenic tyrosine kinases induce expression of Bcl-x<sub>L</sub> and enhance protein stability. Among these tyrosine kinases is EGFR1, which has been described to up-regulate Bcl-x<sub>L</sub> in other tumor models<sup>[26]</sup>. In line with this observation, we show a significant upregulation of Bcl-x<sub>L</sub> in CRC cells upon treatment with EGF in this study. Since overexpression of EGFR1 is a frequent finding in CRC cells, EGFR1 signalling may represent a major cause for the induction of Bcl-x<sub>L</sub> expression in CRC. Another mechanism which triggers Bcl-x<sub>L</sub> activity in tumor cells is suppression of deamidation<sup>[27]</sup>. In a recent immunohistochemical evaluation of human CRC tissues, hypoxia-inducible factor-1 has been discussed to induce expression of Bcl-x<sub>L</sub><sup>[28]</sup>.

We also explored expression of the anti-apoptotic protein Mcl-1 in CRC. In other cancer entities, e.g. in hepatocellular carcinoma, a significant correlation of



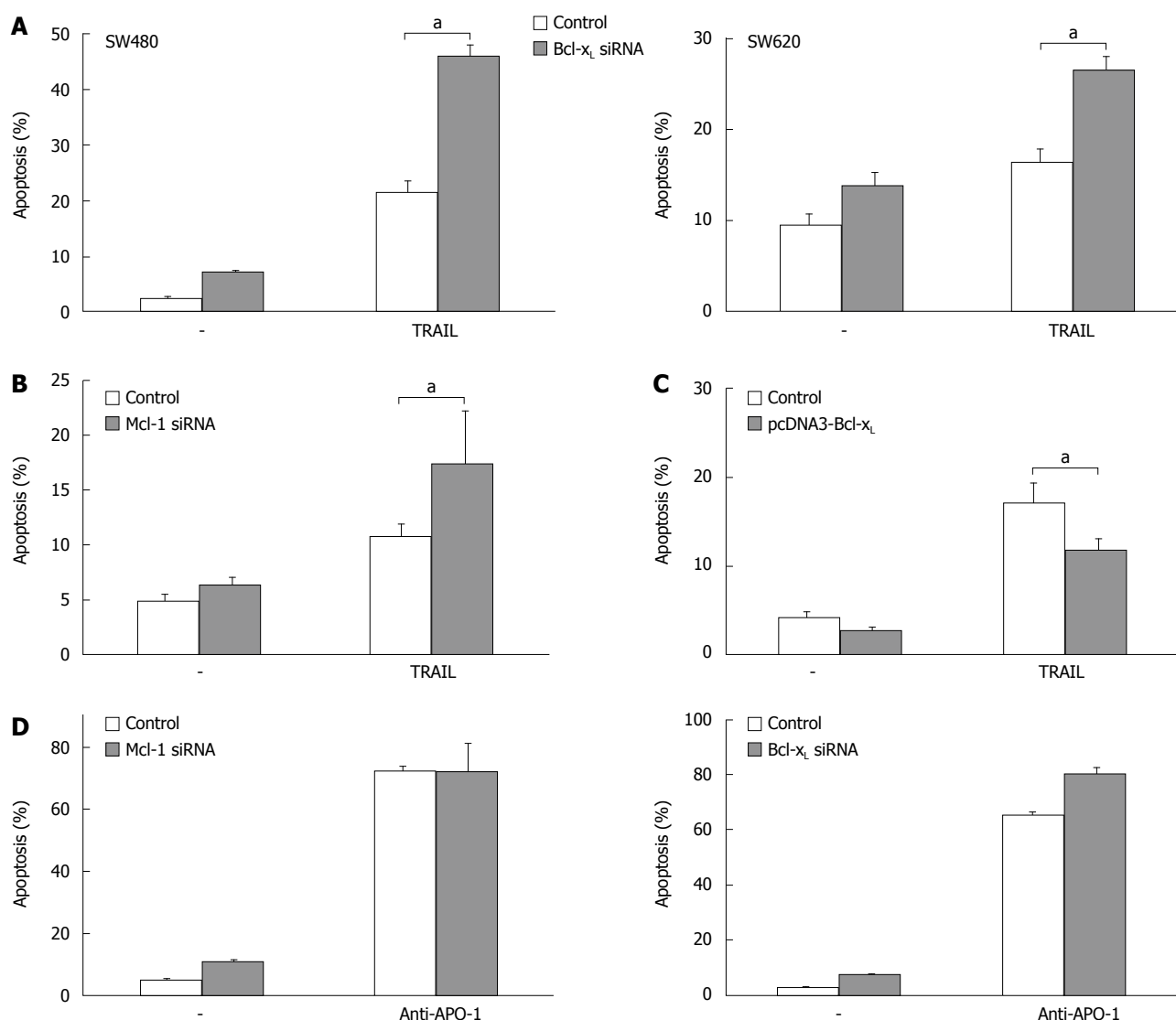
**Figure 5** Bcl-x<sub>L</sub> and Mcl-1 knock down enhances apoptosis induction after EGFR1 inhibition. **A:** SW480 and HT29 cells were treated with EGF (100 ng/mL) for the time indicated. Whole cell lysates were prepared, separated, and immunoblotted with antibodies against Bcl-x<sub>L</sub>, Mcl-1 and  $\alpha$ -tubulin; **B:** SW480 cells were transfected with siRNA specific for Bcl-x<sub>L</sub> (upper panel), or Mcl-1 (lower panel), respectively, or transfected with siRNA specific for GFP as control. 24 h post transfection cells were treated with cetuximab (100  $\mu$ g/mL) or PD168393 (0.7  $\mu$ mol/L) for further 24 h. Cells were then harvested and analyzed for apoptosis induction by flow cytometry. Assays were performed in triplicates and are representative for at least two independent experiments. Values are means  $\pm$  SD, <sup>a</sup> $P < 0.05$ .

Bcl-x<sub>L</sub> and Mcl-1 with apoptosis resistance was observed<sup>[29]</sup>. In our study, *Mcl-1* mRNA expression was significantly lower in CRC tissue compared to non-neoplastic cells. In contrast, no profound difference was observed on protein level in immunohistochemistry. Previous studies, however, observed decreased Mcl-1 expression relative to normal mucosa and adenomas, also in immunohistochemistry<sup>[13]</sup>. At the same time, decrease in Mcl-1 expression has been discussed as a later event in the progression of colorectal tumors, since adenomas show no decreased Mcl-1 expression<sup>[13]</sup>. The significant difference in *Mcl-1* expression we observed on mRNA level, is in line with these previously published data. Another important aspect about Mcl-1 expression is the staining pattern: in contrast to normal mucosa cells, Mcl-1 has been described to be diffusely expressed in tumour samples in previous studies<sup>[30]</sup>. Such a diffuse expression could also be detected in our study.

Next, we confirmed that Mcl-1 has a relatively short

half-life in CRC cells. Inhibition of translational events in different CRC cell lines resulted in a much more rapid decrease in Mcl-1 compared to Bcl-x<sub>L</sub> expression. This is in line with the well-known fact that Mcl-1 provides short-term cell viability protection against cell death during critical transitions in the cell fate<sup>[6]</sup>. The rapid decrease of Mcl-1 expression can be explained mainly by efficient proteasomal degradation. In addition, we could show that Mcl-1 expression is induced upon treatment with EGF. This might be mediated at least in part by activation of the MEK/ERK-pathway. In lung cancer, it has been shown that EGF enhanced Mcl-1 protein level in an ERK-dependent manner<sup>[8]</sup>. In our study, EGF also induced expression of Bcl-x<sub>L</sub> and ERK might also be involved in this context. Chemotherapeutic agents such as oxaliplatin have been shown to decrease anti-apoptotic proteins like Bcl-x<sub>L</sub> in SW480 cells<sup>[31]</sup>. This effect at least in part explains the apoptosis-inducing capacity of oxaliplatin. However, other chemotherapeutic agents such as





**Figure 6** TRAIL- and CD95-induced apoptosis after modulation of Mcl-1 and Bcl-x<sub>L</sub> expression. **A:** SW480 or SW620 cells were transfected with siRNA specific for Bcl-x<sub>L</sub> or GFP as control. 24 h post transfection cells were treated with TRAIL (0.1 µg/mL) for further 24 h. Cells were then harvested and analyzed for apoptosis induction by flow cytometry; **B:** SW480 cells were transfected with siRNA specific for Mcl-1 or GFP as control. 24 h post transfection cells were treated with TRAIL (0.01 µg/mL) for further 24 h. Cells were then harvested and analyzed for apoptosis induction by flow cytometry; **C:** SW480 cells were transfected with pcDNA3 Bcl-x<sub>L</sub> or pcDNA3 empty vector as control. 24 h post transfection, cells were treated with TRAIL (0.1 µg/mL) for further 12 h. Cells were then harvested and analyzed for apoptosis induction by flow cytometry; **D:** SW480 cells were transfected with siRNA specific for Mcl-1 (left panel) or Bcl-x<sub>L</sub> (right panel) or with control siRNA. 24 h post transfection cells were treated with anti-APO-1 (0.1 µg/mL) for further 24 h. Cells were then harvested and analyzed for apoptosis induction by flow cytometry. All assays were performed in triplicates and are representative for at least three independent experiments. Values are mean ± SD, <sup>a</sup>*P* < 0.05.

5-FU and paclitaxel did not downregulate Bcl-x<sub>L</sub> in CRC cells in previous studies<sup>[32]</sup>.

Our results demonstrate that a specific knock down of Bcl-x<sub>L</sub> and, to a lower extent, knock down of Mcl-1 by RNAi sensitize CRC cells to chemotherapeutic drugs frequently applied in CRC therapy (5-FU, oxaliplatin and irinotecan). Notably, Bcl-x<sub>L</sub> knock down alone already significantly induces apoptosis in untreated cells. Thus, Bcl-x<sub>L</sub> expression is important for the survival of CRC cells. Our results extend studies which have already shown that knock down of Bcl-x<sub>L</sub> effectively blocks proliferation of CRC cells<sup>[33]</sup>. On the other hand, overexpression of Bcl-x<sub>L</sub> counteracts chemotherapeutic drug-induced apoptosis. In previous studies, overexpression of Bcl-x<sub>L</sub> has been reported to enhance resistance to various chemotherapeutic agents in leukaemia cells<sup>[34]</sup>.

However, in a previous study, resistance of CRC cells was not observed in Bcl-x<sub>L</sub> overexpressing cells treated with 5-FU or TRAIL<sup>[33]</sup>. In our study, Bcl-x<sub>L</sub> overexpression only reduced apoptosis rates in CRC cells treated with 5-FU, irinotecan and oxaliplatin.

Bcl-x<sub>L</sub> exerts anti-apoptotic effects in cancer cells mainly by its interaction with pro-apoptotic Bcl-2 family members, e.g. Bax and Bak. Activation of Bax and Bak commit the cell to apoptosis by permeabilizing the outer mitochondrial membrane. However, interaction with Bcl-2 proteins such as Bcl-x<sub>L</sub> and Mcl-1 ablates pro-survival functions of Bax and Bak<sup>[35]</sup>. Bax expression is not reduced in CRC tissues<sup>[25]</sup>. Thus, Bcl-x<sub>L</sub> silencing might induce apoptosis *via* release of Bax and concomitant mitochondrial permeabilization. However, more studies are required to fully understand the roles of the

Bcl-2 proteins and how they cooperate to regulate CRC cell survival.

The apoptosis-sensitizing effect of Mcl-1 modulation was less pronounced (e.g. for oxaliplatin and irinotecan-induced apoptosis) or not significant (e.g. for 5-FU-induced apoptosis), respectively. The reason might be the relatively low expression of Mcl-1 in CRC cells, which may at least in part be compensated by a higher Bcl-x<sub>L</sub> expression. Nevertheless, Mcl-1 knock down has sensitizing effects in our study. This effect may also be explained by the fact that Mcl-1, like Bcl-x<sub>L</sub>, guards Bax and Bak and thereby prevents them to activate mitochondria.

Notably, Bcl-x<sub>L</sub> knock down by siRNA also enhances death receptor-mediated apoptosis in CRC cells. CD95-mediated apoptosis was increased by Bcl-x<sub>L</sub> knock down in SW480 cells. However, this effect is supposed to be less relevant for the *in vivo* situation, since no CD95 expression has been detected in CRC tissues in previous studies<sup>[30]</sup>. Remarkably, Bcl-x<sub>L</sub> knock down considerably enhanced tumor necrosis factor alpha (TNF-alpha)-related apoptosis-inducing ligand (TRAIL)-induced apoptosis of SW480 as well as SW620 cells. TRAIL is a member of the TNF family, which has been reported to induce apoptosis in various tumor cells, but not in normal cells, thus representing a promising anticancer agent<sup>[36]</sup>. Agonistic TRAIL receptor antibodies have already entered clinical trials<sup>[37]</sup>. Recently, we have shown that treatment with TRAIL alone or in combination with chemotherapeutic drugs (with the exception of cisplatin) is not toxic for human hepatocytes<sup>[38]</sup>. In our study, treatment of CRC cells with TRAIL resulted in relatively low apoptosis rates. This is in line with previous studies on CRC cells, e.g. on SW620 cells<sup>[39]</sup> or HT29 cells<sup>[40]</sup>. The reason for restricted apoptosis induction is that TRAIL signalling also involves activation of anti-apoptotic pathways including PI3K/Akt, NF-κB and MEK/ERK<sup>[41]</sup>. NF-κB activation, for example, induces anti-apoptotic proteins such as Mcl-1 in HT29 cells, contributing to TRAIL resistance<sup>[39]</sup>. Bcl-x<sub>L</sub> has also been shown to be upregulated by NF-κB activity<sup>[42]</sup>.

Several approaches have been exploited to sensitize cancer cells towards TRAIL. An important strategy, also pursued in this study, is downregulation of anti-apoptotic Bcl-2 proteins. In the present study, Bcl-x<sub>L</sub> knock down by siRNA efficiently sensitized CRC cells towards TRAIL-induced apoptosis. In contrary, Mcl-1 knock down only slightly sensitized CRC cells to TRAIL. These data correspond to previous studies of our group and others on hepatocellular carcinoma, where Mcl-1 knock down did not sensitize towards TRAIL<sup>[9]</sup>. However, in cholangiocellular carcinoma, where high Mcl-1 expression is frequently found, Mcl-1 knock down renders cancer cells susceptible to TRAIL<sup>[11]</sup>. Since TRAIL-induced apoptosis in cancer cells is hampered by NF-κB activation, inhibition of NF-κB is likely to augment TRAIL-induced death of CRC cells. Approaches to block NF-κB are, among others, peptidomimetic compounds that disrupt the IKK complex or multikinase inhibitors, such as sorafenib<sup>[43]</sup>.

The epidermal growth factor receptor (EGFR1) is

a receptor tyrosine kinase of the ErbB family that is abnormally activated in many epithelial tumors, such as CRC. EGFR1 is involved in survival signalling, cell migration, metastasis formation, angiogenesis, and reduced responses to chemotherapy. Clinical and survival benefits with anti-EGFR1 agents have been demonstrated in tumor patients (for review<sup>[44]</sup>). Monoclonal antibodies to EGFR1 are among promising novel targeted therapies being explored in CRC. One such agent that inhibits EGFR1 signalling by interfering with ligand-binding is cetuximab. Cetuximab is a human-mouse chimeric therapeutic monoclonal antibody that competitively binds to the extracellular domain of EGFR1. EGFR1 tyrosine kinase inhibitors, such as PD168393, also block EGFR1 signalling. In this study, knock down of Mcl-1 slightly sensitized CRC cells towards cetuximab and PD168393. Moreover, knock down of Bcl-x<sub>L</sub> sensitized CRC cells towards cetuximab as well as PD168393. These findings suggest that combining EGFR1 blockage with agents that directly destabilize or disable Bcl-x<sub>L</sub> and Mcl-1 will have therapeutic benefits.

The development of siRNA technology has made it possible to suppress the function of specific molecules and helps to develop new treatment strategies for cancer<sup>[45]</sup>. Our study suggests that Bcl-x<sub>L</sub> and Mcl-1 are suitable targets to sensitize CRC cells to death. The delivery of siRNA *in vivo* including specific uptake in tumor cells remains a challenging issue<sup>[46]</sup>. Many approaches use plasmid and viral vectors for transcription of short-hairpin RNAs, both *in vitro* and *in vivo*. However, human trials are still on the way to optimize delivery techniques. Another approach to specifically knock down Bcl-x<sub>L</sub> expression is by antisense oligonucleotides. Bispecific antisense oligonucleotides inhibiting both Bcl-2 and Bcl-x<sub>L</sub> may be useful to induce apoptosis of tumor cells<sup>[47]</sup>. Other promising strategies to downregulate Bcl-x<sub>L</sub> or Mcl-1 are application of small-molecule inhibitors. ABT-737 is an example of one of the first small-molecule inhibitors of Bcl-2/Bcl-x<sub>L</sub> shown to be efficacious *in vivo*, causing complete regression in small-cell lung carcinoma tumour xenografts in mice<sup>[22]</sup>. TW-37 has recently been described to simultaneously inhibit Bcl-2, Bcl-x<sub>L</sub> and Mcl-1 in lymphoma cells by targeting the BH3-binding groove of these Bcl-2 proteins<sup>[48]</sup>. Apart from direct suppression of Bcl-x<sub>L</sub> by siRNA or small-molecule inhibitors, suppression of oncogenic tyrosine kinases, such as Src kinases, which trigger Bcl-x<sub>L</sub> expression<sup>[26]</sup>, is another promising approach to induce killing of CRC cells.

In conclusion, our findings clearly implicate the anti-apoptotic activity of Bcl-2 family members, such as Bcl-x<sub>L</sub> and, to a lower extent, Mcl-1, as important components of the treatment resistance of CRC cells. Efficacy of chemotherapy, EGFR1 blockage and treatment with TRAIL, might be substantially improved by co-suppression of the anti-apoptotic protein Bcl-x<sub>L</sub>.

## COMMENTS

### Background

Colorectal carcinoma (CRC) is a very common malignancy with an increasing

incidence in recent decades. Defects in apoptosis signalling contribute to the resistance of CRC cells towards different treatment regimens. Thus, one of the main goals for oncologic treatment of patients suffering from CRC is to overcome resistance of tumor cells towards apoptosis.

### Research frontiers

Decreased sensitivity of mitochondria towards apoptosis stimuli, such as chemotherapy, is a key mechanism for apoptosis resistance of CRC cells. Mitochondrial activation is determined by the interaction of pro- and anti-apoptotic Bcl-2 family proteins, such as Bcl-x<sub>L</sub> and Mcl-1. In CRC, anti-apoptotic Bcl-2 family proteins are highly expressed, thus contributing to apoptosis resistance.

### Innovations and breakthroughs

In previous articles, the interaction of anti- and pro-apoptotic members of the Bcl-2 protein family and their role for the apoptosis sensitivity of carcinoma cells has been extensively studied. It has been shown that anti-apoptotic Bcl-2 family members are capable of blocking pro-apoptotic members of the family. Approaches to block the activity of anti-apoptotic Bcl-2 proteins, e.g. by RNA interference, have been evaluated and proven to be likely effective for the treatment of cancer patients.

### Applications

In this article, authors show an important role of Bcl-x<sub>L</sub> and Mcl-1 for the apoptosis resistance of CRC cells. Thus, downregulation of these anti-apoptotic proteins is a promising approach for the treatment of patients with CRC. Here authors show that the use of RNA interference can effectively downregulate Bcl-x<sub>L</sub> and Mcl-1 expression in CRC cells. After downregulation, CRC cells are sensitized to chemotherapy and target therapy approaches. Other ways to downregulate these proteins is application of so called "BH3-only mimetics". These drugs can interact with Bcl-x<sub>L</sub> and Mcl-1 and thereby induce the release of pro-apoptotic Bcl-2 proteins. "BH3-only mimetics" have already entered clinical trials in cancer patients.

### Terminology

Apoptosis is also depicted as programmed cell death. It is characterized by typical morphological alterations, e.g. the condensation of chromatin in the nucleus. Bcl-2 proteins are a large family of proteins, which can be sub-divided in anti-apoptotic members, multidomain pro-apoptotic members and BH3-only pro-apoptotic members. Bcl-x<sub>L</sub> and Mcl-1 are both anti-apoptotic members of the Bcl-2 family. Receptors for epidermal growth factor (EGF) contribute to the growth of cancer cells. Therapeutic approaches in patients with CRC target this receptor (e.g. antibodies binding to the EGF receptor as well as small molecules which inhibit the kinase domain of the receptor) and have been proven to be effective anti-cancer reagents in clinical studies.

### Peer review

This study shows that Mcl-1 and Bcl-x<sub>L</sub> are important anti-apoptotic factors in CRC. Downregulation of Bcl-x<sub>L</sub> is proven to be a promising approach to sensitize CRC towards chemotherapy and targeted therapy. Thus, a translational idea for the treatment of CRC is provided. This is a well written paper and the results are important.

## REFERENCES

- Majer M, Akerley W, Kuwada SK. Oncologists' current opinion on the treatment of colon carcinoma. *Anticancer Agents Med Chem* 2007; **7**: 492-503
- Schulze-Bergkamen H, Krammer PH. Apoptosis in cancer--implications for therapy. *Semin Oncol* 2004; **31**: 90-119
- Kroemer G, Reed JC. Mitochondrial control of cell death. *Nat Med* 2000; **6**: 513-519
- Kozopas KM, Yang T, Buchan HL, Zhou P, Craig RW. MCL1, a gene expressed in programmed myeloid cell differentiation, has sequence similarity to BCL2. *Proc Natl Acad Sci USA* 1993; **90**: 3516-3520
- Rinkenberger JL, Horning S, Klocke B, Roth K, Korsmeyer SJ. Mcl-1 deficiency results in peri-implantation embryonic lethality. *Genes Dev* 2000; **14**: 23-27
- Craig RW. MCL1 provides a window on the role of the BCL2 family in cell proliferation, differentiation and tumorigenesis. *Leukemia* 2002; **16**: 444-454
- Fleischer B, Schulze-Bergkamen H, Schuchmann M, Weber A, Biesterfeld S, Muller M, Krammer PH, Galle PR. Mcl-1 is an anti-apoptotic factor for human hepatocellular carcinoma. *Int J Oncol* 2006; **28**: 25-32
- Song L, Coppola D, Livingston S, Cress D, Haura EB. Mcl-1 regulates survival and sensitivity to diverse apoptotic stimuli in human non-small cell lung cancer cells. *Cancer Biol Ther* 2005; **4**: 267-276
- Schulze-Bergkamen H, Fleischer B, Schuchmann M, Weber A, Weinmann A, Krammer PH, Galle PR. Suppression of Mcl-1 via RNA interference sensitizes human hepatocellular carcinoma cells towards apoptosis induction. *BMC Cancer* 2006; **6**: 232
- Han J, Goldstein LA, Gastman BR, Rabinowich H. Interrelated roles for Mcl-1 and BIM in regulation of TRAIL-mediated mitochondrial apoptosis. *J Biol Chem* 2006; **281**: 10153-10163
- Taniai M, Grambihler A, Higuchi H, Werneburg N, Bronk SF, Farrugia DJ, Kaufmann SH, Gores GJ. Mcl-1 mediates tumor necrosis factor-related apoptosis-inducing ligand resistance in human cholangiocarcinoma cells. *Cancer Res* 2004; **64**: 3517-3524
- Nijhawan D, Fang M, Traer E, Zhong Q, Gao W, Du F, Wang X. Elimination of Mcl-1 is required for the initiation of apoptosis following ultraviolet irradiation. *Genes Dev* 2003; **17**: 1475-1486
- Krajewska M, Moss SF, Krajewski S, Song K, Holt PR, Reed JC. Elevated expression of Bcl-X and reduced Bak in primary colorectal adenocarcinomas. *Cancer Res* 1996; **56**: 2422-2427
- Kumar R, Mandal M, Lipton A, Harvey H, Thompson CB. Overexpression of HER2 modulates bcl-2, bcl-XL, and tamoxifen-induced apoptosis in human MCF-7 breast cancer cells. *Clin Cancer Res* 1996; **2**: 1215-1219
- Rashmi R, Kumar S, Karunakaran D. Ectopic expression of Bcl-XL or Ku70 protects human colon cancer cells (SW480) against curcumin-induced apoptosis while their down-regulation potentiates it. *Carcinogenesis* 2004; **25**: 1867-1877
- Zhang L, Yu J, Park BH, Kinzler KW, Vogelstein B. Role of BAX in the apoptotic response to anticancer agents. *Science* 2000; **290**: 989-992
- Zangemeister-Wittke U, Schenker T, Luedke GH, Stahel RA. Synergistic cytotoxicity of bcl-2 antisense oligodeoxynucleotides and etoposide, doxorubicin and cisplatin on small-cell lung cancer cell lines. *Br J Cancer* 1998; **78**: 1035-1042
- Chen C, Edelstein LC, Gelinas C. The Rel/NF-kappaB family directly activates expression of the apoptosis inhibitor Bcl-x(L). *Mol Cell Biol* 2000; **20**: 2687-2695
- Ashkenazi A, Pai RC, Fong S, Leung S, Lawrence DA, Marsters SA, Blackie C, Chang L, McMurtrey AE, Hebert A, DeForge L, Koumenis IL, Lewis D, Harris L, Bussiere J, Koeppen H, Shahrokh Z, Schwall RH. Safety and antitumor activity of recombinant soluble Apo2 ligand. *J Clin Invest* 1999; **104**: 155-162
- Nicoletti I, Migliorati G, Pagliacci MC, Grignani F, Riccardi C. A rapid and simple method for measuring thymocyte apoptosis by propidium iodide staining and flow cytometry. *J Immunol Methods* 1991; **139**: 271-279
- Merino D, Lalaoui N, Morizot A, Solary E, Micheau O. TRAIL in cancer therapy: present and future challenges. *Expert Opin Ther Targets* 2007; **11**: 1299-1314
- Stauffer SR. Small molecule inhibition of the Bcl-X(L)-BH3 protein-protein interaction: proof-of-concept of an in vivo chemopotentiator ABT-737. *Curr Top Med Chem* 2007; **7**: 961-965
- Sinicrope FA, Ruan SB, Cleary KR, Stephens LC, Lee JJ, Levin B. bcl-2 and p53 oncoprotein expression during colorectal tumorigenesis. *Cancer Res* 1995; **55**: 237-241
- Fisher DE. Apoptosis in cancer therapy: crossing the threshold. *Cell* 1994; **78**: 539-542
- Maurer CA, Friess H, Buhler SS, Wahl BR, Graber H, Zimmermann A, Buchler MW. Apoptosis inhibiting factor Bcl-xL might be the crucial member of the Bcl-2 gene family in colorectal cancer. *Dig Dis Sci* 1998; **43**: 2641-2648
- Karni R, Jove R, Levitzki A. Inhibition of pp60c-Src reduces

- Bcl-XL expression and reverses the transformed phenotype of cells overexpressing EGF and HER-2 receptors. *Oncogene* 1999; **18**: 4654-4662
- 27 **Zhao R**, Yang FT, Alexander DR. An oncogenic tyrosine kinase inhibits DNA repair and DNA-damage-induced Bcl-xL deamidation in T cell transformation. *Cancer Cell* 2004; **5**: 37-49
  - 28 **Wincewicz A**, Sulkowska M, Koda M, Sulkowski S. Cumulative expression of HIF-1-alpha, Bax, Bcl-xL and P53 in human colorectal cancer. *Pathology* 2007; **39**: 334-338
  - 29 **Sieghart W**, Losert D, Strommer S, Cejka D, Schmid K, Rasoul-Rockenschaub S, Bodingbauer M, Crevenna R, Monia BP, Peck-Radosavljevic M, Wacheck V. Mcl-1 overexpression in hepatocellular carcinoma: a potential target for antisense therapy. *J Hepatol* 2006; **44**: 151-157
  - 30 **Backus HH**, Van Groenigen CJ, Vos W, Dukers DF, Bloemena E, Wouters D, Pinedo HM, Peters GJ. Differential expression of cell cycle and apoptosis related proteins in colorectal mucosa, primary colon tumours, and liver metastases. *J Clin Pathol* 2002; **55**: 206-211
  - 31 **Fujie Y**, Yamamoto H, Ngan CY, Takagi A, Hayashi T, Suzuki R, Ezumi K, Takemasa I, Ikeda M, Sekimoto M, Matsuura N, Monden M. Oxaliplatin, a potent inhibitor of survivin, enhances paclitaxel-induced apoptosis and mitotic catastrophe in colon cancer cells. *Jpn J Clin Oncol* 2005; **35**: 453-463
  - 32 **Wu S**, Zhu H, Gu J, Zhang L, Teraishi F, Davis JJ, Jacob DA, Fang B. Induction of apoptosis and down-regulation of Bcl-XL in cancer cells by a novel small molecule, 2[[3-(2,3-dichlorophenoxy)propyl]amino]ethanol. *Cancer Res* 2004; **64**: 1110-1113
  - 33 **Zhu H**, Guo W, Zhang L, Davis JJ, Teraishi F, Wu S, Cao X, Daniel J, Smythe WR, Fang B. Bcl-XL small interfering RNA suppresses the proliferation of 5-fluorouracil-resistant human colon cancer cells. *Mol Cancer Ther* 2005; **4**: 451-456
  - 34 **Schmitt E**, Cimoli G, Steyaert A, Bertrand R. Bcl-xL modulates apoptosis induced by anticancer drugs and delays DEVDase and DNA fragmentation-promoting activities. *Exp Cell Res* 1998; **240**: 107-121
  - 35 **Adams JM**, Cory S. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 2007; **26**: 1324-1337
  - 36 **Walczak H**, Miller RE, Ariail K, Gliniak B, Griffith TS, Kubin M, Chin W, Jones J, Woodward A, Le T, Smith C, Smolak P, Goodwin RG, Rauch CT, Schuh JC, Lynch DH. Tumoricidal activity of tumor necrosis factor-related apoptosis-inducing ligand in vivo. *Nat Med* 1999; **5**: 157-163
  - 37 **Marini P**, Denzinger S, Schiller D, Kauder S, Welz S, Humphreys R, Daniel PT, Jendrossek V, Budach W, Belka C. Combined treatment of colorectal tumours with agonistic TRAIL receptor antibodies HGS-ETR1 and HGS-ETR2 and radiotherapy: enhanced effects in vitro and dose-dependent growth delay in vivo. *Oncogene* 2006; **25**: 5145-5154
  - 38 **Ganten TM**, Koschny R, Sykora J, Schulze-Bergkamen H, Buchler P, Haas TL, Schader MB, Untergasser A, Stremmel W, Walczak H. Preclinical differentiation between apparently safe and potentially hepatotoxic applications of TRAIL either alone or in combination with chemotherapeutic drugs. *Clin Cancer Res* 2006; **12**: 2640-2646
  - 39 **Vaculova A**, Hofmanova J, Soucek K, Kozubik A. Different modulation of TRAIL-induced apoptosis by inhibition of pro-survival pathways in TRAIL-sensitive and TRAIL-resistant colon cancer cells. *FEBS Lett* 2006; **580**: 6565-6569
  - 40 **Tillman DM**, Izeradjene K, Szucs KS, Douglas L, Houghton JA. Rottlerin sensitizes colon carcinoma cells to tumor necrosis factor-related apoptosis-inducing ligand-induced apoptosis via uncoupling of the mitochondria independent of protein kinase C. *Cancer Res* 2003; **63**: 5118-5125
  - 41 **Falschlehner C**, Emmerich CH, Gerlach B, Walczak H. TRAIL signalling: decisions between life and death. *Int J Biochem Cell Biol* 2007; **39**: 1462-1475
  - 42 **Bos JL**, Fearon ER, Hamilton SR, Verlaan-de Vries M, van Boom JH, van der Eb AJ, Vogelstein B. Prevalence of ras gene mutations in human colorectal cancers. *Nature* 1987; **327**: 293-297
  - 43 **Ricci MS**, Kim SH, Ogi K, Plastaras JP, Ling J, Wang W, Jin Z, Liu YY, Dicker DT, Chiao PJ, Flaherty KT, Smith CD, El-Deiry WS. Reduction of TRAIL-induced Mcl-1 and cIAP2 by c-Myc or sorafenib sensitizes resistant human cancer cells to TRAIL-induced death. *Cancer Cell* 2007; **12**: 66-80
  - 44 **Mendelsohn J**, Baselga J. Epidermal growth factor receptor targeting in cancer. *Semin Oncol* 2006; **33**: 369-385
  - 45 **Hannon GJ**. RNA interference. *Nature* 2002; **418**: 244-251
  - 46 **Wall NR**, Shi Y. Small RNA: can RNA interference be exploited for therapy? *Lancet* 2003; **362**: 1401-1403
  - 47 **Zangemeister-Wittke U**, Leech SH, Olie RA, Simoes-Wüst AP, Gautschi O, Luedke GH, Natt F, Haner R, Martin P, Hall J, Nalin CM, Stahel RA. A novel bispecific antisense oligonucleotide inhibiting both bcl-2 and bcl-xL expression efficiently induces apoptosis in tumor cells. *Clin Cancer Res* 2000; **6**: 2547-2555
  - 48 **Mohammad RM**, Goustin AS, Aboukameel A, Chen B, Banerjee S, Wang G, Nikolovska-Coleska Z, Wang S, Al-Katib A. Preclinical studies of TW-37, a new nonpeptidic small-molecule inhibitor of Bcl-2, in diffuse large cell lymphoma xenograft model reveal drug action on both Bcl-2 and Mcl-1. *Clin Cancer Res* 2007; **13**: 2226-2235

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## Staging of portal hypertension and portosystemic shunts using dynamic nuclear medicine investigations

Mircea Dragoteanu, Ioan A Balea, Liliana A Dina, Cecilia D Piglesan, Ioana Grigorescu, Stefan Tamas, Sabin O Cotul

Mircea Dragoteanu, Cecilia D Piglesan, Stefan Tamas, Sabin O Cotul, Department of Nuclear Medicine, "Professor, Dr. Octavian Fodor" Clinical Emergency Hospital, 19-21 Croitorilor Street, Cluj-Napoca 400162, Romania

Ioan A Balea, Resident doctor in nuclear medicine, "Professor, Dr. Octavian Fodor" Clinical Emergency Hospital, 19-21 Croitorilor Street, Cluj-Napoca 400162, Romania

Liliana A Dina, Ioana Grigorescu, Department of Internal Medicine, "Professor, Dr. Octavian Fodor" Clinical Emergency Hospital, 19-21 Croitorilor Street, Cluj-Napoca 400162, Romania

**Author contributions:** Dragoteanu M headed the investigation team, designed and coordinated the study, made the interpretation of the results, introduced the new parameters and classification, worked on the preparation and revision of the manuscript and on the statistical analysis of data; Balea IA assisted with the manuscript preparation and revision and the statistical analysis of data; Dina LA participated in the selection and follow up of the patients, the statistical analysis of the data and the evaluation of the per-rectal portal scintigraphy classical method based on the per-rectal portal shunt index; Piglesan CD, Tamas S as physicists were members of the investigation team; Grigorescu I participated in the selection and follow up of the patients and assisted with the statistical analysis of the data; Cotul SO is a retired honorary professor. As chief of laboratory before 2002 he introduced the classic per-rectal portal scintigraphy and liver angioscintigraphy into practice in this hospital and was a member of the investigation team.

**Correspondence to:** Dr. Mircea Dragoteanu, PhD, Department of Nuclear Medicine, Clinical Emergency Hospital "Prof dr Octavian Fodor", str. Croitorilor 19-21 Cluj-Napoca, 400162, Romania. [dragoteanu@yahoo.co.uk](mailto:dragoteanu@yahoo.co.uk)

Telephone: +40-722-381851 Fax: +40-264-455995

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lation time between right heart and liver (RHLT). LTT for each lobe was used to evaluate the early portal hypertension. RHLT is useful in cirrhosis to detect liver areas missing portal inflow. We calculated the classical per-rectal portal shunt index (PRSI) at PRPS and the hepatic perfusion index (HPI) at LAS.

**RESULTS:** The normal LTT value was  $24 \pm 1$  s. Abnormal LTT had PPV = 100% for CLD. Twenty-seven non-cirrhotic patients had LTT increased up to 35 s (median 27 s). RHLT ( $42 \pm 1$  s) was not related to liver disease. Cirrhosis could be excluded in all patients with PRSI < 5% ( $P < 0.01$ ). PRSI > 30% had PPV = 100% for cirrhosis. Based on PRPS and LAS we propose the classification of CLD in 5 hemodynamic stages. Stage 0 is normal (LTT = 24 s, PRSI < 5%). In stage 1, LTT is increased, while PRSI remains normal. In stage 2, LTT is decreased between 16 s and 23 s, whereas PRSI is increased between 5% and 10%. In stage 3, PRSI is increased to 10%-30%, and LTT becomes undetectable by PRPS due to the portosystemic shunts. Stage 4 includes the patients with PRSI > 30%. RHLT and HPI were used to subtype stage 4. In our study stage 0 had NPV = 100% for CLD, stage 1 had PPV = 100% for non-cirrhotic CLD, stages 2 and 3 represented the transition from chronic hepatitis to cirrhosis, stage 4 had PPV = 100% for cirrhosis.

**CONCLUSION:** LTT allows the detection of early portal hypertension and of opening of transhepatic shunts. PRSI is useful in CLD with extrahepatic portosystemic shunts. Our hemodynamic model stages the evolution of portal hypertension and portosystemic shunts. It may be of use in the selection of patients for interferon therapy.

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### Abstract

**AIM:** To explore portal hypertension and portosystemic shunts and to stage chronic liver disease (CLD) based on the pathophysiology of portal hemodynamics.

**METHODS:** Per-rectal portal scintigraphy (PRPS) was performed on 312 patients with CLD and liver angioscintigraphy (LAS) on 231 of them. The control group included 25 healthy subjects. We developed a new model of PRPS interpretation by introducing two new parameters, the liver transit time (LTT) and the circu-

**Key words:** Chronic liver disease; Portal hypertension; Portosystemic shunts; Per-rectal portal scintigraphy; Angioscintigraphy

**Peer reviewers:** Edoardo G Giannini, Assistant Professor, Department of Internal Medicine, Gastroenterology Unit, Viale Benedetto XV, No. 6, Genoa, 16132, Italy; Stefano Bellentani, Professor, Fondo Studio Malattie Fegato-ONLUS, Sezione di Campogalliano, Via R. Luxemburg, 29/N, 41011 Campogalliano (MO), Italy

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## INTRODUCTION

The most frequent causes of chronic liver disease (CLD) are viral infections, ethanol, autoimmune, enzymatic and metabolic disorders<sup>[1,2]</sup>. Cirrhosis is the final stage of CLD<sup>[3]</sup>. Liver biopsy is still an important diagnostic tool in CLD<sup>[4]</sup>.

Portal hypertension is a major complication which appears during CLD evolution. It is defined as an increase of portal blood pressure over 5-10 mmHg. Portosystemic shunts open when the venous portal-liver gradient becomes higher than 10-12 mmHg<sup>[5,6]</sup>.

There are inferior, superior, anterior and posterior portosystemic shunts which could communicate with either inferior or superior vena cava territories. The existence of shunts as well as their blood flow is correlated to the severity and prognosis of CLD<sup>[7-9]</sup>.

Investigation of portal pressure and portosystemic shunts may be performed by invasive and non-invasive methods. Invasive techniques offer the most correct data because of the direct measure of portal pressure. They have however a limited clinical use because of risks and costs<sup>[10]</sup>.

Among the non-invasive methods, ultrasonography and upper digestive endoscopy are those currently used. The main parameters measured by ultrasonography to evaluate the effects of increased blood pressure in the portal territory are the diameters of portal, splenic and superior mesenteric veins, together with spleen size and portal flow velocity. However, dilation of portal territory veins can be seen in only 50% of cases<sup>[11,12]</sup> and only 35%-80% of cirrhotic patients present esophageal varices at upper digestive endoscopy.

Nuclear medicine offers noninvasive static and dynamic procedures to investigate portal hypertension in CLD and to estimate the existence of portosystemic shunts<sup>[13]</sup>.

A classic method is the liver scintigraphy using labeled colloid (planar and SPECT), which offers data regarding portal hypertension by calculating the capture ratio between liver and spleen, respectively between the right and left liver lobes<sup>[14,15]</sup>. Increased colloid capture in the bone marrow is characteristic for advanced stages of portal hypertension.

Per-rectal portal scintigraphy (PRPS) investigates the hemodynamic importance of portosystemic shunts. Radio-tracer absorbed from rectum passes through inferior mesenteric vein into portal vein-liver-right heart<sup>[16-18]</sup>. The per-rectal portal shunt index (PRSI) was introduced by the classic works of Shiomi and co-authors as the main parameter calculated at PRPS by analyzing the dynamic curves raised on liver and heart areas<sup>[19,20]</sup>.

Liver angioscintigraphy (LAS) uses the hepatic per-

fusion index (HPI) to estimate the ratio between the hepatic artery inflow and total liver perfusion, arterial plus portal. Increased HPI (> 40%) in CLD shows the decrease of portal inflow with reactive increase of the flow through the hepatic artery by activation of the buffer response firstly described by Lautt<sup>[21,22]</sup>. The decrease of portal inflow in advanced CLD is mainly determined by the quantity of blood deviated through portosystemic shunts<sup>[23-25]</sup>. In cirrhosis, HPI > 100% highlights the reversion of portal flow.

In this study we improved the interpretation of classical PRPS technique allowing a better characterization and staging of portal hypertension and portosystemic shunts.

## MATERIALS AND METHODS

PRPS with <sup>99m</sup>Tc-pertechnetate was performed in 312 consecutive patients there were 116 females and 196 males between 18 years and 80 years old. Their CLD diagnosis was based on clinical, laboratory, imaging and morphological data. The final diagnoses of the study population are shown in Table 1. The etiology of CLD in 291 patients is presented in Table 2.

LAS was performed on 231 patients with CLD, randomly selected from the 312 explored by PRPS. There were 141 males and 90 females, between 22 years and 77 years old.

Two hundred and four of the 312 patients who underwent PRPS were also investigated by upper digestive endoscopy: 175 of them had cirrhosis. One hundred and eleven of the cirrhotic patients who presented with esophageal varices at upper digestive endoscopy were followed up for 6 mo after investigation.

The control group for PRPS included 25 healthy subjects, 11 females and 14 males, between 18 years and 80 years old. The control group for LAS was composed of 25 healthy subjects, 10 females and 15 males, between 20 years and 78 years old.

PRPS was also performed on one group of 12 patients with complete thrombosis of portal vein in order to calculate RHLT and to compare its values with those in healthy subjects. This group included 6 females and 6 males, between 34 years and 73 years old.

All the patients were fasted for at least 12 h before LAS and PRSI.

Nuclear medicine investigations were made by using a SPECT Orbiter Siemens gamma-camera with high-resolution, low-energy, parallel collimator connected to a Power Macintosh computer, using ICON dedicated software.

For PRPS we used <sup>99m</sup>Tc-pertechnetate eluted from Drygen generators (General Electric-Amersham, UK). The colloid used for LAS was Hepatate (General Electric-Amersham, UK) labeled with <sup>99m</sup>Tc.

Two enemas were performed in each patient for PRPS: the first on the evening before the exam, and the second two hours prior to the examination.

The patients were positioned at PRPS with the camera detector in the anterior view including the liver

**Table 1** Features of the study population according to the final diagnosis (n)

Type of disease	Number of patients
Steatosis	17
Chronic hepatitis	69
Cirrhosis	202
Unknown	3
CLD infirmed	21
Total	312

**Table 2** Etiology for the patients with chronic liver disease (n)

Etiology	Number of patients
Viral	139
Alcohol	74
Mix (viral + alcohol)	6
Unknown	72
Total	291

and heart areas. A solution containing 2 milliliters of  $^{99m}\text{Tc}$ -pertechnetate (296-370 MBq) was introduced into the upper part of the rectum, followed by 15 milliliters of air under pressure. Serial scintigrams were recorded every 2 s for 3 min. Radioactivity curves were built on liver and heart areas to show the dynamics of radio-tracer absorbed from the rectum.

LAS was performed after antecubital i.v. bolus injection of 370-440 MBq of  $^{99m}\text{Tc}$  radio-colloid, with the patients lying down, in anterior-posterior view. Collimator area included abdomen and lower part of thorax. Sequential images were recorded 1/s for 1 min. Right kidney, right liver lobe and spleen dynamic curves were built. HPI was calculated using the Sarper's method<sup>[26]</sup>.

The results were compared with clinical diagnosis, liver biopsy and upper digestive endoscopy. For statistical evaluation of PRSI we used the Kruskal-Wallis and Mann-Withney non-parametric tests.

### New approach to the per-rectal portal scintigraphy

At the beginning of the research we used for the first 100 patients the classic interpretation of PRPS, based on the calculation of PRSI for the global area of the liver<sup>[16]</sup>. All information was stored in the computer.

In the second stage we considered the possibility of acquiring more useful data in PRPS, by developing a new model of interpretation. Pertechnetate is not significantly captured by liver or heart, so that at PRPS the first-passage histograms built for these organs represent transit curves, not accumulation curves. This suggests the importance of time-related parameters. Transit type of PRPS liver and heart curves is highlighted by dynamic curves built on the inferior mesenteric vein area. These curves have the same ascending aspect as the histograms on liver and heart thereafter produced at first passage by the same radiotracer flow absorbed from the upper rectum.

We introduced LTT as a new parameter useful for the early phases of CLD where PRSI offers not enough information. LTT is the time interval between entrance into the liver and subsequent entrance into the right heart of the radiotracer absorbed from the upper rectum, after passing through the mesenteric and portal veins. LTT was separately measured for each liver lobe as the time interval between the liver and heart dynamic histograms. The normal value is  $24 \pm 1$  s. This parameter is useful for patients without extra-hepatic shunts. In patients with portosystemic extrahepatic shunts, LTT cannot be correctly determined by PRPS because the tracer absorbed from the rectum may arrive at the right heart faster by passing through shunts than following the physiological pathway.

PRSI equal to 10% corresponds to LTT equal to  $16 \pm 1$  s. LTT decreased between 16 s and 23 s corresponds to a PRSI increased up to 10% and approximates the interval in which only the transhepatic shunts are open. A PRSI equal to 30% corresponds to a mean time of 8 s between the liver and heart curves, but this interval does not have the significance of LTT because of the flow passing through the extra-hepatic portosystemic shunts, which arrives at the heart faster than the tracer passing through the liver.

Liver areas perfused only through the hepatic artery may be seen in cirrhosis. One lobe or both have, in such cases, abolished or insignificant portal inflow and the time between PRPS curves on the heart and on the area(s) without portal inflow has a maximum value, equal to RHLT. This is a constant time interval (not related to CLD) between the entrance of the tracer into the right heart and its subsequent arrival to the liver following the route: right heart-lungs-left heart-aorta-hepatic artery. We measured RHLT in the patients with complete portal thrombosis as the time interval between heart and liver curves. In healthy controls, RHLT was measured as the time interval between the arrival of tracer into the right heart and the ascending inflexion on the liver histogram determined by the subsequent arrival of tracer through the hepatic artery. The value of RHLT was  $42 \pm 1$  s for both methods.

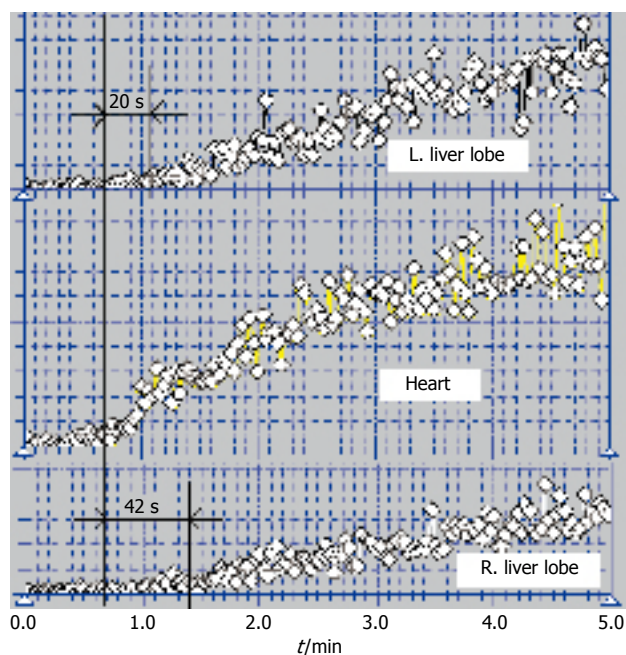
We performed separate PRPS analyses of the two liver lobes, which are autonomous in relation to the blood inflow (Figure 1). No differences between the two lobes could be found in the control group. We compared PRPS curves for the two lobes in order to distinguish subtypes, respectively stages of portal hypertension evolution. Figure 1 shows the case of a cirrhotic patient in which time intervals between the heart histogram and the curves on the two liver lobes are different.

Our PRPS model is based on the two new time parameters, the classic calculation of PRSI and the separate evaluation of the two liver lobes.

## RESULTS

The distribution for the 312 patients of the PRSI calculated using Shiomi's formula is presented in Table 3.





**Figure 1** Per-rectal portal scintigraphy dynamic curves. Separate analyses of the two liver lobes for a cirrhotic patient (stage 4b).

There were no correlations of PRPS and LAS parameters with sex or age ( $P < 0.01$ ).

As many as 202 of 312 patients investigated with the PRPS were diagnosed as having a cirrhosis. One hundred and seventy-five of these underwent upper digestive endoscopy. Twenty seven patients with advanced cirrhosis (all of them with PRSI  $> 30\%$  at PRPS) could not be explored by endoscopy as deemed too risky. Using Child-Pugh classification, the 175 cirrhotic patients investigated by upper digestive endoscopy were classified as follows: 99 in class A, 38 in class B and 38 in class C.

Only 16 patients from the 93 with PRSI between 5% and 30% had esophageal varices. Thirty two additional patients with PRSI  $< 30\%$  but without varices were also diagnosed with cirrhosis. As a result, we had a total number of 48 cirrhotic patients with PRSI  $< 30\%$ . Five patients with cirrhosis had discordant low PRSI values ranging from 5% to 10%, but no cirrhotic patient had normal PRSI ( $< 5\%$ ). All the patients with PRSI  $> 30\%$  had cirrhosis, so we used PRSI = 30% as an upper limit value for chronic hepatitis.

The PRSI was significantly higher in cirrhotic patients than in chronic hepatitis ( $P < 0.01$ ). The median value for PRSI was 5% for the control group, 5% for the patients with steatosis, 6% for the patients with chronic hepatitis and 73.5% for the cirrhotic patients. The sums of ranks for the PRSI values based on Kruskal-Wallis test for the healthy subjects and stages of CLD are shown in Table 4 ( $P = 0.000$ ). Using the Mann-Whitney test we showed that there was a significant statistical difference between PRSI for patients with chronic hepatitis and the controls ( $P = 0.0003$ ), between patients with cirrhosis and those with chronic hepatitis ( $P = 0.0000$ ) and respectively between patients with cirrhosis and controls ( $P = 0.0000$ ).

**Table 3** Distribution of all the patients and of those with cirrhosis according to the per-rectal portal shunt index and to our classification in 5 stages

Per-rectal portal shunt index (%)	Number of patients (n)	Cirrhosis	Stage in our classification
0-5	65	0	Stages 0 & 1
5-10	13	5	Stage 2
10-20	42	43	Stage 3
20-30	38		
30-40	17	154	Stage 4
40-50	10		
50-60	11		
60-70	10		
70-80	18		
80-90	40		
90-100	48		
Total	312	202	

**Table 4** Statistical analysis of the per-rectal portal shunt index for healthy subjects and for the different stages of chronic liver disease using Kruskal-Wallis test

Disease	Number of patients (n)	Sum of ranks
CLD infirmed	21	759.5
Steatosis	17	1201.5
Chronic hepatitis	69	5004.5
Cirrhosis	202	40929.5

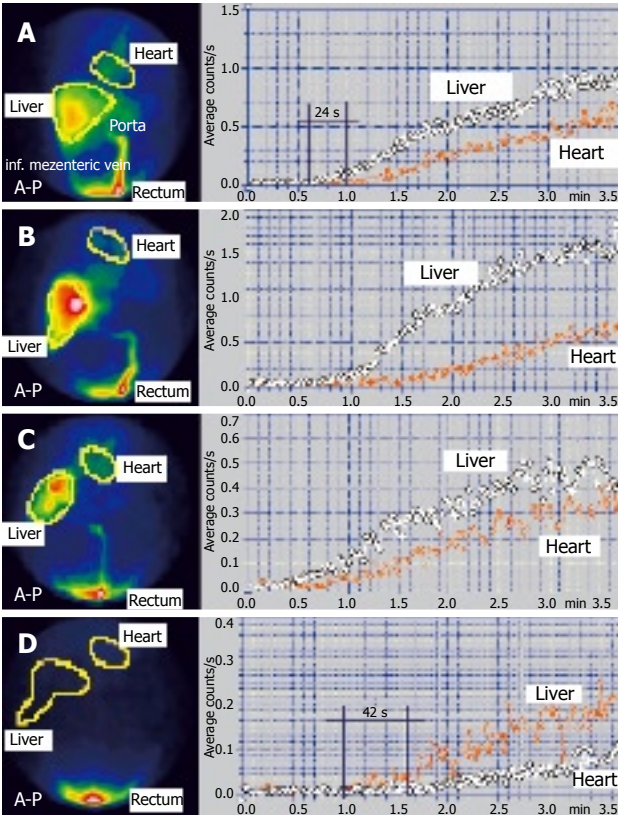
Kruskal-Wallis test:  $H(3, n = 309) = 169.5024, P = 0.000$ .

One hundred and eleven patients with esophageal varices and PRSI  $> 30\%$  were followed up for 6 mo; 51 of these had experienced previous upper digestive bleeding. During the follow up, 17 of these patients had an episode of upper digestive bleeding (11 as first time, 6 as recurrence). All our patients with upper digestive bleeding had PRSI  $> 70\%$  (mean of 88%). The 94 patients from the group of 111 with esophageal varices who had not upper digestive bleeding during the follow up had a mean value of PRSI equal to 46.75%. We had no patients with upper digestive bleeding among those with PRSI  $< 30\%$ , even if they had esophageal varices at upper digestive endoscopy.

These results show the diagnostic value of the classic parameter PRSI. A PRSI  $< 5\%$  had a NPV = 100% for cirrhosis, while a PRSI  $> 30\%$  had PPV = 100% for cirrhosis. A PRSI  $> 70\%$  was associated with a high risk of upper digestive bleeding ( $P < 0.01$ ), while with a PRSI  $< 30\%$  no variceal bleeding was encountered. However, using only PRSI is not always possible to make the differential diagnosis between chronic hepatitis and cirrhosis or between chronic hepatitis and healthy subjects.

Using LTT parameter originally introduced by us at PRPS we were able to find more data about the early stages of portal hypertension, when PRSI cannot offer enough information. In healthy subjects, LTT was  $24 \pm 1$  s. The distribution of LTT in patients with PRSI smaller than 10% (LTT  $> 16$  s) is shown in Table 5. For the 27 non-cirrhotic CLD patients who had prolonged LTT





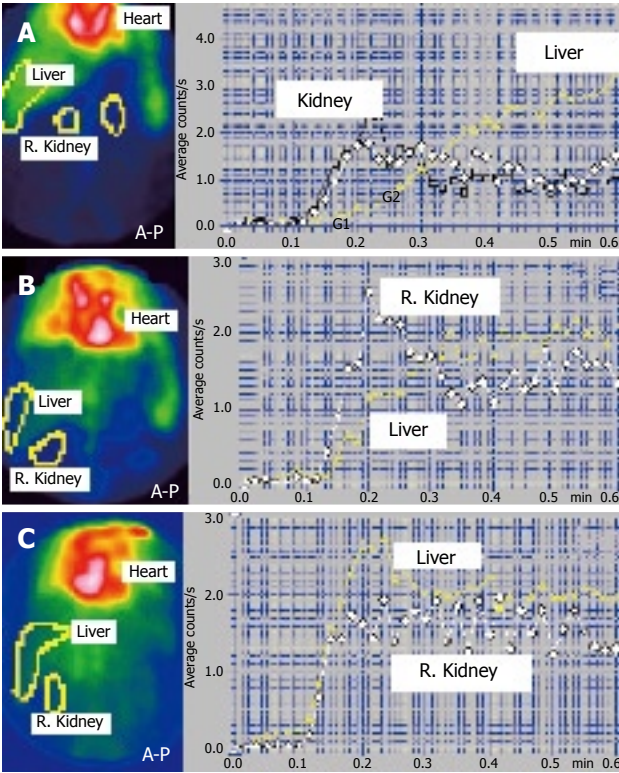
**Figure 2** Per-rectal portal scintigraphy. **A:** Stage 0, normal aspect. LTT = 24 s, PRSI < 5%; **B:** Stage 1. LTT increased (33 s). PRSI < 5%; **C:** Stage 2. Decreased LTT (18 s), PRSI slightly increased (8%); **D:** Stage 4c. Time between heart and liver curves is equal to RHTL = 42 s. Cirrhosis with undetectable portal inflow to the both lobes.

Table 5 Liver transit time and distribution in stages of the patients with the per-rectal portal shunt index lower than 10%					
Per-rectal shunt index	Mean value of liver transit time (s)		Etiology	Number of patients (n)	Stage in our classification
	Right lobe	Left lobe			
< 5%	24		-	38	Stage 0
	25	28	viral	10	Stage 1a
	31	24	alcoholic	6	Stage 1b
	31.5	29	viral + alcoholic	7	Stage 1c
5%-10%	19.5			13	Stage 2

(> 25 s), the median value was 27 s. LTT determined at PRPS has hemodynamic significance as it shows the time required at first-passage by the main part of portal inflow of radio-tracer to arrive to right heart through the liver.

RHLT measured in the control group and in patients with complete portal thrombosis is 42 ± 1 s. We used it to identify cirrhotic patients with undetectable portal inflow to one liver lobe or both. Other causes of portal flow interruption (thromboses, compressions) were excluded by ultrasonography.

HPI was used in our study to show the reactive increase of arterial flow due to the decreasing of portal



**Figure 3** Liver angioscintigraphy. **A:** Normal aspect. HPI = 30%. G1 = arterial inflow segment; G2 = portal inflow segment; **B:** Cirrhotic patient. HPI = 60%; **C:** Cirrhosis with reversed portal flow. HPI = 130%.

inflow (HPI > 40%) and to identify those cirrhotic patients with reversed portal flow (HPI > 100%).

**Staging of portal hypertension and portosystemic shunts**

Using the above shown data we propose a hemodynamic model in 5 steps to stage the evolution of portal hypertension and portosystemic shunts on physiopathological basis using nuclear medicine dynamic investigations (PRPS and LAS).

The 5 stages of portal hypertension and portosystemic shunts proposed by us are the following: (1) Stage 0 is normal: PRSI < 5%, LTT = 24 ± 1 s (Figure 2A), HPI < 40% (Figure 3A); (2) Stage 1 is characterized by PRSI < 5% (normal), but LTT is increased over 25 s at least on one lobe (Figure 2B). HPI is normal. For stage 1 we found 3 subtypes according to the liver lobe(s) with prolonged LTT: (a) Subtype 1a, with increased LTT for the left lobe, but with normal or slightly decreased LTT for the right lobe; (b) Subtype 1b, with increased LTT for the right lobe and with normal or slightly decreased LTT for the left lobe; (c) Subtype 1c, with increased LTT for the both lobes; (3) Stage 2 is characterized by decreased LTT for both lobes, between 16 s and 23 s. PRSI is slightly increased, between 5% and 10% (Figure 2C). HPI calculated at LAS is currently at the upper normal limit or slightly increased, up to 45%; (4) Stage 3 is characterized by a moderately increased PRSI, between 10%-30%. Time interval between the hepatic and heart curves is decreased between 8 s and 16 s, but it is no more equal to LTT due

to the shunts. HPI is moderately increased, usually up to 50%-55%; (5) Stage 4 is characterized by PRSI > 30%. HPI is increased over 60%-70%. Liver curve precedes heart curve with less than 8 s or heart curve precedes liver curve (when PRSI > 50%). We found 4 subtypes of stage 4, according to the lobe(s) with undetectable or reversed portal flow: (a) Subtype 4a: heart curve precedes the hepatic histograms on both lobes with less than 42 s. Both lobes have portal inflow; (b) Subtype 4b: Cardiac curve precedes right liver lobe histogram with 42 s and the left liver lobe histogram with less than 42 s. There is still a portal flow to the left lobe, but the portal inflow to the right lobe is undetectable (Figure 1); (c) Subtype 4c: PRSI > 95%. Time between heart and both liver lobes curves is equal to RHLT = 42 s (Figure 2D). PRPS cannot detect portal inflow to any of the hepatic lobes. For subtypes 4a, 4b and 4c, the HPI is increased over 60%, but smaller than 100% (Figure 3B); (d) Subtype 4d: HPI at LAS is higher than 100% (Figure 3C). The time between PRPS heart curve and both liver lobes histograms is 42 s. Portal flow is reversed.

## DISCUSSION

Stage 0 includes the subjects without CLD. There are no portal flow changes. In our study, stage 0 had NPV = 100% for CLD. The tracer absorbed at PRPS from the rectum reaches the liver through the physiological pathway (inferior mesenteric and portal veins).

In stage 1, it is possible to detect the earliest changes that affect either one lobe or both, determined by the increased resistance opposed by liver to portal inflow. Portal flow velocity decreases and LTT is consequently increased. In our study stage 1 had PPV = 100% for non-cirrhotic CLD. In this stage the transhepatic and extrahepatic shunts are not open and the arterial inflow is normal. We encountered subtype 1a in patients with chronic viral hepatitis, subtype 1b in alcoholic etiology and subtype 1c in viral and mix (viral + alcohol) etiologies.

Stage 2 theoretically corresponds to the dilation of part of transhepatic pathways between portal and hepatic veins as a result of the portal pressure which is increased at higher values than in stage 1. The blood passes faster through these dilated transhepatic shunts than through sinusoids<sup>[27,28]</sup>. LTT is consequently decreased. Our threshold PRSI = 10% between stage 2 and stage 3 was selected based on the correspondence of this PRSI value with LTT =  $16 \pm 1$  s. Extrahepatic shunts are not open and arterial inflow remains normal (hepatic artery buffer response is not activated). Stage 2 theoretically appears when the increased resistance opposed by liver to the portal inflow produces a higher portal pressure which is able to enlarge transhepatic pathways, but is not high enough to open extrahepatic shunts. As stage 1, stage 2 has PPV = 100% for CLD, but stage 2 includes not only chronic hepatitis, but also cirrhotic patients, showing a more advanced stage of portal hypertension than stage 1. 38.46% of our 13 patients in stage 2 had cirrhosis, the other 61.54% had chronic hepatitis.

A steady state between stage 1 and stage 2 may

appear in cases with the resistance (and LTT) increased on one more affected liver lobe and with redirecting of an increased percentage of the portal inflow through the other lobe. In such cases, the lobe less affected may encounter opening of transhepatic shunts due to its higher portal inflow (with consequently slightly decreased LTT, like in stage 2), while the more affected lobe has prolonged LTT (characteristic for stage 1).

Stage 3 is theoretically characterized by the opening of extrahepatic shunts, added to the transhepatic dilated pathways already present from stage 2. Low flow extrahepatic shunts appear when the transhepatic shunts are no more able to compensate the higher values of portal pressure. The inferior per-rectal portosystemic shunts are in most cases the first extrahepatic shunts which open. The cause is the pressure gradient between inferior mesenteric vein and inferior vena cava territories, which is lower than the pressure gradient between portal vein and superior vena cava. The portal inflow to the liver decreases due to shunted flow and PRSI increases over 10%. A reactive increase of the arterial liver inflow due to the activation of the buffer mechanism of the hepatic artery is reflected at LAS by HPI > 40%. 53.75% of our 80 patients in stage 3 had cirrhosis, the other 46.25% had chronic hepatitis.

Stage 4 in our study had PPV = 100% for cirrhosis. Theoretically, stage 4 involves shunts open to the territory of superior vena cava, which has a higher diameter and increased flow<sup>[29]</sup>. PRPS shows diminished or abolished portal inflow to one or both liver lobes, with PRSI > 30%. In stage 4, the tracer absorbed at PRPS from the rectum usually arrives faster to the right heart (through the portosystemic shunts and caval veins) than to the liver through the physiological pathway. Inverted order of heart and liver curves may be thus seen in advanced cases, corresponding to PRSI > 50%. In patients with undetectable portal inflow to one or both lobes (subtypes 4b, 4c, 4d), the tracer absorbed from the rectum reaches those liver areas only through the hepatic artery and time interval between the heart and liver curves is equal to RHLT = 42 s. Subtype 4b appeared more frequently in alcoholic CLD. In our study, we did not have patients with abolished inflow to the left lobe and maintaining portal inflow to the right lobe, but such cases may theoretically exist.

Figure 1 shows a case where the interval between heart and left liver lobe curves was equal to 20 s (left lobe maintained a low portal inflow) while the time interval between heart and right liver lobe histograms was equal to RHLT = 42 s (the right lobe received tracer only through hepatic artery).

In our group, the number of patients in early stages (1 and 2) was lower than in advanced stages (3 and 4). This could be explained by the fact that few patients in early CLD stages were hospitalized and/or proposed for nuclear medicine dynamic liver investigations. Moreover, stage 2 is theoretically an intermediate stage in the natural history of CLD and of the portal hypertension, so it lasts a short time compared to the evolution of the disease.



Our experience confirmed that at PRSI > 70% the risk of upper digestive hemorrhage is increased<sup>[17,30]</sup>.

The classical parameter PRSI allows only a rough characterization of non-cirrhotic patients. PRSI gives no information about early increased resistance opposed by liver to the portal inflow or about the existence of transhepatic shunts, the first that open due to the portal hypertension. Another diagnostic limitation in using PRSI alone is the fact that there are patients with chronic hepatitis but who have normal PRSI, lower than 5%.

Our model using LTT besides PRSI allows a very early diagnosis of portal hypertension, represented by stages 1 and 2. Changes of liver dynamic resistance opposed to portal inflow could be shown in these early stages, at a moment when morphological effects on the portohepatic circulation are not detectable. The diagnosis of early hemodynamic changes determined by portal hypertension could be the basis for an appropriate therapy in a stage when the disease is reversible. Thus, we propose LTT as the main parameter of PRPS in the evaluation of early stages of portal hypertension. PRSI remains a very useful parameter for portal hypertension and portosystemic shunts in advanced stages of CLD.

Staging of portal hypertension has implications in the selection of patients for the treatment. Hemodynamic pathophysiology information offered by nuclear medicine dynamic investigations may improve also the selection of patients for interferon therapy.

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## COMMENTS

### Background

Portal hypertension and portosystemic shunts are severe complications of chronic liver disease (CLD). Their evaluation could be considered a dynamic marker of the progression of the disease. Ultrasonography and upper digestive endoscopy are usually performed to evaluate their existence and hemodynamic importance. Nuclear medicine techniques like per-rectal portal scintigraphy (PRPS) and liver angioscintigraphy (LAS) can offer valuable supplementary information.

### Research frontiers

Doppler ultrasonography and MRI are continuously increasing their accuracy in exploring portal hypertension and portosystemic shunts. PRPS is usually performed to investigate the advanced stages of CLD. The classical PRPS parameter per-rectal portal shunt index (PRSI) is useful in cases with open portosystemic shunts. Our research improves the diagnosis possibilities of PRPS especially in early stages of CLD by introducing two new time parameters. The early diagnosis of CLD and the therapy (including the selection of patients for interferon therapy) may be improved using dynamic scintigraphy data.

### Innovations and breakthroughs

We introduced two new parameters at PRPS, respectively liver transit time (LTT) and right heart to liver transit time (RHLT). LTT is useful in early stages of portal hypertension, before the opening of extrahepatic portosystemic shunts. LTT allows the diagnosis of early increase of liver resistance opposed to portal inflow and of the opening of transhepatic shunts. RHLT is useful in advanced CLD

stages to detect liver areas missing portal inflow. We propose the classification of portal hypertension and portosystemic shunts in 5 hemodynamic stages, characteristic for the progression of the disease. We introduce the separate evaluation of the two liver lobes at PRPS, used to subtype the stages 1 and 4.

### Applications

Using LTT as a basic parameter, PRSI allows the detection of early stages of portal hypertension, which are reversible under proper therapy. Our method can also distinguish between the 1<sup>st</sup> stage of portal hypertension, with increased resistance opposed by liver to portal inflow, without shunts, and the 2<sup>nd</sup> stage, characterized by the opening of transhepatic shunts. The new parameter RHLT and the hepatic perfusion index (HPI) calculated at LAS allow a better characterization of liver hemodynamics in advanced cirrhosis. We confirm the results of other studies showing that at PRSI > 70% the risk for upper digestive bleeding increases. The classification of portal hypertension and portosystemic shunts in 5 hemodynamic stages is useful for clinicians, in order to have a more accurate view of the patients with CLD. A better understanding of hemodynamic status of border-line cases between chronic hepatitis and cirrhosis may also improve the selection of patients for interferon therapy. Patients with PRSI between 5%-30% (stages 2 and 3 in our classification) require a precise evaluation in order to choose an adequate therapy. Correlation of dynamic nuclear medicine techniques with other non-invasive methods makes it possible to avoid liver biopsy for guiding the treatment in border-line patients between chronic hepatitis and cirrhosis. Dynamic follow-up of patients under interferon-treatment may be useful to adjust the therapy. Based on calculation of LTT, PRPS may be used to determine whether the early portal pressure reducing effect of anti-viral therapy is maintained in the long term, especially in sustained viral responders. It can be also helpful to evaluate whether long-term use of anti-viral therapy may delay the appearance and decrease the severity of portal hypertension manifestations.

### Terminology

*Per-rectal portal scintigraphy (PRPS)* is a dynamic nuclear medicine technique which investigates the existence and hemodynamic importance of portal hypertension and portosystemic shunts. A radiotracer introduced in the upper rectum is absorbed and follows the next pathway: inferior mesenteric vein-portal vein-liver-right heart. Dynamic curves built on liver and heart allow the calculation of specific parameters. *Liver transit time (LTT)* determined at PRPS is the time interval between entrance into the liver and subsequent entrance into the right heart of the radiotracer absorbed from the rectum, after passing through mesenteric and portal veins. *Right heart to liver transit time (RHLT)* represents at PRPS a constant time interval (not related to liver disease) between the entrance of the tracer into the right heart and its subsequent arrival to the liver following the next route: right heart-lungs-left heart-aorta-hepatic artery. *Per-rectal portal shunt index (PRSI)* is a parameter calculated at PRPS by analysis of dynamic curves built on liver and heart areas. *Liver angioscintigraphy (LAS)* is a dynamic nuclear medicine method based on i.v. antecubital administration of a radio-tracer and subsequent analyses of the liver dynamic curve which is determined at first passage by both hepatic artery and portal inflows of tracer. *Hepatic perfusion index (HPI)* is a parameter calculated at LAS which estimates the ratio between hepatic artery inflow and total liver perfusion, arterial plus portal.

### Peer review

This is a well done study probably not completely well presented where Dragoteanu and coworkers used per-rectal portal scintigraphy and liver angioscintigraphy in 312 and 231 CLD patients and 25 controls to calculate hepatic perfusion index (HPI) and other new hemodynamic parameters and classify portal hypertension and porto-caval shunts in 5 hemodynamic stages, which are specifically for the progression of CLD.

## REFERENCES

- 1 Yip WW, Burt AD. Alcoholic liver disease. *Semin Diagn Pathol* 2006; **23**: 149-160
- 2 Hui AX, Sung JJ. Advances in chronic viral hepatitis. *Curr Opin Infect Dis* 2005; **18**: 400-406
- 3 Schuppan D, Afdhal NH. Liver cirrhosis. *Lancet* 2008; **371**: 838-851
- 4 Theise ND. Liver biopsy assessment in chronic viral hepatitis: a personal, practical approach. *Mod Pathol* 2007; **20** Suppl 1: S3-S14
- 5 Blei AT. Portal hypertension and its complications. *Curr*

- Opin Gastroenterol* 2007; **23**: 275-282
- 6 **Bosch J**, Garcia-Pagan JC. Complications of cirrhosis. I. Portal hypertension. *J Hepatol* 2000; **32**: 141-156
  - 7 **D'Albuquerque LA**, de Oliveira e Silva A, Pinto Junior PE, de Miranda MP, Genzini T, Gama-Rodrigues JJ. [Surgical treatment of portal hypertension in patients with liver cirrhosis] *Arq Gastroenterol* 1988; **25**: 218-223
  - 8 **Rice TL**. Treatment of esophageal varices. *Clin Pharm* 1989; **8**: 122-131
  - 9 **Wolff M**, Hirner A. Current state of portosystemic shunt surgery. *Langenbecks Arch Surg* 2003; **388**: 141-149
  - 10 **Whalley S**, Puvanachandra P, Desai A, Kennedy H. Hepatology outpatient service provision in secondary care: a study of liver disease incidence and resource costs. *Clin Med* 2007; **7**: 119-124
  - 11 **Gorg C**, Riera-Knorrenschild J, Dietrich J. Pictorial review: Colour Doppler ultrasound flow patterns in the portal venous system. *Br J Radiol* 2002; **75**: 919-929
  - 12 **Badea R**, Lupsor M, Stefanescu H, Nedevschi S, Mitrea D, Serban A, Vasile T. Ultrasonography contribution to the detection and characterization of hepatic restructuring: is the "virtual biopsy" taken into consideration? *J Gastrointestin Liver Dis* 2006; **15**: 189-194
  - 13 **Dragoteanu M**, Cotul SO, Tamas S, Piglesan C. Nuclear medicine dynamic investigations of diffuse chronic liver diseases and portal hypertension. *Rom J Gastroenterol* 2004; **13**: 351-357
  - 14 **Mostbeck A**, Kroiss A. [Nuclear-medical methods in hepatology] *Dtsch Z Verdau Stoffwechselkr* 1981; **41**: 1-13
  - 15 **Cotul S**. [The current posture on radioisotope exploration in chronic diffuse hepatopathies] *Rev Med Interna Neurol Psihiatr Neurochir Dermatovenerol Med Interna* 1988; **40**: 211-216
  - 16 **Shiomi S**, Kuroki T, Kurai O, Kobayashi K, Ikeoka N, Monna T, Ochi H. Portal circulation by technetium-99m pertechnetate per-rectal portal scintigraphy. *J Nucl Med* 1988; **29**: 460-465
  - 17 **Chitapanarux T**, Praisontarangkul OA, Thongsawat S, Pisespongsa P, Leerapun A. Per rectal portal scintigraphy as a useful tool for predicting esophageal variceal bleeding in cirrhotic patients. *World J Gastroenterol* 2007; **13**: 791-795
  - 18 **Kawamura E**, Habu D, Hayashi T, Oe A, Kotani J, Ishizu H, Torii K, Kawabe J, Fukushima W, Tanaka T, Nishiguchi S, Shiomi S. Natural history of major complications in hepatitis C virus-related cirrhosis evaluated by per-rectal portal scintigraphy. *World J Gastroenterol* 2005; **11**: 3882-3886
  - 19 **Shiomi S**, Sasaki N, Habu D, Takeda T, Nishiguchi S, Kuroki T, Tanaka T, Ochi H. Natural course of portal hemodynamics in patients with chronic liver diseases, evaluated by per-rectal portal scintigraphy with Tc-99m pertechnetate. *J Gastroenterol* 1998; **33**: 517-522
  - 20 **Shiomi S**, Kuroki T, Ueda T, Takeda T, Ikeoka N, Nishiguchi S, Nakajima S, Kobayashi K, Ochi H. Clinical usefulness of evaluation of portal circulation by per rectal portal scintigraphy with technetium-99m pertechnetate. *Am J Gastroenterol* 1995; **90**: 460-465
  - 21 **Lautt WW**. Mechanism and role of intrinsic regulation of hepatic arterial blood flow: hepatic arterial buffer response. *Am J Physiol* 1985; **249**: G549-G556
  - 22 **Gulberg V**, Haag K, Rossle M, Gerbes AL. Hepatic arterial buffer response in patients with advanced cirrhosis. *Hepatology* 2002; **35**: 630-634
  - 23 **Dragoteanu M**, Cotul SO, Piglesan C, Tamas S. Liver angioscintigraphy: clinical applications. *Rom J Gastroenterol* 2004; **13**: 55-63
  - 24 **Santambrogio R**, Bruno S, Opocher E, Galeotti F, Zatta G, Grugni M, Macri M, Pisani A, Tarolo G, Spina G. Angioscintigraphic assessment of hemodynamic effects of penbutolol in cirrhotics with portal hypertension. A double-blind, randomized, controlled study. *Hepatogastroenterology* 1990; **37**: 398-402
  - 25 **Zatta G**, Santambrogio R, Boccolari S, Mana O, Gattoni F, Baldini U, Galeotti F, Opocher E, Spina GP, Tarolo GL. Angioscintigraphic assessment of arterial and portal liver blood flow: comparison with splanchnic angiography. *Nuklearmedizin* 1987; **26**: 83-86
  - 26 **Sarper R**, Tarcan YA. An improved method of estimating the portal venous fraction of total hepatic blood flow from computerized radionuclide angiography. *Radiology* 1983; **147**: 559-562
  - 27 **Huet PM**, Pomier-Layrargues G, Villeneuve JP, Varin F, Viallet A. Intrahepatic circulation in liver disease. *Semin Liver Dis* 1986; **6**: 277-286
  - 28 **Chin N**, Ohnishi K, Iida S, Nomura F. Role of intrahepatic portal-systemic shunts in the reduction of portal blood supply to liver cells in cirrhosis. *Am J Gastroenterol* 1988; **83**: 718-722
  - 29 **Tiani C**, Abralles JG, Bosch J. Portal hypertension: pre-primary and primary prophylaxis of variceal bleeding. *Dig Liver Dis* 2008; **40**: 318-327
  - 30 **Hartleb M**, Boldys H, Rudzki K, Nowak A, Nowak S. Portal shunting in inferior mesenteric vein in cirrhosis: correlation with hemorrhage from esophageal varices. *Am J Gastroenterol* 1994; **89**: 863-867

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## Combination of small interfering RNAs mediates greater suppression on hepatitis B virus cccDNA in HepG2.2.15 cells

Xiao-Min Xin, Gui-Qiu Li, Ying-Yu Jin, Min Zhuang, Di Li

Xiao-Min Xin, Gui-Qiu Li, Ying-Yu Jin, Department of Laboratory Diagnosis, the First Affiliated Hospital of Harbin Medical University, Harbin 150081, Heilongjiang Province, China

Min Zhuang, Di Li, Department of Microbiology, Harbin Medical University, Harbin 150081, Heilongjiang Province, China

**Author contributions:** Xin XM and Li GQ contributed equally to this work; Xin XM, Li GQ designed and performed the research; Jin YY, Li D, Zhuang M analyzed the data.

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**Correspondence to:** Di Li, Department of Microbiology, Harbin Medical University, Harbin 150081, Heilongjiang Province, China. [lq7566@126.com](mailto:lq7566@126.com)

**Telephone:** +86-451-86685722 **Fax:** +86-451-86685722

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expression in HepG2.2.15 cells, especially on cccDNA amplification.

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**Key words:** Combination of small interfering RNAs; Covalently closed circular DNA; Hepatitis B virus; RNA interference; HepG2.2.15 cells

**Peer reviewers:** Akihito Tsubota, Assistant Professor, Institute of Clinical Medicine and Research, Jikei University School of Medicine, 163-1 Kashiwa-shita, Kashiwa, Chiba 277-8567, Japan; Jian Wu, Associate Professor of Medicine, Internal Medicine/Transplant Research Program, University of California, Davis Medical Center, 4635 2nd Ave. Suite 1001, Sacramento CA 95817, United States; Fumio Imazeki, MD, Department of Medicine and Clinical Oncology, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8670, Japan; Kazuhiro Hanazaki, MD, Professor and Chairman, Department of Surgery, Kochi Medical School, Kochi University, Kohasu, Okohcho, Nankoku, Kochi 783-8505, Japan

### Abstract

**AIM:** To observe the inhibition of hepatitis B virus (HBV) replication and expression in HepG2.2.15 cells by combination of small interfering RNAs (siRNAs).

**METHODS:** Recombinant plasmid psil-HBV was constructed and transfected into HepG2.2.15 cells. At 48 h, 72 h and 96 h after transfection, culture media were collected and cells were harvested for HBV replication assay. HBsAg and HBeAg in the cell culture medium were detected by enzyme-linked immunosorbent assay (ELISA). Intracellular viral DNA and covalently closed circular DNA (cccDNA) were quantified by real-time polymerase chain reaction (PCR). HBV viral mRNA was reverse transcribed and quantified by reverse-transcript PCR (RT-PCR).

**RESULTS:** siRNAs showed marked anti-HBV effects. siRNAs could specifically inhibit the expression of HBsAg and the replication of HBV DNA in a dose-dependent manner. Furthermore, combination of siRNAs, compared with individual use of each siRNA, exerted a stronger inhibition on antigen expression and viral replication. More importantly, combination of siRNAs significantly suppressed HBV cccDNA amplification.

**CONCLUSION:** Combination of siRNAs mediates a stronger inhibition on viral replication and antigen

Xin XM, Li GQ, Jin YY, Zhuang M, Li D. Combination of small interfering RNAs mediates greater suppression on hepatitis B virus cccDNA in HepG2.2.15 cells. *World J Gastroenterol* 2008; 14(24): 3849-3854 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3849.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3849>

### INTRODUCTION

Hepatitis B virus (HBV) is a major cause for acute and chronic hepatitis in humans. Although recombinant vaccines are widely available, about 400 million people have chronic HBV infection worldwide. Chronic infection may also have serious consequences, and nearly 25% people with chronic HBV infection would die due to untreatable liver cancer<sup>[1]</sup>. The deaths of liver cancer patients resulting from chronic HBV infection exceeds one million per year worldwide<sup>[2]</sup>. Therefore, it is important to develop effective strategies for the treatment of HBV-infected patients.

Nucleotide analogues, such as lamivudine, can effectively inhibit HBV DNA synthesis<sup>[3,4]</sup> and are widely used in the treatment of HBV-infected patients. However, analysis of viral kinetics during lamivudine therapy revealed that a prolonged treatment is required since

lamivudine does not completely inhibit viral replication and the rate of clearance for infected hepatocytes is slow<sup>[5]</sup>. HBV is not a cytopathogenic virus and hepatocytes are normally long-lived and their half-life is estimated to be 6-12 mo or longer, explaining the requirement for a long-term antiviral treatment course, which is associated with the selection of drug-resistant mutants<sup>[6]</sup>.

During HBV replication, viral covalently closed circular DNA (cccDNA) serves as the template for viral transcription and its production is regulated and amplified by an intra-cellular pathway in which newly synthesized genomic DNA is recycled to the nuclei<sup>[7,8]</sup>. This process establishes a steady pool of nuclear cccDNA, which is maintained during the life of infected hepatocytes. It is likely that cccDNA may reactivate synthesis of viral transcript and protein, leading to a rebound of active viral replication. Thus, elimination of cccDNA from infected hepatocytes still remains a challenge in therapy for HBV-infected patients. A potent agent used in anti-HBV therapy should be evaluated with special emphasis on its inhibitory effect against the amplification of cccDNA.

Small interfering RNAs (siRNA) are double-stranded RNA molecules, approximately 21 nucleotides in length that hybridize to a homologous mRNA target and result in degradation of mRNA<sup>[9]</sup>. This posttranscriptional gene silencing process, called RNA interference (RNAi), is evolutionarily conserved in both plants and eukaryotic cells<sup>[10,11]</sup>. Due to the high sequence specificity and efficiency, RNAi can be used in functional genomic studies such as human immunodeficiency virus (HIV), hepatitis C virus (HCV), and influenza virus, *etc*<sup>[12-14]</sup>. HBV is one of the major candidates for RNAi, as its pregenomic RNA is a key intermediate for maintaining DNA replication *via* reverse transcription in the viral life cycle. Many studies have demonstrated that siRNA targeting different regions of the HBV genome can block viral replication and antigen expression<sup>[15-19]</sup>. Our previous study showed that siRNA targeting HBV nuclear localization signal (NLS) can inhibit viral DNA synthesis and cccDNA amplification<sup>[20]</sup>. As HBV infection is a cellular infection involving multiple genes, we hypothesize that combination of siRNAs targeting different sequences along the HBV NLS mediates a greater inhibitory efficacy on cccDNA amplification. If this is the case, it would be of significance in the treatment of HBV infection. In this study, we analyzed the kinetics of HBV genome replication during siRNA therapy and tested the antiviral capacity of combination therapy in comparison to monotherapy in HepG2.2.15 cells, showing that combination of siRNAs exhibits better effects on the inhibition of HBV replication, especially at viral cccDNA level.

## MATERIALS AND METHODS

### Materials

Dulbecco's modified Eagle's medium (DMEM) and G418 were purchased from GIBCO BRL (USA). HepG2.2.15 cells were maintained in our laboratory.

Plasmid psi/U6 was supplied by Wuhan JS Biotech (China). All polymerase chain reaction (PCR) primers were synthesized by Shanghai Boya Biological Company (China). Trizol, M-MLV reverse transcriptase, Lipofection 2000 reagent were purchased from Invitrogen Company (USA). Restriction enzymes were purchased from New England Bio-laboratory (Beijing, China).

### Construction of siRNA express vector

siRNA expression plasmid was generated as previously described<sup>[21]</sup>. Briefly, 21-nucleotide long inverted sequences were cloned into the plasmid pGenesil/U6. Five thymidines were inserted into the downstream anti-sense strand to provide a stop signal for the polymerase III RNA polymerase. The sense strand of hairpin was homologous to the target mRNA (NLS region) as analyzed by BLAST in the NCBI database. Oligonucleotides used to code for the sense strand of siRNA included siRNA1 (5'-AAG ATCTCAATCTCGGGAATC-3'), siRNA2 (5'-CAGGT CCCCTAGAAGAAGAAC-3'), and control siRNAHK (5'-ACTACCGTTGTATAGGTG-3'). All the plasmids constructed were confirmed by endonuclease digestion and DNA sequencing.

### Cell culture and transfection

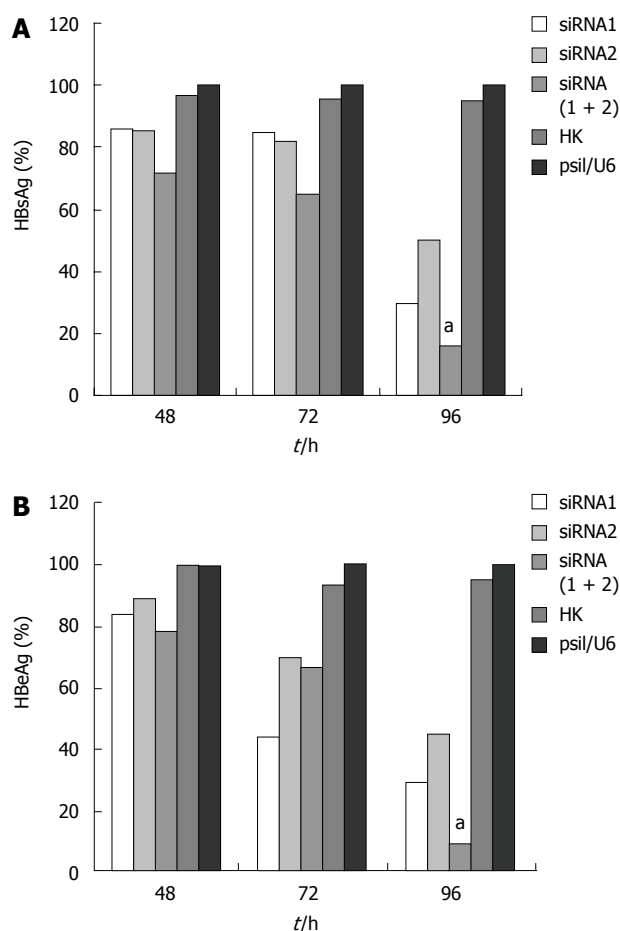
The human hepatoma cell line, HepG2.2.15, was maintained in Dubecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum and 200 µg/mL G418 in 5% CO<sub>2</sub>-humidified air as previously described<sup>[22]</sup>. Cells were cultured at a density of  $3 \times 10^5$  cells per well in 6-well plates. Twenty-four hours after incubation, the cells were transfected with 4 µg siRNA-expressed plasmid using the Lipofection 2000 reagent. We removed the medium, washed the cells with warmed PBS, and added fresh medium every 24 h. At 48, 72 and 96 h after transfection, culture media were collected and cells were harvested for HBV replication assay. All experiments were performed in triplicate and divided into five groups.

### Detection of HBsAg and HBeAg

To assess the effects of RNAi on viral antigen expression, HBsAg and HBeAg levels in culture medium were measured with an enzyme-linked immunosorbent assay (ELISA) kit following its manufacture's instructions.

### Assay of HBV mRNA

Antiviral activities were evaluated by reverse-transcript PCR (RT-PCR). Total RNA was extracted directly from the transfected cells using Trizol reagent. Then, RNA was denatured for 5 min at 70°C, immediately cooled in ice water, reverse transcribed using M-MLV reverse transcriptase. A RT-PCR experiment targeting β-actin gene was run as an internal control. The primer sequences used are HBV forward (5'-ACCTC TGCCTAATCATCTC-3') and reverse (5'-GTAAG ACAGGAAATGTGAAAC-3'), β-actin forward (5'-GTCGGTGTGAACGGATT-3') and reverse (5'-ACTCCACGACGTACTCAGC-3'). The PCR



**Figure 1** Relative expression of HBsAg (A) and HBeAg (B). <sup>a</sup> $P < 0.05$  vs siRNA1 or siRNA2.

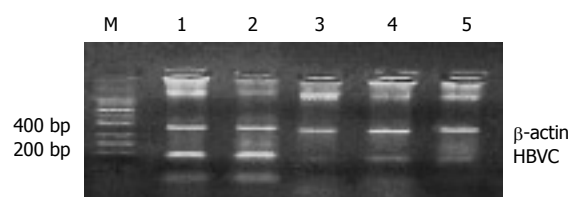
amplification product was analyzed by 1.2% agarose gel electrophoresis.

### Quantitative real-time PCR

Real-time PCR was performed to quantify the HBV DNA or cccDNA using a HBV fluorescence detection kit following its manufacturer's protocol. For measurement of viral DNA, DNA was extracted from the culture supernatant using a QIAamp DNA mini kit. cccDNA was isolated from the transfection cells to quantify its level and examined every 24 h post-transfection according to its manufacture's protocol. Reactions with no reverse transcriptase enzyme added were performed in parallel. The inhibition ratio of HBV DNA was calculated according to the formula:  $1 - \log(\text{treated sample fluorescent intensity}) / \log(\text{control fluorescent intensity}) \times 100\%$ .

### Dose-dependent inhibitory effect of single siRNA

To evaluate the dose-dependent effects of siRNA on HBV gene expression, a series of experiments were conducted in HepG2.2.15 cells transfected with different plasmids at the concentration of 2  $\mu\text{g}$ , 3  $\mu\text{g}$  and 4  $\mu\text{g}$ , respectively. The transfection cells were harvested and cell culture supernatant was collected 96 h post-transfection for further examination. Several parameters



**Figure 2** Reduction in HBV mRNA level after treatment with siRNA. M: marker, lane 1: HK, lane 2: psil/U6, lane 3: siRNAs 1 and 2, lane 4: siRNA2, lane 5: siRNA1.

of HBV were measured including HBsAg, HBV DNA as described above.

### Statistical analysis

Statistical analysis was performed using the SPSS 12.0 software. The results were expressed as mean  $\pm$  SD and compared using *F*-test and one-way ANOVA.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Inhibition of the expression of HBsAg and HBeAg

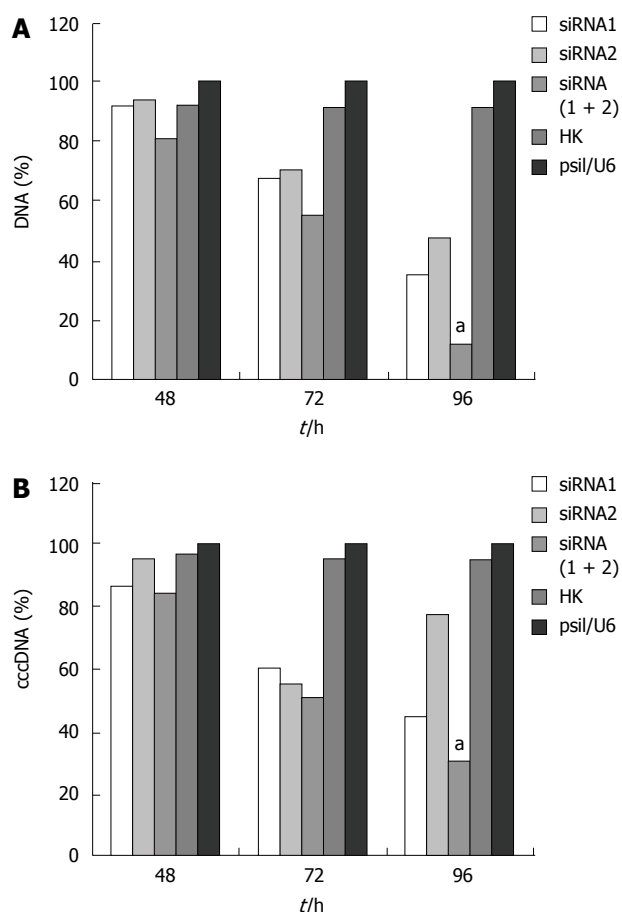
To test whether siRNA could effectively inhibit the expression of viral proteins in HepG2.2.15 cells, HBsAg and HBeAg in culture media were detected by ELISA. For HBsAg, cells transfected with the control siRNA produced an equal secretion with those treated with empty vector ( $P < 0.05$ ) as expected in the absence of any specific silencing response, whereas cells expressing HBV siRNA gave a very different result. The HBsAg level in all the cells integrated with three siRNAs was significantly reduced compared with those transfected with empty vector, and the greatest reduction rate was 83.89% in the combined therapy group 96 h post-transfection (Figure 1A). HBeAg was also reduced in the selected siRNA-transfected cells, and the greatest reduction rate was 91.07% in the combined therapy group 96 h post-transfection (Figure 1B). For both HBsAg and HBeAg, combination therapy for siRNAs was more potent than any individual therapy.

### Reduction of HBV mRNA level

To determine whether viral mRNA is efficiently degraded by siRNA, RT-PCR was performed 48 h, 72 h and 96 h after transfection. The results demonstrated that the mRNA level of each tested siRNA was markedly reduced, whereas the empty plasmid had no effect on mRNA level (Figure 2). Combination therapy with all the siRNAs produced a stronger inhibition on viral transcripts compared with the therapy with one siRNA. The HepG2.2.15 cells treated with combined siRNAs for 96 h did not contain any detectable mRNA, whereas mRNA was abundant in cells treated with empty vector.

### Reduction of copies of viral DNA and cccDNA

The effect of siRNA silencing on HBV DNA was investigated by quantitative real-time PCR, which can detect  $10^4$ - $10^8$  copies of HBV DNA. The level of viral



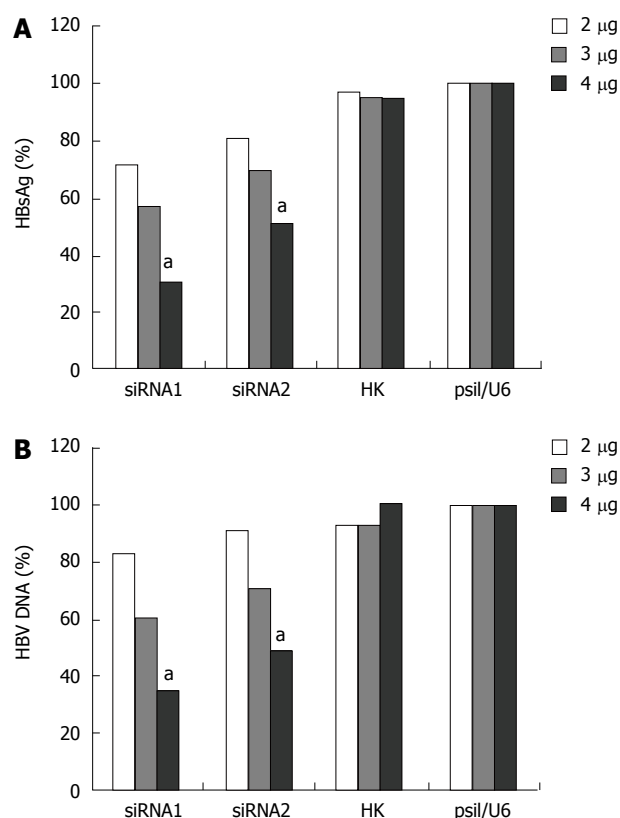
**Figure 3** Inhibition of HBV DNA (A) and cccDNA (B) amplification after treatment with siRNA. <sup>a</sup> $P < 0.05$  vs siRNA1 or siRNA2.

DNA in cells transfected with combined siRNAs was significantly lower than that in cells treated with empty vector. Treatment with irrelevant control siRNA slightly decreased the level of viral DNA in cells transfected with siRNA (Figure 3A). Furthermore, combined siRNA1 and siRNA2 showed a greater inhibitory effect (88.6%) on viral DNA replication than siRNA1 or siRNA2 alone ( $P < 0.05$ ).

HBV cccDNA is an important parameter in the therapy for chronic HBV infection. To evaluate if combination of siRNAs can effectively inhibit viral cccDNA, we isolated viral cccDNA from the transfected cells every 24 h post-transfection. Quantitative assay revealed that HBV cccDNA levels were decreased by 50.4%, 22.31%, and 69.83% in the cells transfected with siRNA1, siRNA2 and siRNA (1 + 2), respectively, compared to the level of cells treated with empty plasmid 96 h post-transfection. Meanwhile, HBV cccDNA levels did not significantly change in cells treated with control siRNA and empty vector. As expected, the use of siRNA1 and siRNA2 synergistically suppressed the viral cccDNA activities compared with siRNA1 or siRNA2 alone ( $P < 0.05$ , Figure 3B). These results were reproducibly observed in three independent experiments.

#### Dose-dependent effect of siRNA

As shown in Figure 4, siRNA had an obvious dose-



**Figure 4** Dose-dependent inhibitory effects of siRNA on HBV antigen HBsAg expression (A) and HBV replication (B). <sup>a</sup> $P < 0.05$  vs 2 μg or 3 μg.

dependent effect on HBV replication and antigen expression 96 h post-transfection. When the cells were transfected with a low concentration of siRNA, siRNA had almost no effect on HBV replication and antigen expression. However, a more significant inhibition of HBsAg and DNA in the cells treated at the concentration of 4 μg was observed. The level of HBsAg was decreased by 29%, 43% or 70% after siRNA1 treatment, and 9%, 30% or 50% after siRNA2 treatment, respectively, at the concentrations of 2 μg, 3 μg, 4 μg (Figure 4A). The level of HBV DNA was reduced by 17%, 39% or 65% after siRNA1 treatment, and 9%, 29% or 51% after siRNA2 treatment, respectively, at the concentrations of 2 μg, 3 μg, 4 μg (Figure 4B).

## DISCUSSION

Chronic HBV infection is one of the most serious diseases threatening human health. RNAi technology provides an alternative strategy to combat HBV infection<sup>[23,24]</sup>. It was reported that viral mutations could escape single synthetic siRNA treatments, such as HBV, HCV and HIV<sup>[25-27]</sup>. One strategy to address the problem is to generate multiple siRNA molecules that can target different sites on the viral genome. The other is to choose target sequences in the relatively conserved region. Recently, combination therapy has emerged as a new approach to the treatment of chronic HBV infection with the objective to decrease the viral load



to the lowest possible levels<sup>[28,29]</sup>. This is then followed by a continued chemotherapy with another nucleotide analog or IFN- $\alpha$  in order to eliminate the remained viral load. Following the same strategy, we also investigated the effect of combination of siRNAs in comparison to monotherapy in HepG2.2.15 cells. As HBV infection is a pathological process involving multiple genes, it will be ideal to develop a combination strategy for siRNAs that can knock down the expression of multiple pathogenic viral antigens as well as inhibit viral replication. During the progress of combination therapy, several siRNAs may block multiple sites in viral genome and make it difficult to repair immediately, thus achieving greater suppression on HBV infection than a single siRNA.

The HepG2.2.15 cell line, a derivative of human HepG2 hepatoma cells, has been used as an *in vitro* stable HBV-producing model<sup>[22]</sup>. The cell line can be transformed with a head-tail dimer of HBV DNA. Using this method, we could simulate the natural condition in which cells can still stably produce mRNA, antigen and viral particles. Specific siRNA targeting different sites on HBV NLS region were transfected and monitored in HepG2.2.15 cells. As a result, the load of HBsAg, HBeAg, and mRNA was inhibited to some extent. Meanwhile, real-time PCR revealed that the number of viral DNA and cccDNA copies was markedly decreased. This inhibition was highly selective, sequence-specific and dose-dependent since control siRNA had almost no inhibitory effect on the expression or replication of HBV.

The selected siRNAs showed marked anti-HBV effects. Surprisingly, combination of siRNAs, compared with a single siRNA, exerted a stronger inhibition on antigen expression and viral replication, even though the final concentration of siRNA used in the therapy was the same. More importantly, combination therapy significantly suppressed HBV cccDNA amplification.

In summary, combination of siRNAs targeting various regions of HBV NLS inhibits viral replication and suppresses cccDNA amplification and can be used in the treatment of HBV infection.

## COMMENTS

### Background

Hepatitis B virus (HBV) is a major cause for acute and chronic hepatitis in humans. The development of an effective therapy for HBV infection is still a challenge. As HBV infection is a cellular infection involving multiple genes, progress in RNA interference (RNAi) has shed light on developing a new anti-HBV strategy.

### Research frontiers

siRNA targeting HBV nuclear localization signal (NLS) can inhibit viral DNA synthesis and cccDNA amplification. In this study, the authors hypothesize that combination of siRNAs targeting different sequences along HBV NLS mediates greater inhibitory effects on cccDNA. If this is the case, it would be of significance in the treatment of HBV infection.

### Innovations and breakthroughs

In this study, the selected siRNA showed marked anti-HBV effects. Surprisingly, combination of siRNAs, compared with a single siRNA, exerted a stronger inhibition on antigen expression and viral replication, even though the final concentration of siRNA used in the therapy was the same. More importantly, combination therapy significantly suppressed HBV cccDNA amplification.

## Applications

Combination of siRNAs targeting various regions of HBV NLS not only inhibits viral replication, but also suppresses cccDNA amplification, indicating that it can be used in the treatment of HBV infection.

## Peer review

The effect of combination of siRNAs on suppressing HBsAg and HBeAg expression, and levels of HBV mRNA, DNA and cccDNA *in vitro* was studied in this study. This manuscript provides some important information about the treatment of chronic HBV infection. It is very interesting paper and has certain value.

## REFERENCES

- 1 **Kao JH**, Chen DS. Global control of hepatitis B virus infection. *Lancet Infect Dis* 2002; **2**: 395-403
- 2 **Seeger C**, Mason WS. Hepatitis B virus biology. *Microbiol Mol Biol Rev* 2000; **64**: 51-68
- 3 **Ganem D**, Prince AM. Hepatitis B virus infection--natural history and clinical consequences. *N Engl J Med* 2004; **350**: 1118-1129
- 4 **Papatheodoridis GV**, Dimou E, Papadimitropoulos V. Nucleoside analogues for chronic hepatitis B: antiviral efficacy and viral resistance. *Am J Gastroenterol* 2002; **97**: 1618-1628
- 5 **Ayres A**, Bartholomeusz A, Lau G, Lam KC, Lee JY, Locarnini S. Lamivudine and Famciclovir resistant hepatitis B virus associated with fatal hepatic failure. *J Clin Virol* 2003; **27**: 111-116
- 6 **Lau DT**, Khokhar MF, Doo E, Ghany MG, Herion D, Park Y, Kleiner DE, Schmid P, Condreay LD, Gauthier J, Kuhns MC, Liang TJ, Hoofnagle JH. Long-term therapy of chronic hepatitis B with lamivudine. *Hepatology* 2000; **32**: 828-834
- 7 **Tuttleman JS**, Pourcel C, Summers J. Formation of the pool of covalently closed circular viral DNA in hepadnavirus-infected cells. *Cell* 1986; **47**: 451-460
- 8 **Rollier C**, Sunyach C, Barraud L, Madani N, Jamard C, Trepo C, Cova L. Protective and therapeutic effect of DNA-based immunization against hepadnavirus large envelope protein. *Gastroenterology* 1999; **116**: 658-665
- 9 **Brummelkamp TR**, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. *Science* 2002; **296**: 550-553
- 10 **Chuang CF**, Meyerowitz EM. Specific and heritable genetic interference by double-stranded RNA in Arabidopsis thaliana. *Proc Natl Acad Sci USA* 2000; **97**: 4985-4990
- 11 **Matzke M**, Matzke AJ, Kooter JM. RNA: guiding gene silencing. *Science* 2001; **293**: 1080-1083
- 12 **Jacque JM**, Triques K, Stevenson M. Modulation of HIV-1 replication by RNA interference. *Nature* 2002; **418**: 435-438
- 13 **Kapadia SB**, Brideau-Andersen A, Chisari FV. Interference of hepatitis C virus RNA replication by short interfering RNAs. *Proc Natl Acad Sci USA* 2003; **100**: 2014-2018
- 14 **Gitlin L**, Karelsky S, Andino R. Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* 2002; **418**: 430-434
- 15 **Yu JY**, DeRuiter SL, Turner DL. RNA interference by expression of short-interfering RNAs and hairpin RNAs in mammalian cells. *Proc Natl Acad Sci USA* 2002; **99**: 6047-6052
- 16 **Tang N**, Huang AL, Zhang BQ, Yan G, He TC. [Potent and specific inhibition of hepatitis B virus antigen expression by RNA interference] *Zhonghua Yixue Zazhi* 2003; **83**: 1309-1312
- 17 **Uprichard SL**, Boyd B, Althage A, Chisari FV. Clearance of hepatitis B virus from the liver of transgenic mice by short hairpin RNAs. *Proc Natl Acad Sci USA* 2005; **102**: 773-778
- 18 **Shlomai A**, Shaul Y. Inhibition of hepatitis B virus expression and replication by RNA interference. *Hepatology* 2003; **37**: 764-770
- 19 **Wu KL**, Zhang X, Zhang J, Yang Y, Mu YX, Liu M, Lu L, Li Y, Zhu Y, Wu J. Inhibition of Hepatitis B virus gene expression by single and dual small interfering RNA

- treatment. *Virus Res* 2005; **112**: 100-107
- 20 **Li GQ**, Gu HX, Li D, Xu WZ. Inhibition of Hepatitis B virus cccDNA replication by siRNA. *Biochem Biophys Res Commun* 2007; **355**: 404-408
- 21 **Sui G**, Soohoo C, Affar el B, Gay F, Shi Y, Forrester WC, Shi Y. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. *Proc Natl Acad Sci USA* 2002; **99**: 5515-5520
- 22 **Sells MA**, Chen ML, Acs G. Production of hepatitis B virus particles in Hep G2 cells transfected with cloned hepatitis B virus DNA. *Proc Natl Acad Sci USA* 1987; **84**: 1005-1009
- 23 **Fire A**, Xu S, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998; **391**: 806-811
- 24 **Zhang XN**, Xiong W, Wang JD, Hu YW, Xiang L, Yuan ZH. siRNA-mediated inhibition of HBV replication and expression. *World J Gastroenterol* 2004; **10**: 2967-2971
- 25 **Das AT**, Brummelkamp TR, Westerhout EM, Vink M, Madiredjo M, Bernards R, Berkhout B. Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. *J Virol* 2004; **78**: 2601-2605
- 26 **Konishi M**, Wu CH, Kaito M, Hayashi K, Watanabe S, Adachi Y, Wu GY. siRNA-resistance in treated HCV replicon cells is correlated with the development of specific HCV mutations. *J Viral Hepat* 2006; **13**: 756-761
- 27 **Wu HL**, Huang LR, Huang CC, Lai HL, Liu CJ, Huang YT, Hsu YW, Lu CY, Chen DS, Chen PJ. RNA interference-mediated control of hepatitis B virus and emergence of resistant mutant. *Gastroenterology* 2005; **128**: 708-716
- 28 **Li GQ**, Xu WZ, Wang JX, Deng WW, Li D, Gu HX. Combination of small interfering RNA and lamivudine on inhibition of human B virus replication in HepG2.2.15 cells. *World J Gastroenterol* 2007; **13**: 2324-2327
- 29 **Colledge D**, Civitico G, Locarnini S, Shaw T. In vitro antihepadnaviral activities of combinations of penciclovir, lamivudine, and adefovir. *Antimicrob Agents Chemother* 2000; **44**: 551-560

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# Treatment of *Helicobacter pylori* in surgical practice: A randomised trial of triple versus quadruple therapy in a rural district general hospital

Siok Siong Ching, Sivakumaran Sabanathan, Lloyd R Jenkinson

Siok Siong Ching, Clinical Research Fellow in General Surgery, Leeds General Infirmary, Leeds, West Yorkshire LS1 3EX, England, United Kingdom

Sivakumaran Sabanathan, Lloyd R Jenkinson, Department of Surgery, Ysbyty Gwynedd, Bangor, Gwynedd LL57 2PW, Wales, United Kingdom

**Author contributions:** Ching SS contributed to the design and set up of the study, he also analysed and interpreted the data and wrote the draft manuscript; Sabanathan S contributed substantially to the recruitment of patients, acquisition of data for the study, and preparation of the manuscript; Jenkinson LR contributed substantially to the conception, administration support and overall supervision of the study, he also contributed substantially to the recruitment of patients and critically revised the manuscript.

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**Correspondence to:** Lloyd R Jenkinson, Department of Surgery, Ysbyty Gwynedd, Penrhosgarnedd, Bangor, Gwynedd LL57 2PW, Wales, United Kingdom. [lloydjenk@btinternet.com](mailto:lloydjenk@btinternet.com)  
Telephone: +44-124-8384308 Fax: +44-124-8384675

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## Abstract

**AIM:** To compare a lansoprazole-based triple versus quadruple therapy for *Helicobacter pylori* (*H. pylori*) eradication with emphasis on side effect profile, patient compliance and eradication rate at a rural district general hospital in Wales, United Kingdom.

**METHODS:** One hundred one patients with *H. pylori* infection were included in the study. Patients were randomised to receive triple therapy comprising of lansoprazole 30 mg, amoxycillin 1 g, clarithromycin 500 mg, all *b.d.* (LAC), or quadruple therapy comprising of lansoprazole 30 mg *b.d.*, metronidazole 500 mg *t.d.s.*, bismuth subcitrate 240 mg *b.d.*, and tetracycline chloride 500 mg *q.d.s.* (LMBT). Cure was defined as a negative <sup>13</sup>C urea breath test 2 mo after treatment.

**RESULTS:** Seven patients were withdrawn after randomisation. Fifty patients were assigned to LAC group and 44 to LMBT group. The intention-to-treat cure rates were 92% and 91%, whereas the per-protocol cure rates were 92% and 97%, respectively. Side effects were common, with 56% experiencing

moderate to severe symptoms in the LAC group and 59% in the LMBT group. Symptoms of vomiting, diarrhoea and black stools were significantly more common in the LMBT group. Patient compliance was 100% for triple therapy and 86% for quadruple therapy ( $P < 0.01$ ). One-third of patients in both groups were still taking acid-reducing medications at six-month follow-up.

**CONCLUSION:** One-week triple and quadruple therapies have similar intention-to-treat eradication rates. Certain side effects are more common with quadruple therapy, which can compromise patient compliance. Patient education or modifications to the regimen are alternative options to improve compliance of the quadruple regimen.

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**Key words:** *Helicobacter pylori*; Triple therapy; Quadruple therapy; Side effects; Treatment compliance; Eradication rate

**Peer reviewer:** Marco Romano, MD, Professor, Dipartimento di Internistica Clinica e Sperimentale-Gastroenterologia, II Policlinico, Edificio 3, II piano, Via Pansini 5, Napoli 80131, Italy

Ching SS, Sabanathan S, Jenkinson LR. Treatment of *Helicobacter pylori* in surgical practice: A randomised trial of triple versus quadruple therapy in a rural district general hospital. *World J Gastroenterol* 2008; 14(24): 3855-3860 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3855.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3855>

## INTRODUCTION

European studies have shown that quadruple therapy, even though more effective with a cure rate of over 95% by per protocol analysis<sup>[1-3]</sup>, is less popular compared to a standard triple therapy for eradication of *Helicobacter pylori* (*H. pylori*) infection. The reasons for this are the complexity of the regimen and also its side effects. Scheduling drugs four or more times a day reduces compliance<sup>[4,5]</sup>. However, some studies have suggested that quadruple therapy has a similar magnitude of

adherence and adverse effects compared to triple therapies<sup>[6,7]</sup>.

Triple therapies are the mainstay of current treatment but resistance to clarithromycin is reducing its effectiveness. In the presence of resistance to clarithromycin, some studies have shown eradication rate below 80% and even as low as 25%-61% with standard triple therapy containing clarithromycin, amoxycillin and a proton-pump inhibitor<sup>[7-11]</sup>. Clarithromycin resistance is also increasing in our region<sup>[12,13]</sup>.

Quadruple therapy is used mainly as a second-line therapy after failed eradication with triple therapy<sup>[14-18]</sup>. Earlier consensus meeting reports including the Maastricht II Consensus Report on the management of *H pylori* infection have recommended the use of quadruple therapy for 1 wk as second-line therapy for *H pylori* infection<sup>[19-21]</sup>. However, updated reports have now recommended quadruple therapy as an alternative first-line eradication therapy<sup>[22-24]</sup>.

The objective of the study was to compare a standard lansoprazole-based triple therapy (HeliClear®) to a lansoprazole-based quadruple therapy as first-line therapy in a surgical practice in a predominantly Caucasian population in North Wales.

## MATERIALS AND METHODS

We conducted a prospective randomised trial of patients under the care of an upper gastrointestinal surgeon at Ysbyty Gwynedd, a rural District General Hospital in North Wales. The population served by Ysbyty Gwynedd is predominantly (98.8%) white and there are about 120 new cases of *H pylori* each year from a population of around 180 000. Twenty-four percent of strains were resistant to metronidazole, 7% to clarithromycin and 4% to both. There was resistance to tetracycline in 1 out of 363 isolates and none to amoxycillin<sup>[12]</sup>.

The Local Ethics Committee of the participating hospitals approved the study. From June 2001 to November 2005, 101 patients with diagnosis of *H pylori* infection proven by gastric histology or urease test or culture were included in the study. Two positive tests were required for inclusion. The inclusion and exclusion criteria are shown in Table 1.

Patients were recruited into the trial once they had met the criteria and given fully informed written consent. Patients were recruited from the outpatient departments at one district general hospital and a satellite hospital served by the same team of doctors. The patients received a 7-d course of either a triple regimen (LAC) or a quadruple regimen (LMBT) (Table 2).

Randomisation took place at the hospital pharmacies when the patients collected their medications with a note from the recruiting doctor. The pharmacists dispensed the medications adhering to the order on a random list of therapy regimens.

A printed chart showing the names of the drugs, the number of pills to take and the time schedule was given to all participants to improve understanding and

Table 1 Inclusion criteria and exclusion criteria

Criteria	
Inclusion criteria	Dyspeptic symptoms Has recent OGD (duodenal ulcer; gastric ulcer; gastritis or non-ulcer dyspepsia) Positive for <i>H pylori</i> on histology and culture or CLO test or <sup>13</sup> C-urea breath test
Exclusion criteria	Age less than 18 or above 75 yr Symptomatic gallstones Treated with antibiotic or bismuth-containing drugs during the month prior to inclusion Treated with proton pump inhibitor during the week prior to inclusion Disturbed gastrointestinal physiology (gastric surgery; vagotomy; Zollinger-Ellison syndrome; chronic ingestion of NSAIDs) Concomitant serious disease Concomitant medications that may adversely interact with the study drugs (e.g. warfarin, anti-epileptics) Pregnancy and breast-feeding Childbearing age without adequate contraception Allergy to drugs used in the study Mental illness Heavy drinking or abuse of drugs

Table 2 Regimens used in the trial

Triple therapy regimen (LAC)	Quadruple therapy regimen (LMBT)
Lansoprazole (30 mg b.d.)	Lansoprazole (30 mg b.d.)
Amoxycillin (1 g b.d.)	Metronidazole (400 mg t.d.s.)
Clarithromycin (500 mg b.d.)	Bismuth subcitrate (240 mg b.d.) Tetracycline chloride (500 mg q.d.s.)

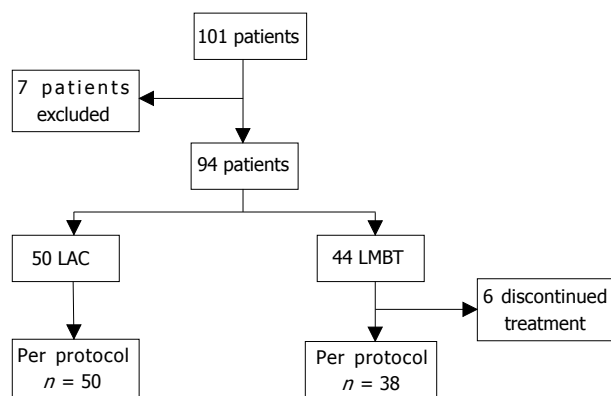
compliance with treatment.

Compliance was evaluated by patient's record of each dosage taken onto the chart during the week of therapy. Any tablet that was not consumed needed to be brought back to the clinic for pill count. The patients were asked to record the reasons for missed dosages. They were also asked to record any side effects and their severity during the therapy. Proton pump inhibitors and other acid-reducing medications were not allowed after treatment. The patients returned for interview at 6 wk after therapy. The efficacy of treatment was evaluated by means of the <sup>13</sup>C-urea breath test performed following the standard European protocol at 8 wk following the start of therapy<sup>[12]</sup>. Patients were reviewed again at 6 mo after therapy to assess symptoms and use of any medications after determining their post therapy *H pylori* status. Patients who tested positive were offered the alternate regimen and retested after a gap of 2 mo.

## Statistical analysis

Proportions were compared using Fisher's Exact Test. Quantitative variables were compared using *t*-test and non-parametric variables were compared using Mann-Whitney *U* test. Non-categorical values are given as the mean ± SD. Calculations were performed using the SPSS for Windows statistical package.





**Figure 1** CONSORT flow diagram showing entries and withdrawals from the study.

**Table 3** Patient characteristics

	Therapy	
	LAC (n = 50)	LMBT (n = 44)
Age	55.2 ± 10.9	53.7 ± 11.4
Gender (male: female)	26:24	27:17
Active smoking	10 (20%)	16 (36%)
NSAID use	4 (8%)	3 (7%)
Ethanol abuse (> 14 U/wk)	4 (8%)	3 (7%)
Previous therapy with antacids	4 (8%)	8 (18%)
Time between treatment and UBT (mo)	2.2 ± 0.7	2.1 ± 0.5
Gastric ulcer	1	1
Duodenal ulcer	3	1
Gastritis	36	33
Duodenitis	6	8
Diagnosis of <i>H pylori</i> infection (Urease: Culture: Biopsy)	42:29:45	37:27:44

NSAID: Non-steroidal anti-inflammatory drug; UBT: Urea breath test.

## RESULTS

One hundred one patients were randomized into the trial but seven patients were withdrawn from the study after randomization (one because of diagnosis of bronchial carcinoma, one because of diagnosis of gallstones, two withdrew from the study and three were non-compliant to study protocol) (Figure 1).

Fifty patients were assigned to the LAC group and 44 to the LMBT group. The demographic and clinical characteristics of the groups were comparable (Table 3).

### Compliance and side-effects

Compliance was excellent in the LAC group with all the patients completing the 7-d therapy. In contrast, 6 patients (14%) in the LMBT group failed to complete the treatment ( $P < 0.01$ ). In spite of this three had a negative breath test.

Four out of the six patients had attributed moderate/severe nausea as the reason for discontinuing treatment. One had severe diarrhoea and another had nausea, vomiting and diarrhoea.

Side effects were reported by vast majority of patients in both groups, 45 patients (90%) in the LAC group and 42 patients (95%) in the LMBT group.

**Table 4** Incidence of side effects n (%)

	Therapy		P-value
	LAC (n = 50)	LMBT (n = 44)	
Nausea	11 (22)	20 (45)	< 0.05
Vomiting	0 (0)	9 (20)	< 0.01
Diarrhoea	14 (28)	25 (57)	< 0.01
Headache	12 (24)	19 (44)	
Dizziness	9 (18)	11 (25)	
Blurred vision	5 (10)	6 (14)	
Itching	5 (10)	5 (11)	
Rash	1 (2)	2 (5)	
Dry mouth	27 (54)	19 (43)	
Sore mouth	4 (8)	0 (0)	
Glossitis	2 (4)	1 (2)	
Black tongue	6 (12)	6 (14)	
Black stool	5 (10)	35 (80)	< 0.01
Taste disturbance	23 (46)	14 (32)	
Arthralgia	3 (6)	1 (2)	

**Table 5** Severity of side-effects (n)

Severity	Therapy	
	LAC	LMBT
None	5	2
Mild	17	19
Moderate	25	13
Severe	3	10
Total	50	44

Severity score: 1 = mild, does not cause any concern; 2 = moderate, but not bad enough to discontinue treatment; 3 = severe or incapacitating, forced to discontinue treatment.

The most frequent symptoms in the LAC group were dry mouth (54%) and taste disturbance (46%). Patients in the LMBT group experienced significantly more nausea (45%), vomiting (20%), diarrhoea (57%) and black stool (80%) (Table 4).

Each symptom was graded as mild, moderate or severe. In the LAC group, mild symptoms were observed in 17 patients (34%), moderate symptoms observed in 25 patients (50%) and severe symptoms observed in 3 patients (6%). In the LMBT group, mild symptoms were observed in 19 patients (43%), moderate symptoms observed in 13 patients (30%) and severe symptoms observed in 10 patients (23%) (Table 5,  $P < 0.05$ ). Despite most of the patients experiencing some side effects, none were severe enough to require hospitalization.

### <sup>13</sup>C-urea breath test

All 94 patients returned for a <sup>13</sup>C-urea breath test 2 mo after eradication therapy. Four patients (8%) from the LAC group and four patients from LMBT (9%) had positive results indicating failure of *H pylori* eradication. Three of the four patients had an incomplete quadruple therapy (Table 6).

All the eight patients who tested positive with <sup>13</sup>C-urea breath test had the alternate regimen. Three of four patients, who had initially LAC and then LMBT therapy, were negative on the second breath test.

Table 6 <sup>13</sup>C-urea breath test results *n* (%)

	Therapy		P-value
	LAC ( <i>n</i> = 50)	LBMT ( <i>n</i> = 44)	
Returned for UBT	50 (100)	44 (100)	-
Completed therapy	50 (100)	38 (86)	< 0.01
UBT result	46 negative, 4 positive	37 negative, 1 positive	
Not completed therapy	0 (0%)	6 (14)	< 0.01
UBT result	-	3 positive, 3 negative	-
Intention-to-treat cure rate	92% (46/50)	91% (40/44)	
Per-protocol cure rate	92% (46/50)	97% (37/38)	

UBT: Urea breath test.

Table 7 Symptomatic outcome at 6-mo follow-up *n* (%)

	Therapy	
	LAC ( <i>n</i> = 50)	LBMT ( <i>n</i> = 44)
Follow-up at 6 mo	46 (92)	40 (91)
Persistent symptoms	4 (8)	7 (16)
Recurrent symptoms	17 (34)	9 (20)
Repeat eradication therapy	1 (2)	1 (2)
Long-term acid-reduction therapy	17 (34)	14 (32)

### Six-month follow-up

Eighty-six patients (91.5%) returned for a 6-mo follow-up. Over one-third of patients had recurrent or persistent symptoms and remained on long-term acid-reduction therapy (with proton-pump inhibitors, H<sub>2</sub>-antagonist or other antacids) even after successful eradication (Table 7).

## DISCUSSION

This study has shown that a lansoprazole-based quadruple therapy is as effective as triple therapy in a predominantly white population in the UK (intention-to-treat rate: 91% *vs* 92% respectively). The resistance to clarithromycin (7%) is beginning to diminish the effectiveness of the triple therapy (92% per protocol eradication) whereas metronidazole resistance (24%) did not affect quadruple therapy (97% per protocol eradication)<sup>[25]</sup>.

Side effects are common in both regimens occurring in around 90% of patients. However, severe side effects occurred more frequently with quadruple therapy (23% *vs* 6%) and this reduced compliance.

Four out of the six patients taking quadruple therapy stopped because of nausea and vomiting, which was probably due to metronidazole. Replacing metronidazole with amoxicillin should reduce these side effects and increase compliance<sup>[26]</sup>. Interestingly, dry mouth was noticed more in the triple therapy group even though lansoprazole was the most likely cause.

The intention-to-treat cure rate of quadruple therapy (LBMT) was comparable to triple therapy (LAC) in spite of lower compliance. Educating patients about the possible common side effects and the importance of

complete eradication should provide a very high cure rate as the per protocol cure rate was 97% for quadruple therapy.

Quadruple therapy is very cost effective and should be considered as a first-line therapy especially when there are economic constraints. Lansoprazole-based quadruple therapy costs £17 as against £38 for the triple therapy for a one-week course<sup>[27]</sup>. The difference of £21 per treatment can be relieved from economic burden for the health service to treat this common condition.

Patients have to be warned that about one sixth of them will have persistent symptoms and about third of them will develop recurrent symptoms with a similar proportion needing long-term treatment with a proton-pump inhibitor, H<sub>2</sub>-antagonist or other antacids.

Modified seven-day quadruple therapy, by reducing the frequency of tetracycline chloride and bismuth subcitrate from four times to three times daily, has also been tried successfully as a first-line treatment with cure rate and compliance rate of over 90%<sup>[2]</sup>. Bateson has shown that a twice-daily quadruple therapy using lansoprazole, tetracycline, clarithromycin and metronidazole is effective (95.5% eradication rate) in UK patients with duodenal ulcer but this pre-dated resistance to clarithromycin and metronidazole<sup>[28]</sup>. Amoxicillin has been shown to improve eradication in resistant patients and perhaps a trial of a twice-daily quadruple therapy substituting amoxicillin for metronidazole should be considered<sup>[26]</sup>. Other approaches to the problem of antibiotic resistance include a sequential therapy that substituted amoxicillin with tinidazole during the first 5 d of a 10-d triple therapy with pantoprazole, amoxicillin and clarithromycin, which has been shown to achieve a significantly higher eradication rate<sup>[29]</sup>. Pretreatment sensitivity testing has been confirmed to be cost effective by significantly improved eradication in a study that used omeprazole and two antibiotics chosen based on susceptibility testing, compared to omeprazole, clarithromycin and metronidazole standard triple therapy<sup>[30]</sup>.

Recent randomised studies that compared triple therapy with quadruple therapy as a first-line treatment option for *H. pylori* and some reports showed superior eradication rates with the quadruple therapy<sup>[6,31,32]</sup> whereas others have shown no difference<sup>[33,34]</sup>. Quadruple therapy is becoming the standard treatment as resistance to clarithromycin, and to a lesser extent metronidazole, is reducing the efficacy of triple therapies. The side effects may be reduced by replacing metronidazole with amoxicillin but patients should be better educated about the side effects in order to improve compliance and cure rates.

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## COMMENTS

### Background

The treatment for *Helicobacter pylori* (*H. pylori*) is becoming less effective as the organism is becoming resistant to the commonly used antibiotics in triple

therapies. Quadruple therapies were less popular because of their side effects but still have good eradication rates.

### Research frontiers

This study compares lansoprazole-based triple and quadruple therapy for *H pylori* infection in white Caucasians in rural Wales, an area with low resistance to Clarithromycin and moderate resistance to metronidazole.

### Innovations and breakthroughs

Both regimens had high eradication rates (> 90%) showing that resistance has not yet significantly affected this UK population. Even better rates (97%) can be achieved with quadruple therapy if patients are able to complete the full course. Patients need to be educated about the side effects and importance of completing the course to achieve the higher eradication rates.

### Applications

Quadruple therapies provide a cost effective and highly successful treatment for *H pylori*. The side effects and compliance may be improved by substituting amoxicillin for metronidazole-an area for future research.

### Terminology

Triple therapy is a regimen of a proton pump inhibitor and two antibiotics. Quadruple therapy is a regimen of a proton pump inhibitor, a bismuth compound and two antibiotics.

### Peer review

The authors compared lansoprazole-based triple and quadruple therapy in the eradication of *H pylori*. They found that both regimens were equally effective and that quadruple therapy was less costly even though 6 patients had to discontinue treatment because of side effects. This is an important study.

## REFERENCES

- 1 de Boer WA, Driessen WM, Potters VP, Tytgat GN. Randomized study comparing 1 with 2 weeks of quadruple therapy for eradicating *Helicobacter pylori*. *Am J Gastroenterol* 1994; **89**: 1993-1997
- 2 Calvet X, Garcia N, Gene E, Campo R, Brullet E, Sanfeliu I. Modified seven-day, quadruple therapy as a first line *Helicobacter pylori* treatment. *Aliment Pharmacol Ther* 2001; **15**: 1061-1065
- 3 de Boer SY, v d Meeberg PC, Siem H, de Boer WA. Comparison of four-day and seven-day pantoprazole-based quadruple therapy as a routine treatment for *Helicobacter pylori* infection. *Neth J Med* 2003; **61**: 218-222
- 4 Buring SM, Winner LH, Hatton RC, Doering PL. Discontinuation rates of *Helicobacter pylori* treatment regimens: a meta-analysis. *Pharmacotherapy* 1999; **19**: 324-332
- 5 Fennerty MB, Lieberman DA, Vakil N, Magaret N, Faigel DO, Helfand M. Effectiveness of *Helicobacter pylori* therapies in a clinical practice setting. *Arch Intern Med* 1999; **159**: 1562-1566
- 6 Laine L, Hunt R, El-Zimaity H, Nguyen B, Osato M, Spenard J. Bismuth-based quadruple therapy using a single capsule of bismuth biskalcitrate, metronidazole, and tetracycline given with omeprazole versus omeprazole, amoxicillin, and clarithromycin for eradication of *Helicobacter pylori* in duodenal ulcer patients: a prospective, randomized, multicenter, North American trial. *Am J Gastroenterol* 2003; **98**: 562-567
- 7 Fischbach LA, van Zanten S, Dickason J. Meta-analysis: the efficacy, adverse events, and adherence related to first-line anti-*Helicobacter pylori* quadruple therapies. *Aliment Pharmacol Ther* 2004; **20**: 1071-1082
- 8 Laine L, Frantz JE, Baker A, Neil GA. A United States multicentre trial of dual and proton pump inhibitor-based triple therapies for *Helicobacter pylori*. *Aliment Pharmacol Ther* 1997; **11**: 913-917
- 9 Comet R, Calvet X, Navarro M, Garcia N, Sanfeliu I. [Seven-day omeprazole, clarithromycin, and amoxicillin for the therapy of *Helicobacter pylori* infection] *Gastroenterol Hepatol* 1998; **21**: 81-83
- 10 Pipkin GA, Williamson R, Wood JR. Review article: one-week clarithromycin triple therapy regimens for eradication of *Helicobacter pylori*. *Aliment Pharmacol Ther* 1998; **12**: 823-837
- 11 Calvet X, Lopez-Lorente M, Cubells M, Bare M, Golvez E, Molina E. Two-week dual vs. one-week triple therapy for cure of *Helicobacter pylori* infection in primary care: a multicentre, randomized trial. *Aliment Pharmacol Ther* 1999; **13**: 781-786
- 12 Elviss NC, Owen RJ, Xerry J, Walker AM, Davies K. *Helicobacter pylori* antibiotic resistance patterns and genotypes in adult dyspeptic patients from a regional population in North Wales. *J Antimicrob Chemother* 2004; **54**: 435-440
- 13 Chisholm SA, Teare EL, Davies K, Owen RJ. Surveillance of primary antibiotic resistance of *Helicobacter pylori* at centres in England and Wales over a six-year period (2000-2005). *Euro Surveill* 2007; **12**: E3-E4
- 14 Gisbert JP, Gisbert JL, Marcos S, Gravalos RG, Carpio D, Pajares JM. Seven-day 'rescue' therapy after *Helicobacter pylori* treatment failure: omeprazole, bismuth, tetracycline and metronidazole vs. ranitidine bismuth citrate, tetracycline and metronidazole. *Aliment Pharmacol Ther* 1999; **13**: 1311-1316
- 15 Lee JM, Breslin NP, Hyde DK, Buckley MJ, O'Morain CA. Treatment options for *Helicobacter pylori* infection when proton pump inhibitor-based triple therapy fails in clinical practice. *Aliment Pharmacol Ther* 1999; **13**: 489-496
- 16 Gomollon F, Ducons JA, Ferrero M, Garcia Cabezo J, Guirao R, Simon MA, Montoro M. Quadruple therapy is effective for eradicating *Helicobacter pylori* after failure of triple proton-pump inhibitor-based therapy: a detailed, prospective analysis of 21 consecutive cases. *Helicobacter* 1999; **4**: 222-225
- 17 Sicilia B, Sierra E, Lago A, Villar M, Garcia S, Gomollon F. [High eradication rates in *Helicobacter pylori* infection in patients with duodenal ulcer who failed previous eradication therapy] *Med Clin (Barc)* 2000; **115**: 641-643
- 18 Boixeda D, Bermejo F, Martin-De-Argila C, Lopez-Sanroman A, Defarges V, Hernandez-Ranz F, Milicua JM, Garcia-Plaza A. Efficacy of quadruple therapy with pantoprazole, bismuth, tetracycline and metronidazole as rescue treatment for *Helicobacter pylori* infection. *Aliment Pharmacol Ther* 2002; **16**: 1457-1460
- 19 Lam SK, Talley NJ. Report of the 1997 Asia Pacific Consensus Conference on the management of *Helicobacter pylori* infection. *J Gastroenterol Hepatol* 1998; **13**: 1-12
- 20 Gisbert JP, Calvet X, Gomollon F, Sainz R. [Treatment for the eradication of *Helicobacter pylori*. Recommendations of the Spanish Consensus Conference] *Med Clin (Barc)* 2000; **114**: 185-195
- 21 Malfertheiner P, Megraud F, O'Morain C, Hungin AP, Jones R, Axon A, Graham DY, Tytgat G. Current concepts in the management of *Helicobacter pylori* infection--the Maastricht 2-2000 Consensus Report. *Aliment Pharmacol Ther* 2002; **16**: 167-180
- 22 Hunt R, Fallone C, Veldhuyzen van Zanten S, Sherman P, Smaill F, Flook N, Thomson A. Canadian *Helicobacter* Study Group Consensus Conference: Update on the management of *Helicobacter pylori*--an evidence-based evaluation of six topics relevant to clinical outcomes in patients evaluated for *H pylori* infection. *Can J Gastroenterol* 2004; **18**: 547-554
- 23 Vilaichone RK, Mahachai V, Graham DY. *Helicobacter pylori* diagnosis and management. *Gastroenterol Clin North Am* 2006; **35**: 229-247
- 24 Malfertheiner P, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, Hunt R, Rokkas T, Vakil N, Kuipers EJ. Current concepts in the management of *Helicobacter pylori* infection: the Maastricht III Consensus Report. *Gut* 2007; **56**: 772-781
- 25 van der Wouden EJ, Thijs JC, van Zwet AA, Sluiter WJ, Kleibeuker JH. The influence of in vitro nitroimidazole resistance on the efficacy of nitroimidazole-containing anti-*Helicobacter pylori* regimens: a meta-analysis. *Am J Gastroenterol* 1999; **94**: 1751-1759

- 26 **Chi CH**, Lin CY, Sheu BS, Yang HB, Huang AH, Wu JJ. Quadruple therapy containing amoxicillin and tetracycline is an effective regimen to rescue failed triple therapy by overcoming the antimicrobial resistance of *Helicobacter pylori*. *Aliment Pharmacol Ther* 2003; **18**: 347-353
- 27 **British Medical Association**, Royal Pharmaceutical Society of Great Britain. British National Formulary. 42nd ed. Wallingford: Pharmaceutical Press, 2001: 41, 43, 169, 286
- 28 **Bateson MC**. Quadruple therapy for symptomatic spontaneous duodenal ulcer disease. *Postgrad Med J* 2001; **77**: 447-450
- 29 **Vaira D**, Zullo A, Vakil N, Gatta L, Ricci C, Perna F, Hassan C, Bernabucci V, Tampieri A, Morini S. Sequential therapy versus standard triple-drug therapy for *Helicobacter pylori* eradication: a randomized trial. *Ann Intern Med* 2007; **146**: 556-563
- 30 **Romano M**, Marmo R, Cuomo A, De Simone T, Mucherino C, Iovene MR, Montella F, Tufano MA, Del Vecchio Blanco C, Nardone G. Pretreatment antimicrobial susceptibility testing is cost saving in the eradication of *Helicobacter pylori*. *Clin Gastroenterol Hepatol* 2003; **1**: 273-278
- 31 **Katellaris PH**, Forbes GM, Talley NJ, Crotty B. A randomized comparison of quadruple and triple therapies for *Helicobacter pylori* eradication: The QUADRATE Study. *Gastroenterology* 2002; **123**: 1763-1769
- 32 **Uygun A**, Kadayifci A, Safali M, Ilgan S, Bagci S. The efficacy of bismuth containing quadruple therapy as a first-line treatment option for *Helicobacter pylori*. *J Dig Dis* 2007; **8**: 211-215
- 33 **Calvet X**, Ducons J, Guardiola J, Tito L, Andreu V, Bory F, Guirao R. One-week triple vs. quadruple therapy for *Helicobacter pylori* infection - a randomized trial. *Aliment Pharmacol Ther* 2002; **16**: 1261-1267
- 34 **Jang HJ**, Choi MH, Kim YS, Seo YA, Baik KH, Baik IH, Eun CS, Kim JB, Kae SH, Kim DJ, Lee MS, Kim HY, Lee J. [Effectiveness of triple therapy and quadruple therapy for *Helicobacter pylori* eradication] *Korean J Gastroenterol* 2005; **46**: 368-372

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liver unit, we selected retrospectively those fulfilling the following criteria: detectable HCV RNA, Knodell score  $\leq 3$  at the first pathological evaluation<sup>[10]</sup>, at least 2 sequential liver biopsies in the absence of antiviral therapy or HIV and HBV co-infection. Kidney transplant recipients or hemodialyzed patients were excluded as well as patients with other causes of chronic liver disease (hepatotoxic drugs, autoimmune chronic hepatitis, hemochromatosis, Wilson's disease and alpha 1 anti-trypsin deficiency). In our center we usually perform a liver biopsy in all HCV-RNA chronic carriers, whatever the transaminase levels. In the case of low pathological lesions, we usually propose therapeutic abstention, a biochemical follow-up twice a year, an abdominal ultrasonography (US) yearly and a pathological follow-up with a liver biopsy every three to five years. In patients infected by blood transfusion or intravenous drug use, duration of HCV infection was estimated as the time elapsed from the year of transfusion or intravenous drug use (IVDU) onset to that of the first liver biopsy. Gender, route of infection, HCV viral genotype and serum viral load, body mass index (BMI), alcohol consumption before the first biopsy, serum ALT and serum glutamyl-transferase (GGT) levels were recorded for each patient.

### Histological analysis

Liver biopsy specimens were fixed, paraffin-embedded, and routinely stained with haematoxylin-eosin and Masson's trichrome and picosirius red for collagen. For each liver biopsy specimen, stage of fibrosis (from 0 to 4) and grade of necro-inflammation including portal inflammation (from 0 to 4), periportal piecemeal necrosis (from 0 to 10) and intralobular inflammation (from 0 to 4) were established according to the Knodell score criteria. Worsening of the necro-inflammation (sum of portal inflammation, periportal piecemeal necrosis and intralobular inflammation scores) and fibrosis were defined by an increase of at least 2 and 1 points, respectively.

### RNA quantification and procedure for HCV genotyping

Serum HCV RNA quantitative detection was performed using the RT-PCR method with a sensitivity limit of 100 copies/mL (Amplicor® Roche, Switzerland). Genotypes were identified using the INNO-LIPA HCV procedure (Innogenetics, Belgium).

### Statistical analysis

SPSS software version 10.0 (SPSS Inc, Chicago, Illinois, USA) was used for statistical analysis. Quantitative variables were compared using Student's *t*-test or non-parametric Mann-Whitney variance analysis (ANOVA). Qualitative variables were compared using the  $\chi^2$  test or the Fischer test when necessary. Multivariate analysis was done using robust logistic regression. A two-tailed *P* value less than 0.05 was considered as significant.

## RESULTS

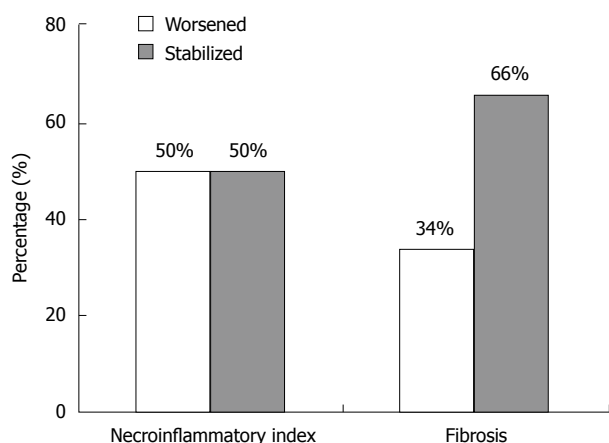
Among our HCV-infected patient group, 410 patients

**Table 1** Demographic and clinical features of HCV patients with initially normal liver

Demographic and clinical features	Data
Number of patients	76
Sex (M/F)	34/42
Age at the first biopsy (mean, yr)	38 $\pm$ 9
Age at Infection (mean, yr)	25 $\pm$ 9
BMI (M/F)	23.5 $\pm$ 3.1/22 $\pm$ 5.1
Route of infection:Transfusion	28 (36.8%)
IVDU	33 (43.4%)
Other or unknown	15 (20%)
Genotype 1/2/3/4/5	40/9/15/6/2
Infection duration, median (95% CI)	13 (1-28)
Times between 2 biopsies, median (95% CI)	4 (2-16)
Alcohol consumption (g/d, mean $\pm$ SD)	22.2 $\pm$ 44
ALT level between 2 biopsies	
Constantly normal	42 (55.3%)
Normal or $< 2$ N	31 (40.8%)
$> 2$ N	3 (3.9%)
Viral load:	
Low (under 350 000 UI/mL)	33 (55%)
Medium	14 (23.3%)
High (more than 700 000 UI/mL)	13 (21.7%)
Knodell score at the first liver biopsy	
Necroinflammatory index (mean $\pm$ SD)	1.7 $\pm$ 0.7
Fibrosis score (mean $\pm$ SD)	0.6 $\pm$ 0.5

had a first liver biopsy with a Knodell score  $\leq 3$ . Only 76 patients, 34 males and 42 females, had at least a second liver biopsy and fulfilled the selection criteria. Their main characteristics are given in Table 1. Mean age at infection and at the first biopsy were 25  $\pm$  9 years and 38  $\pm$  9 years, respectively. Thirty-three patients (43.4%) had been contaminated by intravenous drug use and 28 (36.8%) by transfusions. Forty patients (55.6%) were infected with genotype 1 and 15 (20.8%) with genotype 3. The median duration of infection before the first biopsy was 13 (95% CI: 1-28) years and the median time between paired biopsies was 4 (95% CI: 2-16) years. The mean number of ALT level available between the 2 biopsies was 6  $\pm$  3. During the follow-up period, 33 patients (43.4%) had normal serum ALT activity, 33 (43.4%) patients displayed occasional mild ALT increases (less than 2 times the upper normal limit) and 10 patients (13.2%) had constantly elevated ALT ( $> 3$ N). The mean daily alcohol consumption was 22.2 g/d (95% CI: 0-250). Mean BMI was 23.5  $\pm$  3.1 for males and 22.0  $\pm$  5.1 for females, without significant difference. Forty patients (55.6%) were infected with genotype 1; 9 (12.5%) with genotype 2; 15 (20.8%) with genotype 3; 6 (8.3%) with genotype 4 and 2 (2.8%) with genotype 5. Genotype was unknown for 4 patients. Viral load was low (under 350 000 UI/mL) for 33 patients (55%), medium (350 000 to 700 000 UI/mL) for 14 patients (23.3%) and high (more than 700 000 UI/mL) for 13 patients (21.7%). At the first biopsy, mean values for necro-inflammation and fibrosis were 1.75  $\pm$  0.68 and 0.57  $\pm$  0.5 respectively.

At the last biopsy, a significant increase in necro-inflammation ( $\geq 2$  points) and fibrosis score ( $\geq 1$  point) was observed in 38 (50%) and 26 patients (34%) respectively; 3 patients having a fibrosis equal to 3 and one equal to 4 (Figure 1). The mean difference in the necro-



**Figure 1** Pathological evolution. 38 patients (50%) had progression of necroinflammatory activity (progression  $\geq 2$ ) and 26 (34%) had progression of fibrosis (progression  $\geq 1$ ; 3 patients having a fibrosis score at 3 and one at 4).

inflammation and fibrosis scores between the 2 biopsies was low:  $1.79 \pm 2.23$  and  $0.42 \pm 0.77$ , respectively. Univariate analysis showed there was no difference between activity and fibrosis evolution according to the genotype, viral load or the infection duration. A higher fibrosis progression ( $1.00 \pm 1.3$ ) was observed in patients with BMI  $> 25$  as compared to patients with BMI  $< 25$  ( $0.28 \pm 0.53$ ) ( $P = 0.03$ ). A significant progression in activity (mean =  $1.7 \pm 0.8$  *vs*  $0.4 \pm 0.5$ ) ( $P < 0.05$ ) or fibrosis (mean =  $0.9 \pm 0.3$  *vs*  $0.0 \pm 0.2$ ) ( $P < 0.01$ ) was observed in patients with elevated ALT as compared to patients with normal ALT. There was also a significant correlation between activity progression and fibrosis progression ( $P = 0.003$ ).

By multivariate analysis, factors independently associated with liver fibrosis progression were an elevated ALT (RR = 7.5, CI = 1.4) ( $P = 0.02$ ), BMI  $> 25$  (RR = 4.9, CI = 1.2) ( $P = 0.03$ ) and the interval between the 2 biopsies (RR = 1.8, CI = 1.3) ( $P = 0.001$ ).

## DISCUSSION

The long-term natural history of the so-called healthy carriers of HCV is not clear. Although ALT levels do not reflect the severity of the liver damage<sup>[11]</sup>, patients with persistently normal ALT levels usually have a less severe disease, corresponding to a lower progression of fibrosis<sup>[8,12-15]</sup>. Nevertheless, some reports suggest the presence of significant fibrosis or cirrhosis in some of them<sup>[8,13,16,17]</sup>. Other reports underline the relationship between excess weight and hepatitis C-related fibrosis progression<sup>[18,19]</sup>. This study was designed to evaluate whether HCV-infected subjects with pathologically normal liver had any progression of liver damage after 4 years of follow-up. This might allow the screening of patients for which antiviral treatment would be helpful.

In our study, fibrosis progression only concerns one third of the patients. The short time between two biopsies cannot exclude a later worsening of liver fibrosis, which is probably slow, with a low risk of evolution to cirrhosis. Although ALT levels do not reflect the sever-

ity of the liver disease, elevated ALT was associated with liver fibrosis progression in this population with initially normal liver. Overweight, accordingly to the recent literature was also associated with fibrosis worsening<sup>[19]</sup>. Patients with elevated ALT or BMI may have more necro-inflammatory activity resulting in more pronounced fibrosis progression<sup>[19,21]</sup>.

Our results suggest that ALT level follow-up is necessary whatever the histopathological results. In the subgroup of patients with elevated ALT or BMI, pathological follow-up seems to be useful and weight loss should be proposed. Our study suggests that liver fibrosis progression is correlated with time between biopsies, which probably make histopathological controls necessary. In patients with normal liver, normal ALT level and without co-morbidities such as excess weight, the time between biopsies should be longer than 5 years.

In our study, alcohol consumption was not correlated with fibrosis progression. We recorded the alcohol consumption before the first biopsy. We can suggest as a major hypothesis for this unusual result, that patients reduced their alcohol consumption after knowledge of their HCV status during follow up<sup>[22]</sup>.

Antiviral therapy is usually not used in the so-called “asymptomatic HCV-carriers”<sup>[23,24]</sup>. We can differentiate in these “asymptomatic carriers” a sub-group which is at risk of fibrosis progression. The question is whether and when pegylated interferon and ribavirin should be a therapeutic option? Theoretically, if patients with normal liver tests do not really need treatment, therapy still can be proposed to interested patients with the same response rate as patients with elevated transaminases<sup>[25-29]</sup>. In addition, patients with a higher risk of liver fibrosis progression should be treated, particularly in case of genotype 2 or 3 infection<sup>[30]</sup>.

In conclusion, this study confirms that the “HCV-healthy carrier” state does exist. Fibrosis does not worsen in two thirds of HCV-carriers without histopathological features after 4 years, supporting the concept that the natural history of chronic hepatitis in this group of subjects is characterized by a very slow or no progression. Antiviral therapy is not recommended in these patients with normal ALT or BMI under 25. Overweight, HCV-infected patient should be informed of the risk of liver fibrosis progression and the need of dietetic councils.

## COMMENTS

### Background

Chronic hepatitis C virus (HCV) infection cause liver damage, with a fibrotic scarring, which can progress to cirrhosis. The natural history of the infection varies among patients. For instance, in 10% of the case, the liver appears pathologically normal. Altogether, 10% to 40% of HCV infected patients harbor normal liver tests and the disease progresses very slowly as compared with patients with elevated liver tests. Until now, studies usually differentiate patients with or without liver test abnormalities but rarely patients with or without liver biopsy abnormalities.

### Research frontiers

The goal of this work was to evaluate and differentiate patients for whom treatment will offer a better management of the disease. Among HCV infected



patients without significant liver damage, one-third progress toward fibrosis. This work focuses on early detection of these patients with a view to treatment before fibrosis onset.

### Innovations and breakthroughs

In two thirds of HCV infected patients without significant liver damage, there is no fibrosis progression. In these patients, fibrosis progression is associated with abnormal liver tests and elevated BMI.

### Applications

These observations may be helpful to suggest if a patient should receive an antiviral therapy. A treatment should be counselled to the patients with abnormal liver test and elevated BMI.

### Terminology

Liver fibrosis is the excessive accumulation of a scarred tissue that occurs in most types of chronic liver diseases. This fibrosis can progress to cirrhosis. The transaminases are a group of liver enzymes including alanine aminotransferase (ALT). Elevated transaminases can be an indicator of liver damage.

### Peer review

Sobesky *et al* present an interesting study describing the features and development of individuals with chronic hepatitis C infection and almost normal histological findings. The paper is properly written.

## REFERENCES

- 1 Seymour CA. Asymptomatic infection with hepatitis C virus. *BMJ* 1994; **308**: 670-671
- 2 Everhart JE, Stolar M, Hoofnagle JH. Management of hepatitis C: a national survey of gastroenterologists and hepatologists. *Hepatology* 1997; **26**: 78S-82S
- 3 Okanoue T, Yasui K, Sakamoto S, Minami M, Nagao Y, Itoh Y, Kagawa K, Kashima K. Circulating HCV-RNA, HCV genotype, and liver histology in asymptomatic individuals reactive for anti-HCV antibody and their follow-up study. *Liver* 1996; **16**: 241-247
- 4 Collier JD, Woodall T, Wight DG, Shore S, Gimson AE, Alexander GJ. Predicting progressive hepatic fibrosis stage on subsequent liver biopsy in chronic hepatitis C virus infection. *J Viral Hepat* 2005; **12**: 74-80
- 5 Mathurin P, Moussalli J, Cadranet JF, Thibault V, Charlotte F, Dumouchel P, Cazier A, Huraux JM, Devergie B, Vidaud M, Opolon P, Poynard T. Slow progression rate of fibrosis in hepatitis C virus patients with persistently normal alanine transaminase activity. *Hepatology* 1998; **27**: 868-872
- 6 Martinot-Peignoux M, Boyer N, Cazals-Hatem D, Pham BN, Gervais A, Le Breton V, Levy S, Degott C, Valla DC, Marcellin P. Prospective study on anti-hepatitis C virus-positive patients with persistently normal serum alanine transaminase with or without detectable serum hepatitis C virus RNA. *Hepatology* 2001; **34**: 1000-1005
- 7 Persico M, Persico E, Suozzo R, Conte S, De Seta M, Coppola L, Palmentieri B, Sasso FC, Torella R. Natural history of hepatitis C virus carriers with persistently normal aminotransferase levels. *Gastroenterology* 2000; **118**: 760-764
- 8 Pradat P, Alberti A, Poynard T, Esteban JL, Weiland O, Marcellin P, Badalamenti S, Trepo C. Predictive value of ALT levels for histologic findings in chronic hepatitis C: a European collaborative study. *Hepatology* 2002; **36**: 973-977
- 9 Persico M, Perrotta S, Persico E, Terracciano L, Folgori A, Ruggeri L, Nicosia A, Vecchione R, Mura VL, Masarone M, Torella R. Hepatitis C virus carriers with persistently normal ALT levels: biological peculiarities and update of the natural history of liver disease at 10 years. *J Viral Hepat* 2006; **13**: 290-296
- 10 Knodell RG, Ishak KG, Black WC, Chen TS, Craig R, Kaplowitz N, Kiernan TW, Wollman J. Formulation and application of a numerical scoring system for assessing histological activity in asymptomatic chronic active hepatitis. *Hepatology* 1981; **1**: 431-435
- 11 Persico M, Romano M. Alanine aminotransferase measurements and histological disease in hepatitis C. *Lancet* 1993; **342**: 1369-1370
- 12 Ghany MG, Kleiner DE, Alter H, Doo E, Khokar F, Promrat K, Herion D, Park Y, Liang TJ, Hoofnagle JH. Progression of fibrosis in chronic hepatitis C. *Gastroenterology* 2003; **124**: 97-104
- 13 Puoti C, Magrini A, Stati T, Rigato P, Montagnese F, Rossi P, Aldegheri L, Resta S. Clinical, histological, and virological features of hepatitis C virus carriers with persistently normal or abnormal alanine transaminase levels. *Hepatology* 1997; **26**: 1393-1398
- 14 Zarski JP, Mc Hutchison J, Bronowicki JP, Sturm N, Garcia-Kennedy R, Hodaj E, Truta B, Wright T, Gish R. Rate of natural disease progression in patients with chronic hepatitis C. *J Hepatol* 2003; **38**: 307-314
- 15 Zylberberg H, Pol S, Thiers V, Chaix ML, Lagorce D, Brechot C, Nalpas B, Berthelot P. Significance of repeatedly normal aminotransferase activities in HCV-infected patients. *J Clin Gastroenterol* 1999; **29**: 71-75
- 16 Pasquale G, Sagnelli E, Coppola N, Scarano F, Scolastico C, Bellomo PF, Lettieri A, Piccinino F. Is liver biopsy necessary for hepatitis C virus carriers with persistently normal aminotransferase levels? *Eur J Gastroenterol Hepatol* 2003; **15**: 831-833
- 17 Rumi MG, De Filippi F, Donato MF, Del Ninno E, Colombo M. Progressive hepatic fibrosis in healthy carriers of hepatitis C virus with a transaminase breakthrough. *J Viral Hepat* 2002; **9**: 71-74
- 18 Ortiz V, Berenguer M, Rayon JM, Carrasco D, Berenguer J. Contribution of obesity to hepatitis C-related fibrosis progression. *Am J Gastroenterol* 2002; **97**: 2408-2414
- 19 Perumalswami P, Kleiner DE, Lutchman G, Heller T, Borg B, Park Y, Liang TJ, Hoofnagle JH, Ghany MG. Steatosis and progression of fibrosis in untreated patients with chronic hepatitis C infection. *Hepatology* 2006; **43**: 780-787
- 20 Bedossa P, Moucari R, Chelbi E, Asselah T, Paradis V, Vidaud M, Cazals-Hatem D, Boyer N, Valla D, Marcellin P. Evidence for a role of nonalcoholic steatohepatitis in hepatitis C: a prospective study. *Hepatology* 2007; **46**: 380-387
- 21 Moucari R, Asselah T, Cazals-Hatem D, Voitot H, Boyer N, Ripault MP, Sobesky R, Martinot-Peignoux M, Maylin S, Nicolas-Chanoine MH, Paradis V, Vidaud M, Valla D, Bedossa P, Marcellin P. Insulin resistance in chronic hepatitis C: association with genotypes 1 and 4, serum HCV RNA level, and liver fibrosis. *Gastroenterology* 2008; **134**: 416-423
- 22 Nalpas B, Martin S, Fontaine H, Fabbro-Peray P, Brechot C, Pol S. Impact of medical recommendations on alcohol consumption in HCV positive patients. *J Hepatol* 2001; **35**: 312-313
- 23 Verslype C, Michielsens P, Adler M, Orlent H, Sprengers D, Delwaide J, D'heygere F, Langlet P, Brenard R, Colle I, Reynaert H, Starkel P, Henrion J. The management of patients with mild hepatitis C. *Acta Gastroenterol Belg* 2005; **68**: 314-318
- 24 Sangiovanni A, Morales R, Spinzi G, Rumi M, Casiraghi A, Ceriani R, Colombo E, Fossati M, Prada A, Tavani E, Minoli G. Interferon alfa treatment of HCV RNA carriers with persistently normal transaminase levels: a pilot randomized controlled study. *Hepatology* 1998; **27**: 853-856
- 25 Bini EJ, Mehandru S. Sustained virological response rates and health-related quality of life after interferon and ribavirin therapy in patients with chronic hepatitis C virus infection and persistently normal alanine aminotransferase levels. *Aliment Pharmacol Ther* 2006; **23**: 777-785
- 26 Hasan F, Asker H, Al-Khalid J, Al-Mekhaizeem K, Al-Shamali M, Siddique I, Al-Nakib B. Interferon-alpha in combination with ribavirin for the treatment of chronic hepatitis C in patients with persistently normal aminotransferase levels. *Digestion* 2002; **65**: 127-130
- 27 Jacobson IM, Ahmed F, Russo MW, Lebovics E, Dieterich DT, Esposito SP, Bach N, Klion F, Tobias H, Antignano L, Brown RS Jr, Gabbaiadeh D, Geders J, Levendoglu H. Interferon alfa-2b [correction of alpha-2b] and ribavirin for



- patients with chronic hepatitis C and normal ALT. *Am J Gastroenterol* 2004; **99**: 1700-1705
- 28 **Rossini A**, Ravaggi A, Biasi L, Agostinelli E, Bercich L, Gazzola GB, Callea F, Radaeli E, Cariani E. Virological response to interferon treatment in hepatitis C virus carriers with normal aminotransferase levels and chronic hepatitis. *Hepatology* 1997; **26**: 1012-1017
- 29 **Shiffman ML**, Stewart CA, Hofmann CM, Contos MJ, Luketic VA, Sterling RK, Sanyal AJ. Chronic infection with hepatitis C virus in patients with elevated or persistently normal serum alanine aminotransferase levels: comparison of hepatic histology and response to interferon therapy. *J Infect Dis* 2000; **182**: 1595-1601
- 30 **Bacon BR**. Treatment of patients with hepatitis C and normal serum aminotransferase levels. *Hepatology* 2002; **36**: S179-S184

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RAPID COMMUNICATION

## Nuclear $\beta$ -catenin expression as a prognostic factor in advanced colorectal carcinoma

Adam Elzagheid, Abdelbaset Buhmeida, Eija Korkeila, Yrjö Collan, Kari Syrjänen, Seppo Pyrhönen

Adam Elzagheid, Department of Pathology, Faculty of Medicine Al-Arab Medical University, Benghazi, Libya  
Abdelbaset Buhmeida, Eija Korkeila, Kari Syrjänen, Seppo Pyrhönen, Department of Oncology and Radiotherapy, Turku University Hospital, Savitehtaankatu 1 PB 52, FIN-20521, Turku, Finland

Yrjö Collan, Department of pathology, University of Turku, Kiinamyllynkatu 10, FIN-20540, Turku, Finland

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Correspondence to: Dr. Adam Elzagheid, MD, PhD, Department of Pathology, University of Turku, Kiinamyllynkatu 10, FIN-20520, Turku, Finland. [adibel@utu.fi](mailto:adibel@utu.fi)

Telephone: +35-8-2-3133966 Fax: +35-8-2-3133965

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### Abstract

**AIM:** To investigate the changing pattern of  $\beta$ -catenin expression and its prognostic value in advanced colorectal cancer (CRC).

**METHODS:** Archival tumor samples were analyzed for  $\beta$ -catenin using immunohistochemistry (IHC) in 95 patients with advanced CRC.

**RESULTS:** Membranous  $\beta$ -catenin expression was found in the normal colorectal epithelium. Almost 100% of CRC cases showed membranous and cytoplasmic expression, and 55 (58%) cases showed nuclear expression. In univariate (Kaplan-Meier) survival analysis, only the nuclear index (NI) was a significant predictor of disease-free survival (DFS) ( $P = 0.023$ ;  $n = 35$ ), with a NI above the median associated with longer DFS (34.2 mo) than those with a NI below the median (15.5 mo) ( $P = 0.045$ , ANOVA). The other indices were not significant predictors of DFS, and none of the three tested indices (for membranous, cytoplasmic, or nuclear expression) predicted disease-specific survival (DSS). However, when dichotomized as positive or negative nuclear expression, the former was a significant predictor of more favorable DFS ( $P = 0.041$ ) and DSS ( $P = 0.046$ ).

**CONCLUSION:** Nuclear  $\beta$ -catenin expression provides additional information in predicting patient outcome in advanced CRC.

### INTRODUCTION

$\beta$ -catenin is a 92-kDa multifunctional protein that, in its membrane location, links the intracellular part of the E-cadherin complex to actin cytoskeleton, which is a critical step in morphogenesis and maintenance of tissue integrity<sup>[1]</sup>. Alternatively, through Wnt signaling-mediated stabilization,  $\beta$ -catenin may act as a down-stream transcriptional trans-activator of several target genes<sup>[2]</sup>. Alterations in  $\beta$ -catenin protein expression levels and genetic rearrangement located in  $\beta$ -catenin exon 3 have been shown to contribute to the malignant character of various carcinomas and are likely to affect both intercellular adhesion and signal transduction, which are believed to be two independent functions of  $\beta$ -catenin protein<sup>[3]</sup>.

The rare occurrence of mutations in  $\beta$ -catenin exon 3 has been previously documented in ulcerative colitis-related neoplastic progression<sup>[4]</sup> and in colorectal cancer (CRC) as well<sup>[5]</sup>. Immunohistochemical studies suggest that the observed accumulation in  $\beta$ -catenin protein is probably due to genomic alterations in  $\beta$ -catenin coding regions, particularly in exon 3.

The impact of aberrations in  $\beta$ -catenin expression on the clinical outcome of CRC is controversial. Some studies reported prognostic value for cytoplasmic rather than nuclear expression<sup>[6]</sup>, whereas others showed that nuclear accumulation of  $\beta$ -catenin may be an independent marker of unfavorable prognosis<sup>[7,8]</sup>. The aim of this study was to evaluate the possible role of  $\beta$ -catenin expression as a predictor of clinical outcome in advanced CRC patients.

## MATERIALS AND METHODS

### Study material

Ninety-five patients with advanced colorectal carcinoma (CRC), enrolled consecutively from CRC patients attending our clinic for therapeutic procedures during the late 1990s, were included in our study. Of these 95 patients, 60 had metastases at diagnosis (Stage IV disease), while the remaining 35 patients (with stage II and III disease at baseline) subsequently developed a metastatic disease during the mean follow-up (FU) time of  $25.1 \pm 27.8$  (SD) mo. All patients were treated at the Department of Oncology and Radiotherapy, Turku University Hospital, according to the protocols in routine use for the treatment of CRC patients with stage II, III or IV disease at that time. The 95 patients included in the present study were enrolled into the study cohort between October 1998 and August 2003. All patients were prospectively followed-up until death or until their last clinical visit (March 2007), with the median FU-time of 27.6 (range 3-150) mo. The study was approved by the TUH Ethics Committee and was conducted in accordance with the Declaration of Helsinki. Samples were collected with the endorsement of the National Authority for Medico-legal Affairs.

Key clinical data for these patients are shown in Table 1. Of the 95 cases, 38 were women and 57 were men. The mean age was 61.5 (range 24-78) years. The majority ( $n = 39$ ) of the tumors were localized in the left colon, followed in the order of frequency by the right colon ( $n = 24$ ), rectum ( $n = 24$ ), and colon transversum ( $n = 7$ ). At the time of diagnosis, 15 patients were Stage II, 20 were Stage III and 60 patients were Stage IV. Accordingly, the majority ( $n = 63$ , 66.3%) had T3 tumors, and almost half of the patients had lymph node involvement at the time of diagnosis ( $n = 46$ ). The patients were selected for the cohort on the basis of both the diagnosis and treatment they received, and each patient was assigned to one of two treatment arms: (1) 20 were treated with irinotecan alone, and (2) 75 received a combination of irinotecan and 5-fluorouracil (5-FU). The chemotherapy regimen the patients received was included in a previous study investigating irinotecan combined with bolus 5-fluorouracil and folinic acid<sup>[9]</sup>.

### $\beta$ -catenin immunostaining

Formalin-fixed, paraffin-embedded primary colorectal tumor tissue was obtained from 95 patients. Sections were cut serially at 5  $\mu$ m for routine haematoxylin and eosin staining and for immunohistochemical (IHC) analysis. An experienced pathologist confirmed all histological diagnoses. IHC analysis was done using an automatic system (BenchMark XT, Ventana Medical Systems, Inc. Tucson, Arizona, USA). This fully automated processing of bar code labeled slides included baking of the slides, solvent free deparaffinization, antigen retrieval in a cell conditioning buffer CC2 (Mild: 36 min conditioning, and standard: 60 min conditioning), and incubation with the monoclonal mouse  $\beta$ -catenin antibody (clone CAT-5H10, isotype IgG1-kappa, Zymed

Table 1 Key characteristics of patients and their tumors

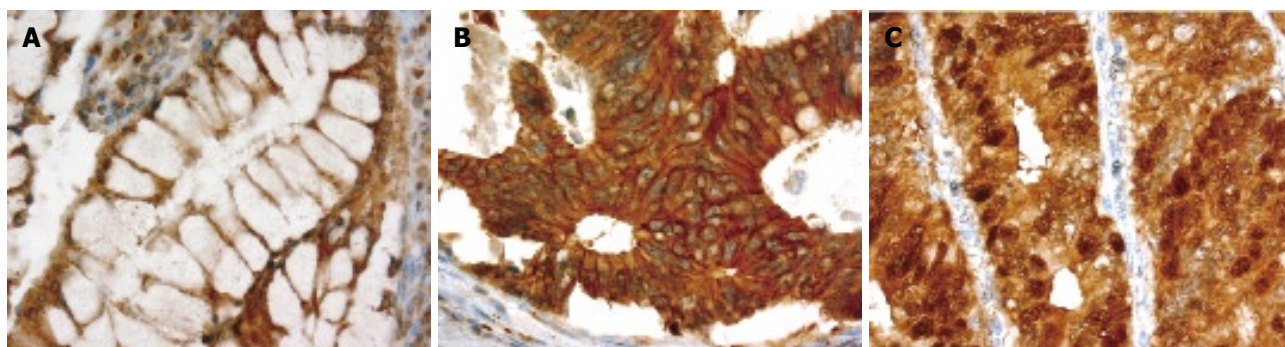
Variable	No. or value	% <sup>1</sup>
Patients	95	
Male	57	60.0
Female	38	40.0
Age (yr)		
Median (range)	60.7 (24-80)	
Primary tumour status <sup>2</sup>	95	
T1	1	1.1
T2	6	6.3
T3	63	66.3
T4	17	17.9
Tx	8	8.5
Primary nodal status <sup>2</sup>	95	
N0	25	26.3
N+	46	48.4
Nx	24	25.3
Metastases at diagnosis <sup>2</sup>	95	
M0	35	36.8
M1	60	63.2
Histological grade	95	
Gr I	12	12.6
Gr II	62	65.3
Gr III	18	19.0
NA	3	3.2
Stage	95	
Stage II	15	15.8
Stage III	20	21.0
Stage IV	60	63.2
Survival (mo)		
From primary diagnosis Median (range)	27.3 (3-150)	
From metastasis Median (range)	21.4 (3-80)	

<sup>1</sup>When applicable; <sup>2</sup>TNM classification; Tx: Unknown, Nx: Unknown, NA: Not available.

Laboratories, San Francisco, CA) at a dilution 1:200 (32 min, 37°C). The dilution of the primary antibody was based on previous dilution experiments. UltraView™ Universal DAB (a biotin-free, Multimer-based detection system for the specific and sensitive detection of mouse IgG, mouse IgM, and rabbit IgG primary antibodies) was used. UltraView DAB includes: ultraView Universal HRP, ultraView Universal DAB Inhibitor, ultraView Universal DAB Chromogen, ultraView Universal DAB H<sub>2</sub>O<sub>2</sub>, and ultraView Universal DAB Copper. Counterstaining with haematoxylin (2021) was done for 4 min, and post-counterstaining with a blueing reagent (2037) was done for 4 min as well. After staining, the sections were dehydrated in ethanol, cleared in xylene, and covered with Mountex and cover slips.

### Evaluation of $\beta$ -catenin staining

$\beta$ -catenin staining was evaluated using regular light microscopy by an observer who was blind to the clinical data (AB). All membranous, cytoplasmic, and nuclear staining were evaluated separately. For cell membrane staining, four categories were used (+++, ++, +, -), starting from equivalent to normal to entirely negative<sup>[10]</sup>. The cytoplasmic staining was also graded into four categories: (0) Negative, no detectable staining, (1) Weak, but still detectable staining, (2) Moderate, clearly positive but still weak, (3) Heavy staining, intense<sup>[11]</sup>.



**Figure 1** Different immunohistochemical (IHC) staining patterns for  $\beta$ -catenin in colorectal carcinomas. **A:** In normal colonic epithelium,  $\beta$ -catenin is predominantly expressed in the cell membrane; **B:** A medium-powered view of a colonic adenocarcinoma showing membranous and cytoplasmic expression of  $\beta$ -catenin; **C:** This case shows intense nuclear expression of  $\beta$ -catenin.

The nuclear staining index (NI) was also graded to into four categories (+++, ++, +, -): (0) Negative, only blue staining seen, (1) Weak, blue staining clearly seen through brown staining, (2) Moderate, blue scarcely seen through brown staining, nuclei appear darker than the cytoplasm, (3) Heavy staining, no blue seen through brown staining, nuclei appear darker than the cytoplasm. Three staining indexes were calculated: the membrane index (MI), cytoplasmic index (CI), and nuclear index (NI). These indices were calculated with both the intensity of staining and the fraction of positively-stained cells taken into account using the following formula:

$$I = 0 * f_0 + 1 * f_1 + 2 * f_2 + 3 * f_3$$

where I is the staining index and  $f_0$ - $f_3$  are the fractions of the cells showing a defined level of staining intensity (from 0 to 3). Theoretically, index scores could vary between 0 and 3<sup>[12]</sup>. The reproducibility of the evaluation of the  $\beta$ -catenin staining indices was tested by employing two observers (AE, AB), and the estimations showed good correlation and reproducibility (Pearson's r: MI, CI, and NI, were 0.77, 0.91, and 0.90, respectively).

### Statistical analysis

Statistical analyses were performed using SPSS® (SPSS, Inc., Chicago, USA) and STATA (Stata Corp., Texas, USA) software packages (SPSS for Windows, version 14.0.1 and STATA/SE 9.2). Frequency tables were analyzed using the Chi-square test, which included the likelihood ratio (LR) or Fischer's exact test to assess the significance of the correlation between the categorical variables. Odds ratios and their 95% confidence intervals (95% CI) were calculated where appropriate, using the exact method. Differences in the means of continuous variables were analyzed using non-parametric tests (Mann-Whitney or Kruskal-Wallis) for 2- and K-independent samples, respectively. ANOVA (analysis of variance) was only used for deriving the mean values (and their SD) for each individual category. Bivariate correlation (Spearman rho) and scatterplots were used to check the correlations between two continuous variables (MI, CI *vs* DFS, DSS), controlled by linear regression analysis (R and R<sup>2</sup>) for linearity. Univariate survival (life-table) analysis for the outcome measure (DSS, DFS) was based on Cox's method (indices treated as continuous

variables), and/or using Kaplan-Meier analysis (indices with Median as cut-off). Multivariate survival analysis was carried out using Cox's proportional hazards model in a backward stepwise manner with the log-likelihood ratio (L-R) significance test, using the default values for enter and exclusion criteria. The assumption of proportional hazards was controlled by log-minus-log (LML) survival plots. In all tests, the values  $P < 0.05$  were regarded statistically.

## RESULTS

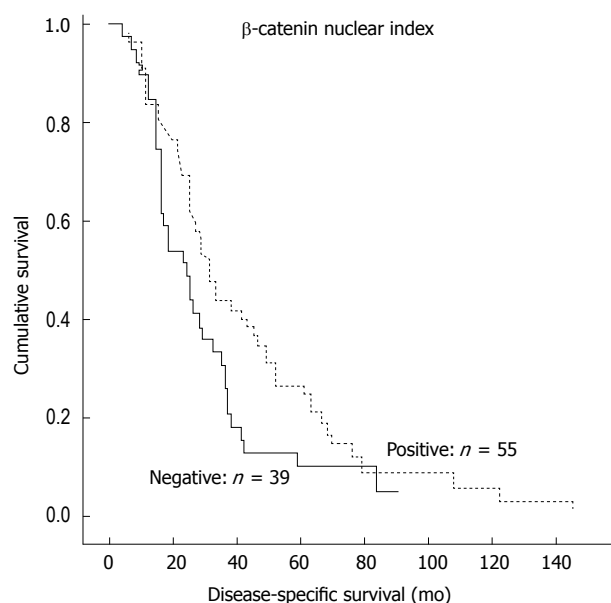
$\beta$ -catenin expression patterns are illustrated in Figure 1. The expression pattern of  $\beta$ -catenin was predominantly membranous and weakly cytoplasmic in normal colonic epithelium but the pattern was cytoplasmic, membranous, or nuclear in the tumor tissue. Almost 100% of the cases showed membranous and cytoplasmic  $\beta$ -catenin expression, with nuclear expression being observed in 55 (58%) cases. The mean values of the three  $\beta$ -catenin staining indices (MI, CI, and NI) were 1.14, 1.26, and 0.80, respectively, and the median values were 1.20, 1.30, and 0.77, respectively.

We analyzed the three  $\beta$ -catenin staining indices in relation to all available clinical variables and tumor characteristics in univariate analyses. Using the median cut-off point, there was no correlation between  $\beta$ -catenin expression and most of the clinical variables (age, sex, stage, and grade). However,  $\beta$ -catenin expression (CI and NI) was borderline or significantly related ( $P = 0.06$ ,  $P = 0.04$ , respectively) to the localization of the primary tumor, with expression being more intense in descending colon and rectum carcinomas than in lesions of the ascending and transverse colon.

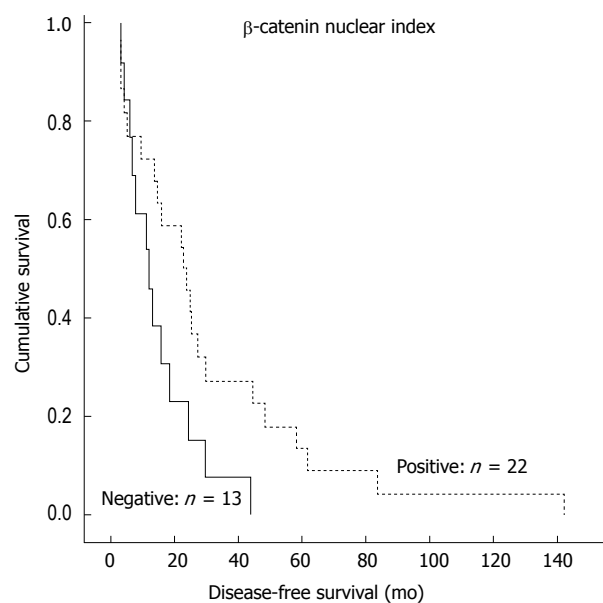
There was also a marginal relation ( $P = 0.086$ ) between NI and response to treatment in that the patients with a NI below the median had a response rate (24/48, 50%) better than those with a NI above the median (16/47, 34.0%). A direct relationship ( $P = 0.068$ ) was found between the MI and response to treatment when the 75th percentile was used; patients with a MI  $> 75\%$  had a higher response rate (9/16; 56.3%) than patients with a MI  $< 75\%$  (31/79, 39.2%).

In univariate (Kaplan-Meier) survival analysis





**Figure 2** Disease-specific survival predicted by the nuclear index (NI) of colorectal tumors. The stippled line: nuclear expression positive. The continuous line: nuclear expression negative. The Kaplan-Meier, log rank test;  $P = 0.046$ . One patient died of another cause and was excluded from analyses.



**Figure 3** Disease-free survival predicted by the nuclear index (NI) of colorectal tumors. The stippled line: nuclear expression positive. The continuous line: nuclear expression negative. The Kaplan-Meier, log rank test;  $P = 0.041$ .

(calculable for 35 patients with stage II or III disease) with the median as the cut-off, only the NI was a significant predictor of more favorable disease-free survival (DFS) ( $P = 0.023$ ). The patients with a NI above the median had longer DFS (34.2 mo) than those with a NI below the median (15.5 mo) ( $P = 0.045$ , ANOVA). The other indices were not significant predictors of DFS, and none of the three indices predicted disease-specific survival (DSS) in univariate analysis (Cox or Kaplan-Meier).

When the patients were stratified into two groups: nuclear expression positive ( $n = 55$ ) and nuclear expression negative ( $n = 39$ ), the former was a significant predictor of more favorable DSS ( $P = 0.046$ ) (Figure 2) and DFS ( $P = 0.041$ ) (Figure 3).

When  $\beta$ -catenin expression was analyzed jointly with E-cadherin expression<sup>[13]</sup> as a potential predictor of disease outcome, the combined (E-cadherin and  $\beta$ -catenin) cytoplasmic index did not provide any significant prognostic information.

We also reproduced the grading used by Ougolkov *et al*<sup>[7]</sup>, resulting in 6/46 cases with nuclear expression at the invasive front, 20/46 cases with a diffuse nuclear pattern, and 20/46 tumors with a mixed pattern. When correlated with the treatment response and disease outcome, this grading system did not produce any results with predictive or prognostic value.

## DISCUSSION

As compared with the sub-cellular distribution of  $\beta$ -catenin in normal colonic mucosa, neoplastic cells demonstrated a distinct shift from a membranous localization to a more widespread distribution (membranous, cytoplasmic, and nuclear) in cancer lesions. This is in line

with previous reports describing  $\beta$ -catenin expression in cancer cells with this type of altered pattern<sup>[14,15]</sup>. For example, Wong *et al*<sup>[16]</sup> observed no nuclear  $\beta$ -catenin accumulation in normal tissues, whereas it was present in 8% of polyps, 92% of adenomas, and 100% of carcinomas. In the present series, nuclear expression or accumulation was observed in 48% of the cancer samples, which is in line with several other reports<sup>[7,17]</sup>. Interestingly, in 13% of the cases with nuclear expression,  $\beta$ -catenin was expressed by the tumour cells at the invasion fronts, a figure very similar to the 9% reported by Ougolkov *et al*<sup>[7]</sup>.

We did not find significant correlations between the three  $\beta$ -catenin expression patterns (MI, CI, and NI) and most of the clinical variables recorded (age, sex, grade, and stage), except for tumor localization. Accordingly,  $\beta$ -catenin expression, both CI and NI, was more intense in carcinomas of the descending colon and rectum as compared with lesions localized in the ascending and transverse colon. Similar observations have been reported by previous studies<sup>[18-20]</sup>. There is increasing evidence to suggest that molecular mechanisms and molecular phenotypes differ in carcinomas arising in the proximal and distal segments of the large bowel<sup>[21]</sup>. The involvement of different molecular pathways in colorectal carcinogenesis is exemplified by the fact that cancers of “mutator” phenotypes preferentially occur in the proximal (right side) colon, whereas the adenoma-carcinoma sequence phenotype is characteristic of carcinomas in the distal (left side) colon and rectum<sup>[22,23]</sup>. Corresponding differences have also been shown in association with other potential prognosticators<sup>[24]</sup>.

Interestingly, a marginally significant relation was observed between the NI and MI and response to treatment. Accordingly, the patients who did not respond to treatment had a NI above the median, whereas patients

who had a high MI responded better to treatment. The significance of these observations remains to be elucidated in a larger study. There is an obvious need to identify novel molecular targets for cancer therapeutics, and in this respect, the recent data showing that suppression of  $\beta$ -catenin can inhibit the neoplastic growth of APC-mutant colorectal cancer are of interest<sup>[25]</sup>.

The correlation between  $\beta$ -catenin expression pattern and clinical outcome is a controversial subject. Some studies reported that cytoplasmic rather than nuclear accumulation of  $\beta$ -catenin is significantly related to metastasis-free survival in CRC<sup>[6]</sup>. The same was reported regarding the potential prognostic value of nuclear expression. There are studies reporting that positive nuclear expression at the invasive front of the tumor predicts shorter survival<sup>[7,19]</sup>. However, other workers failed to find any correlation between nuclear expression and survival in CRC<sup>[17,26]</sup>. In contrast, our data show that nuclear expression is a significant predictor of more favorable DSS and DFS (Figures 2 and 3). We also analyzed our samples using the same system as originally described by Ougolkov *et al*<sup>[7]</sup>. In our study, however, this special grading system did not confirm the original observation that nuclear expression at the invasive front of the tumor predicts a shorter survival<sup>[7]</sup>.

There are multiple explanations for the inconsistent and, in part, discrepant results reported in different studies<sup>[6,7,17,19,26]</sup>. Such potential confounding factors might include the size of tissue samples, intrinsic tumor heterogeneity, lack of standardization in the evaluation of positive and negative results, and different immunohistochemical staining and grading methods with varying degree of sensitivity. In addition, our patients represent advanced CRC, with the majority of patients having stage IV disease, as compared with the Ougolkov study, where the majority of patients had stage II CRC. Also, the type of treatment may have played a role in the detected relationships.

Cell-cell adhesion molecules are believed to participate in the processes of invasion, migration and metastasis<sup>[27-29]</sup>. In this regard, the E-cadherin and  $\beta$ -catenin complex plays a critical role in cell-cell adhesion. E-cadherin is a member of the cadherin family that mediates calcium-dependent adhesion to ensure the maintenance of a normal phenotype of epithelial cells<sup>[1,30]</sup>.  $\beta$ -catenin binds directly to the cytoplasmic domain of E-cadherin and to the actin microfilament network of the cellular cytoskeleton. This binding is essential for stable cell-cell adhesion<sup>[31]</sup>.

It can be reasoned that altered expression of  $\beta$ -catenin (i.e., the shift from membranous to cytoplasmic and nuclear sites) might compromise the integrity of the E-cadherin/ $\beta$ -catenin complex and result in weaker cell-cell adhesion in cancer cells. Thus, it seems feasible to assess whether altered co-expression patterns of these two markers is of any predictive value in CRC. For that purpose, we combined both the membranous and cytoplasmic E-cadherin expression with membranous and cytoplasmic  $\beta$ -catenin expression to compare normal (MI/MI) and abnormal (CI/CI)

co-expression, respectively, of these two markers as previously analysed in CRC<sup>[13]</sup>. To our disappointment, however, neither the membranous nor the cytoplasmic E-cadherin/ $\beta$ -catenin index (analyzed in two different modes) provided any useful information as to DFS or DSS. Thus, no added value can be obtained with analyzing E-cadherin expression together with  $\beta$ -catenin expression as compared to the analysis of the latter alone (Figures 2 and 3).

Taken together, the present results confirm that  $\beta$ -catenin expression is markedly altered in the vast majority of colorectal cancers. This shift from normal membranous expression to the cytoplasmic (CI) and nuclear (NI) patterns seems to bear some association with the localization of the primary tumour, being most pronounced in lesions of the descending colon and rectum. Although the association of CI and NI to treatment response remains unclear, NI seems to provide some prognostic value in predicting more favourable DFS and also DSS, when dichotomized as NI+/NI-expression. Many of the issues still remain unanswered, however, and additional clinical and experimental studies are needed to fully elucidate the role of  $\beta$ -catenin in colorectal carcinogenesis and its potential usefulness as an independent predictor of disease outcome.

## REFERENCES

- 1 **Aberle H**, Butz S, Stappert J, Weissig H, Kemler R, Hoschuetzky H. Assembly of the cadherin-catenin complex in vitro with recombinant proteins. *J Cell Sci* 1994; **107** (Pt 12): 3655-3663
- 2 **Barth AI**, Nathke IS, Nelson WJ. Cadherins, catenins and APC protein: interplay between cytoskeletal complexes and signaling pathways. *Curr Opin Cell Biol* 1997; **9**: 683-690
- 3 **Fujimori M**, Ikeda S, Shimizu Y, Okajima M, Asahara T. Accumulation of beta-catenin protein and mutations in exon 3 of beta-catenin gene in gastrointestinal carcinoid tumor. *Cancer Res* 2001; **61**: 6656-6659
- 4 **Nilbert M**, Rambech E. Beta-catenin activation through mutation is rare in rectal cancer. *Cancer Genet Cytogenet* 2001; **128**: 43-45
- 5 **Aust DE**, Terdiman JP, Willenbacher RF, Chang CG, Molinaro-Clark A, Baretton GB, Loehrs U, Waldman FM. The APC/beta-catenin pathway in ulcerative colitis-related colorectal carcinomas: a mutational analysis. *Cancer* 2002; **94**: 1421-1427
- 6 **Maruyama K**, Ochiai A, Akimoto S, Nakamura S, Baba S, Moriya Y, Hirohashi S. Cytoplasmic beta-catenin accumulation as a predictor of hematogenous metastasis in human colorectal cancer. *Oncology* 2000; **59**: 302-309
- 7 **Ougolkov AV**, Yamashita K, Mai M, Minamoto T. Oncogenic beta-catenin and MMP-7 (matrilysin) cosegregate in late-stage clinical colon cancer. *Gastroenterology* 2002; **122**: 60-71
- 8 **Miyamoto S**, Endoh Y, Hasebe T, Ishii G, Kodama K, Goya M, Ono M, Saitoh N, Chiba T, Ochiai A. Nuclear beta-catenin accumulation as a prognostic factor in Dukes' D human colorectal cancers. *Oncol Rep* 2004; **12**: 245-251
- 9 **Glimelius B**, Ristamaki R, Kjaer M, Pfeiffer P, Skovsgaard T, Tveit KM, Linne T, Frodin JE, Boussard B, Oulid-Aissa D, Pyrhonen S. Irinotecan combined with bolus 5-fluorouracil and folinic acid Nordic schedule as first-line therapy in advanced colorectal cancer. *Ann Oncol* 2002; **13**: 1868-1873
- 10 **Elzagheid A**, Kuopio T, Ilmen M, Collan Y. Prognostication of invasive ductal breast cancer by quantification of

- E-cadherin immunostaining: the methodology and clinical relevance. *Histopathology* 2002; **41**: 127-133
- 11 **Elzagheid A**, Algars A, Bendardaf R, Lamlum H, Ristamaki R, Collan Y, Syrjanen K, Pyrhonen S. E-cadherin expression pattern in primary colorectal carcinomas and their metastases reflects disease outcome. *World J Gastroenterol* 2006; **12**: 4304-4309
  - 12 **Lipponen P**, Collan Y. Simple quantitation of immunohistochemical staining positivity in microscopy. *Acta Stereol* 1992; **11**: 125-132
  - 13 **Bendardaf R**, Elzagheid A, Lamlum H, Ristamaki R, Collan Y, Pyrhonen S. E-cadherin, CD44s and CD44v6 correlate with tumour differentiation in colorectal cancer. *Oncol Rep* 2005; **13**: 831-835
  - 14 **Mikami T**, Mitomi H, Hara A, Yanagisawa N, Yoshida T, Tsuruta O, Okayasu I. Decreased expression of CD44, alpha-catenin, and deleted colon carcinoma and altered expression of beta-catenin in ulcerative colitis-associated dysplasia and carcinoma, as compared with sporadic colon neoplasms. *Cancer* 2000; **89**: 733-740
  - 15 **Horkko TT**, Klintrup K, Makinen JM, Napankangas JB, Tuominen HJ, Makela J, Karttunen TJ, Makinen MJ. Budding invasive margin and prognosis in colorectal cancer--no direct association with beta-catenin expression. *Eur J Cancer* 2006; **42**: 964-971
  - 16 **Wong SC**, Lo ES, Lee KC, Chan JK, Hsiao WL. Prognostic and diagnostic significance of beta-catenin nuclear immunostaining in colorectal cancer. *Clin Cancer Res* 2004; **10**: 1401-1408
  - 17 **Roca F**, Mauro LV, Morandi A, Bonadeo F, Vaccaro C, Quintana GO, Specterman S, de Kier Joffe EB, Pallotta MG, Puricelli LI, Lastiri J. Prognostic value of E-cadherin, beta-catenin, MMPs (7 and 9), and TIMPs (1 and 2) in patients with colorectal carcinoma. *J Surg Oncol* 2006; **93**: 151-160
  - 18 **Zhang B**, Ougolkov A, Yamashita K, Takahashi Y, Mai M, Minamoto T. beta-Catenin and ras oncogenes detect most human colorectal cancer. *Clin Cancer Res* 2003; **9**: 3073-3079
  - 19 **Baldus SE**, Monig SP, Huxel S, Landsberg S, Hanisch FG, Engelmann K, Schneider PM, Thiele J, Holscher AH, Dienes HP. MUC1 and nuclear beta-catenin are coexpressed at the invasion front of colorectal carcinomas and are both correlated with tumor prognosis. *Clin Cancer Res* 2004; **10**: 2790-2796
  - 20 **Feng Han Q**, Zhao W, Bentel J, Shearwood AM, Zeps N, Joseph D, Iacopetta B, Dharmarajan A. Expression of sFRP-4 and beta-catenin in human colorectal carcinoma. *Cancer Lett* 2006; **231**: 129-137
  - 21 **Chung DC**. The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis. *Gastroenterology* 2000; **119**: 854-865
  - 22 **Loeb LA**. A mutator phenotype in cancer. *Cancer Res* 2001; **61**: 3230-3239
  - 23 **Yashiro M**, Carethers JM, Laghi L, Saito K, Slezak P, Jaramillo E, Rubio C, Koizumi K, Hirakawa K, Boland CR. Genetic pathways in the evolution of morphologically distinct colorectal neoplasms. *Cancer Res* 2001; **61**: 2676-2683
  - 24 **Hilska M**, Roberts PJ, Collan YU, Laine VJ, Kossi J, Hirsimaki P, Rahkonen O, Laato M. Prognostic significance of matrix metalloproteinases-1, -2, -7 and -13 and tissue inhibitors of metalloproteinases-1, -2, -3 and -4 in colorectal cancer. *Int J Cancer* 2007; **121**: 714-723
  - 25 **Green DW**, Roh H, Pippin JA, Drebin JA. Beta-catenin antisense treatment decreases beta-catenin expression and tumor growth rate in colon carcinoma xenografts. *J Surg Res* 2001; **101**: 16-20
  - 26 **Chung GG**, Provost E, Kielhorn EP, Charette LA, Smith BL, Rimm DL. Tissue microarray analysis of beta-catenin in colorectal cancer shows nuclear phospho-beta-catenin is associated with a better prognosis. *Clin Cancer Res* 2001; **7**: 4013-4020
  - 27 **Mareel M**, Boterberg T, Noc V, Van Hoorde L, Vermeulen S, Bruyneel E, Bracke M. E-cadherin/catenin/cytoskeleton complex: a regulator of cancer invasion. *J Cell Physiol* 1997; **173**: 271-274
  - 28 **Nelson WJ**, Nusse R. Convergence of Wnt, beta-catenin, and cadherin pathways. *Science* 2004; **303**: 1483-1487
  - 29 **Bonitsis N**, Batistatou A, Karantima S, Charalabopoulos K. The role of cadherin/catenin complex in malignant melanoma. *Exp Oncol* 2006; **28**: 187-193
  - 30 **Munro SB**, Blaschuk OW. A comprehensive survey of the cadherins expressed in the testes of fetal, immature, and adult mice utilizing the polymerase chain reaction. *Biol Reprod* 1996; **55**: 822-827
  - 31 **Shiozaki H**, Oka H, Inoue M, Tamura S, Monden M. E-cadherin mediated adhesion system in cancer cells. *Cancer* 1996; **77**: 1605-1613

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RAPID COMMUNICATION

## Intrahepatic CD8<sup>+</sup> lymphocyte trapping during tolerance induction using mushroom derived formulations: A possible role for liver in tolerance induction

Mony Shuvy, Tiberiu Hershcovici, Cristina Lull-Noguera, Harry Wichers, Ofer Danay, Dan Levanon, Lidya Zolotarov, Yaron Ilan

Mony Shuvy, Tiberiu Hershcovici, Lidya Zolotarov, Yaron Ilan, Liver Unit, Department of Medicine, Hadassah, Hebrew University Medical Center, Jerusalem IL-91120, Israel  
Cristina Lull-Noguera, Harry Wichers, Wageningen University and Research Center, Wageningen 6706 KN, The Netherlands

Cristina Lull-Noguera, Department of Animal Production and Food Science and Technology, University CEU-Cardenal Herrera, Avenida Seminario s/n, Moncada 46113, Valencia  
Ofer Danay, Dan Levanon, Migal, Kiryat Shmone 11016, Israel

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**Author contributions:** All authors were involved in designing the research and performed the research. Shuvy M and Hershcovici T contributed equally.

**Correspondence to:** Yaron Ilan, MD, Liver Unit, Department of Medicine, Hebrew University-Hadassah Medical Center, P.O.B 12000, Jerusalem IL-91120, Israel. [ilan@hadassah.org.il](mailto:ilan@hadassah.org.il)  
Telephone: +972-2-6778231 Fax: +972-2-6431021

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### Abstract

**AIM:** To determine the immunomodulatory effect of Shiitake (a mushroom extract), we tested its effect on liver-mediated immune regulation in a model of immune-mediated colitis.

**METHODS:** Four groups of mice were studied. Colitis was induced by intracolonic instillation of TNBS in groups A and B. Groups A and C were treated daily with Shiitake extract, while groups B and D received bovine serum albumin. Mice were evaluated for development of macroscopic and microscopic. The immune effects of Shiitake were determined by FACS analysis of intra-hepatic and intrasplenic lymphocytes and IFN- $\gamma$  ELISPOT assay.

**RESULTS:** Administration of Shiitake resulted in marked alleviation of colitis, manifested by significant improvement in the macroscopic and microscopic scores, and by reduction in IFN- $\gamma$ -producing colonies in group A, compared to group B mice (1.5 pfu/mL vs 3.7 pfu/mL, respectively). This beneficial effect was associated with a significant increase in the intra-hepatic CD8<sup>+</sup> lymphocyte trapping, demonstrated

by an increased intrasplenic/intrahepatic CD4/CD8 lymphocyte ratio. These effects were accompanied by a 17% increase in the number of intrahepatic natural killer T (NKT) cells. A similar effect was observed when Shiitake was administered to animals without disease induction.

**CONCLUSION:** Shiitake extract affected liver-mediated immune regulation by altering the NKT lymphocyte distribution and increasing intrahepatic CD8<sup>+</sup> T lymphocyte trapping, thereby leading to alleviation of immune-mediated colitis.

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**Key words:** Mushrooms; Colitis; Immune modulation; Shiitake; Natural killer T cell

**Peer reviewers:** Dr. Wing-Kin Syn, Department of Medicine, Division of Gastroenterology, Duke University MC, GSRB-1, Suite 1073, DUMC 3256, 595 LaSalle Street, Durham 27710, United States; Mario U Mondelli, Prof, Department of Infectious Diseases, Fondazione IRCCS Policlinico San Matteo and University of Pavia, Laboratori Area Infettivologica, Dipartimento di Malattie Infettive, Fondazione IRCCS Policlinico San Matteo, via Taramelli 5, Pavia 27100, Italy

Shuvy M, Hershcovici T, Lull-Noguera C, Wichers H, Danay O, Levanon D, Zolotarov L, Ilan Y. Intrahepatic CD8<sup>+</sup> lymphocyte trapping during tolerance induction using mushroom derived formulations: A possible role for liver in tolerance induction. *World J Gastroenterol* 2008; 14(24): 3872-3878 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3872.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3872>

### INTRODUCTION

Mushrooms have been valued by humans throughout history as a food product and for medical purposes<sup>[1]</sup>. Mushrooms have been used as a medicine in the Far East since ancient times. Extracts and isolated metabolites from mushrooms are known to modulate immune responses<sup>[2]</sup>, resulting in enhanced innate and acquired disease resistance. The major immunomodulatory effects of the active substances derived from mushrooms include mitogenicity and activation of immune effector



cells, such as lymphocytes, macrophages, and natural killer cells. Activation of these cells can result in the production of cytokines, including interleukins (ILs), tumor necrosis factor alpha (TNF- $\alpha$ ), and interferon gamma (INF- $\gamma$ )<sup>[2]</sup>. The ability of select mushroom extracts to modulate the differentiation capacity of CD4<sup>+</sup> T cells into mature Th1 and/or Th2 subsets has been documented recently<sup>[3]</sup>.

Other recent studies have suggested that these extracts may have a profound effect on Th1- or Th2-immune mediated disorders<sup>[4]</sup>. A number of bioactive molecules, mostly polysaccharides with anti-tumor properties have been identified in mushroom-derived formulations<sup>[5]</sup>.

Lentian, a (1-3)- $\beta$ -glucan from *Lentinus edodes* (Shiitake), is licensed as an immunostimulatory drug<sup>[6]</sup>. Pre-treatment of mice with lentian results in increased concentrations of TNF- $\alpha$ , IL-12, and INF- $\gamma$ , as well as an increase in the number of *Listeria monocytogenes*-specific CD8 T cells in the spleen. The bacterial burden in the spleen and liver of mice was reduced significantly during primary and secondary listeria infection after lentian pre-treatment of mice. In addition, *Lentinus edodes* and its active component, the polysaccharide lentian, have been found to be effective against several tumors, including prostate and gastric tumors, and leukemia<sup>[7,8]</sup>. However, the mechanism of action of lentian is not clearly understood and there is currently no data on its effect on immune-mediated diseases.

The role of liver in the pathogenesis of various immune-mediated disorders is well known<sup>[9]</sup>. Liver contains a mixture of lymphocytes including both conventional T and B cells, as well as a distinct population of resident liver lymphocytes. Furthermore, liver is involved in the trapping and destruction of activated T cells<sup>[10]</sup>. As of this writing, the precise role of the liver in immune cell trapping and destruction is not fully understood, although one theory suggests that cells already in the process of apoptosis are sequestered in the liver<sup>[11,12]</sup>. A second theory suggests that the liver plays an active role by destroying activated T cells through a local tolerance mechanism that causes clonal deletion<sup>[13,14]</sup>.

The goal of the present study was to determine the effect of Shiitake on colonic inflammation in a murine model of immune-mediated colitis and to examine the role of liver in systemic tolerance induction in this setting.

## MATERIALS AND METHODS

### Animals

Normal inbred 2-4 mo old C57BL male mice were obtained from Harlan, Israel, and maintained in the Animal Core of the Hadassah-Hebrew University Medical School. Mice were maintained on standard laboratory feed and kept in 12-h light/dark cycles. All of the experiments were performed in accordance with the institute's ethical committee for animal handling.

### Experimental design

Four groups of mice consisting of 10 animals each

were studied. Mice in experimental group A were fed Shiitake extract (50 g/mouse), starting 2 d before (day 2), until 9 d after induction of colitis by intra-rectal administration of trinitrobenzene sulfonic acid (TNBS, day 9) as described previously<sup>[15]</sup>. Mice in group B were fed bovine serum albumin (BSA, 50 g/mouse). Group C was fed with Shiitake extract (50 g/mouse) from day 0 of experiment until day 9. Mice in control group D were fed with BSA, 50 g/mouse from day 0 to day 9. Mice were sacrificed on day 10.

### Preparation and administration of the mushrooms

**Source:** Mushroom spawn (*Lentinus edodes* 4087) used in this study was purchased from Sylvan (France).

**Mushroom culture:** Mushrooms were grown on a 1:1 mixture of cotton and wheat straws. The straws were oven dried at 60°C for 24 h and milled to 1-3 cm particle size. The straw mixture was wetted to 70% water content and packed into 4 kg polypropylene bags containing a microporous filter. The bags were steam sterilized at 100°C for 2 h, then cooled to 25°C for inoculation with 2% spawn w/w. The culture was incubated at 25°C for 30 d. For fruiting, the temperature was reduced to 16°C with a relative humidity of 90%, 12 h daily light and CO<sub>2</sub> concentration of 600-800 ppm. The fruiting bodies were then oven dried at 60°C for 24 h and milled to 1 mm particle size.

**Experimental colitis:** Colitis was induced by a single enema of TNBS (Sigma, USA)<sup>[16]</sup>. Fifty milligrams of TNBS was dissolved in 20% ethanol (total volume 1 mL) and instilled through a rubber catheter 10 cm into the colon *via* the anus, without any bowel preparation. The animals were kept fasting for 24 h prior to the procedure and were anesthetized with Ketalar. After instillation, the mice were maintained in the supine position until recovery from the anesthesia to prevent immediate leakage of the instillate.

**Assessment of colonic damage:** Assessment of colonic damage was performed 10 d following induction of colitis<sup>[15]</sup>. Ten centimeters of the distal colonic tissue was removed and opened by a longitudinal incision and gently washed with saline. The freshly opened colonic segments were examined by an independent observer blinded to the treatment. The extent of the mucosal damage was assessed. Four macroscopic parameters were determined: degree of colonic ulceration, intestinal and peritoneal adhesions, wall thickness, and degree of mucosal edema. Each parameter was graded on a scale from 0 (completely normal) to 4 (most severe).

**Colonic histology:** For each animal, 6 specimens of colonic tissue from the distal 10 cm were removed for histological analysis. The tissues were fixed in formaldehyde, then sliced into 4 to 6 mm pieces, dehydrated in ethanol, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin. The degree of inflammation on microscopic sections of the colon was graded semi-quantitatively from 0 to 4.

Grade 0: Normal with no signs of inflammation; Grade 1: very low level of leukocyte infiltration; Grade 2: Low level of leukocyte infiltration; and Grade 3: High level of infiltration with high vascular density and bowel wall thickening; Grade 4: Transmural infiltration, with loss of goblet cells, high vascular density, wall thickening, and disruption of normal bowel architecture. Two experienced examiners who were blinded to the study group performed the grading.

#### **Evaluation of the role of natural killer T (NKT) lymphocytes and the liver in CD4 trapping**

**Liver and spleen lymphocyte isolation:** Splenocytes and liver lymphocytes were isolated as described previously with the following modifications<sup>[17]</sup>. The inferior vena cava was cut above the diaphragm and the liver was flushed with 5 mL cold PBS until it became pale in color. The connective tissue and the gallbladder were removed, and the liver was placed in a 10 mL dish containing cold sterile PBS. Splenocytes and liver lymphocytes were isolated by crushing the spleen and liver through a stainless mesh (size 60, Sigma Chemical Co., St. Louis MO)<sup>[16]</sup>. The resulting cell suspension was placed in a 50 mL tube for 3 min, washed twice with cold PBS (1250 r/min for 10 min), and all insoluble debris was removed. Cells were re-suspended in PBS, and passed through a nylon mesh pre-soaked in PBS. Unbound cells were collected and washed twice in 45 mL PBS. For liver and spleen lymphocyte isolation, the cells were suspended in a 50 mL tube containing 7 mL PBS, and underlaid with 20 mL Histopaque 1077 (Sigma Diagnostics, St. Louis, MO). The tube was centrifuged at 1640 r/min for 15 min at room temperature. Cells at the density interface were collected, diluted in a 50 mL tube, and washed twice with ice-cold PBS (pelleting at 1250 r/min for 10 min). Approximately  $1 \times 10^6$  cells per mouse liver were recovered. The cell viability, assessed by Trypan blue staining was > 95%. Both splenocytes and liver-associated lymphocytes were isolated from every animal in all the experimental groups.

**Flow cytometry analysis for determination of CD4<sup>+</sup>, CD8<sup>+</sup> and NKT lymphocyte subsets:** Following lymphocyte isolation, triplicate samples of  $2 \times 10^5$  to  $5 \times 10^5$  cells/500  $\mu$ L PBS were placed in Falcon 2052 tubes, incubated with 4 mL 1% BSA for 10 min, and centrifuged at 1400 r/min for 5 min. The cells were resuspended in 10  $\mu$ L FCS with 1:20 FITC-anti mouse CD3 antibody, 1:20 PE-anti mouse CD4 antibody, 1:20 APC-anti mouse CD8 antibody, or 1:20 FITC-anti mouse NK1.1 antibody (NKR-P1C, Pharmingen, USA), and mixed every 10 min for 30 min. The cells were washed twice in 1% BSA, and kept at 4°C until reading. In the control group, only 5  $\mu$ L of 1% BSA was added. Analytical cell sorting was performed on  $1 \times 10^4$  cells from each group with a fluorescence-activated cell sorter (FACSTAR plus, Becton Dickinson). Only live cells were counted, and background fluorescence from non-antibody-treated lymphocytes was subtracted. Gates were set by forward- and side-scatter to exclude dead cells and red blood cells. Data

was analyzed by the Consort 30 two-color contour plot (Becton Dickinson, Oxnard, CA) or Cellquest program.

**Antigen specific IFN ELISPOT assays:** IFN spot forming cells (SFC), were identified using a modified subject-specific, antigen-directed ELISPOT assay (Mabtech, Nacka, Sweden)<sup>[18]</sup>. Filtration plates (96 well), coated with high protein binding hydrophobic PVDF membrane were used (Millipore Corp., Bedford, MA, USA). The plates were coated with 1-D1K anti-IFN coating antibody (15 mg/mL, Mabtech, Nacka, Sweden) for 24 h at 4°C. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll gradient separation of 2 mL whole blood samples, collected in acid citrate dextrose tubes, and processed within 1 h. The PBMC were washed twice in RPMI 1640 with 10% fetal bovine serum. The cells were cultured in 96 well plates ( $1 \times 10^5$  cells/well) with RPMI 1640 and 10% FBS. Triplicate samples were prepared with 2 doses of the study drug from each subject (5 and 10 g/mL) or phytohemagglutinin (PHA, 2.5 g/mL) without antigen. Plates were incubated for 48 h at 37°C under 5% CO<sub>2</sub>. The plates were then washed and 100  $\mu$ L biotinylated antibody (7-B6-1-biotin, Mabtech, Nacka, Sweden) at a concentration of 1  $\mu$ g/mL in filtered PBS with 0.5% FBS. Plates were incubated for 3 h at room temperature. Following washing, 100  $\mu$ L of streptavidin-alkaline phosphatase was added, and the plates were incubated for 90 min at room temperature. The plates were washed and substrate (BioRad, Richmond, CA) was added for 30 min, until reddish-purple spots appeared. Using a dissection microscope, dark spots, reflecting IFN- $\gamma$ -secreting clones, were counted. The results were expressed as mean IFN- $\gamma$ -secreting cells per  $10^5$  PBMC (in triplicate), after subtraction of the mean spots from wells without the study drug.

#### **Statistical analysis**

Statistical analysis was performed using the student's *t* test. *P* < 0.05 was considered significant.

## **RESULTS**

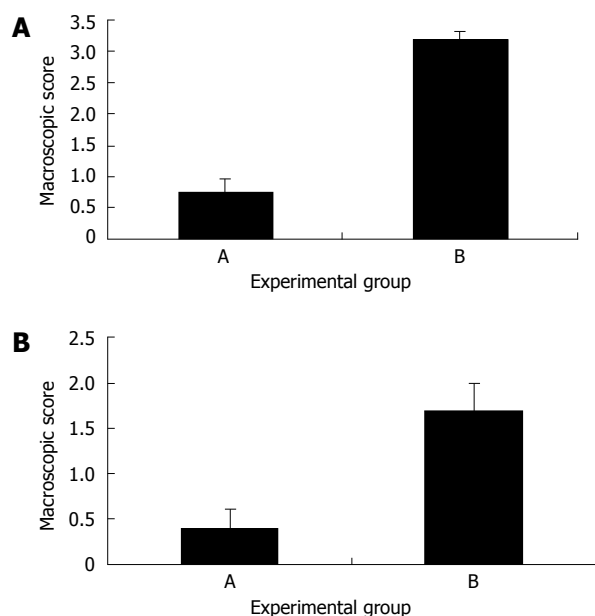
#### **Effect of Shiitake on survival**

Shiitake administration was associated with significant improvement in the survival of animals in group A, compared with the control group B (BSA control). Of the mice in group A, 100% survived *vs* 44% in group B. All mice in control groups C and D survived.

#### **Effect of Shiitake on severity of experimental colitis**

**Macroscopic scoring of colitis:** The severity of colitis in mice treated with Shiitake mushroom showed marked improvement compared with mice in group B, that were fed BSA (mean macroscopic score  $0.75 \pm 0.2$  and  $3.2 \pm 0.1$  for groups A and B, respectively, *P* < 0.005, Figure 1A). No significant signs of colitis were observed in mice in groups C and D.

**Histological assessment of colitis:** Similar results



**Figure 1** A: Effect of Shiitake administration on the macroscopic score of experimental colitis: Shiitake administration significantly alleviated the severity of colitis in group A mice compared with mice in control group B; B: Effect of Shiitake administration on the microscopic score of colitis: Shiitake administration alleviated the severity of colitis in group A mice compared with mice in control group B.

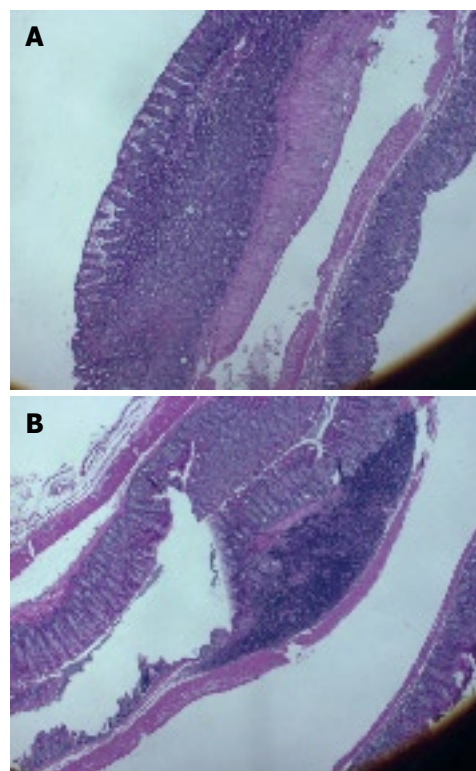
were obtained in the total microscopic score of colitis. The mean microscopic score was  $0.4 \pm 0.2$  in group A *vs*  $1.7 \pm 0.3$  in group B (Figure 1B). Histological evaluation of colonic tissues showed a marked reduction in the inflammatory response in Shiitake fed mice (Figure 2A). By contrast, mice in group B showed severe colitis, manifested by inflammatory infiltration of the mucosa, and patchy necrosis of the mucosa and submucosa with purulent and fibrinoid material extending up to the muscle layer. The muscle and serosal layers showed infiltration by acute and chronic inflammatory cells (Figure 2B).

#### Effect of Shiitake on NKT lymphocyte distribution

The number of intra-hepatic NKT cells was determined in all experimental groups. The number of liver NKT cells was increased in group A, compared to group B (29.1% *vs* 18.6%, Figure 3A). Liver NKT cells were also increased in group C compared with group D (32.1% *vs* 24.1%). The intrasplenic NKT cell counts, reflecting the total systemic pools of NKT cells, were also determined. A decrease in NKT cell number was observed in group A compared with group B, and in group C compared with group D (1.1 *vs* 2.4 and 1.6 *vs* 3.3, respectively). To determine whether the intra-hepatic NKT increase was part of a systemic increase in NKT cells, the intrasplenic/intrahepatic NKT cell ratio was calculated. This ratio was significantly reduced in the Shiitake fed mice (groups A and C *vs* groups B and D, Figure 3B).

#### Effect of Shiitake on the intrahepatic and systemic CD4/CD8 ratios

The intra-hepatic CD4/CD8 ratio was reduced signifi-



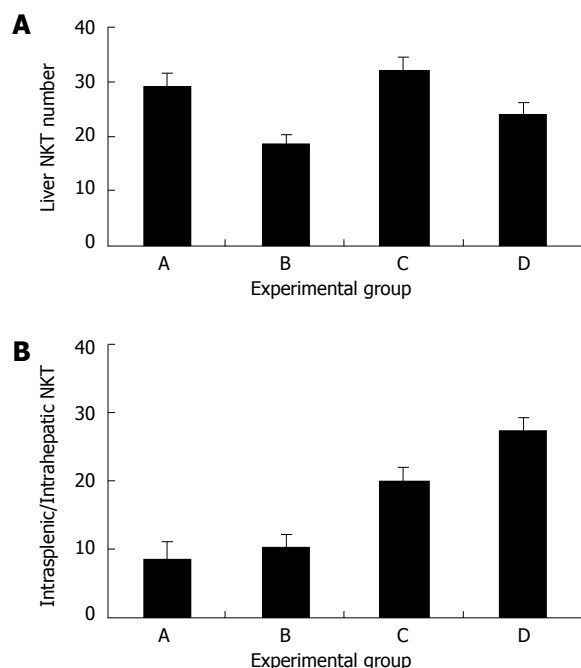
**Figure 2** Histological evaluation of colonic tissues showed a marked reduction in the inflammatory response in Shiitake fed mice (A). By contrast, severe colitis was observed in mice in group B, manifested by inflammatory infiltration of the mucosa, and patchy necrosis of the mucosa and submucosa, with purulent and fibrinoid material extending to the muscle layer (B).

cantly in mice that were fed Shiitake compared with those fed BSA (1.5 for group A *vs* 2.4 in group B,  $P < 0.005$ , Figure 4). A marked decrease in the intra-hepatic CD4/CD8 ratio was also noted in group C compared with the naïve group D (1.5 *vs* 2,  $P < 0.005$ , Figure 4). Interestingly, an opposite effect was noted when the intrasplenic CD4/CD8 ratios were calculated. Intra-hepatic CD8 trapping in Shiitake fed mice was associated with an increase in the peripheral (intrasplenic) CD4/CD8 ratio compared with BSA fed mice (4 and 2.1 for groups A and B, respectively;  $P < 0.005$ ; Figure 4). However, even in mice without colitis, feeding Shiitake caused the peripheral CD4/CD8 ratio to decrease compared with naïve mice (2.3 in group C *vs* 3.9 in group D,  $P < 0.005$ , Figure 4). The intrasplenic/intrahepatic CD4/CD8 ratio was also calculated. This ratio was increased in groups A, C, D, while it was reduced significantly in group B (2.6, 1.5, 1.80 *vs* 0.9, respectively;  $P < 0.005$  for all; Figure 5). The increased peripheral/liver CD4/CD8 ratio suggests CD8 lymphocyte trapping in the liver during systemic tolerance induction.

#### Effect of Shiitake on antigen-directed IFN ELISPOT

The number of antigen specific T-cell colonies secreting IFN- $\gamma$  was reduced significantly in group A compared with group B (1.5 pfu/mL *vs* 3.7 pfu/mL,  $P < 0.005$ ), suggesting a specific effect of Shiitake on T cells involved in Th1-mediated immune colitis.



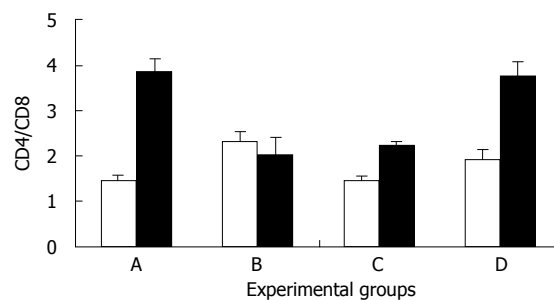


**Figure 3** A: The effect of Shiitake administration on intrahepatic NKT cells. Liver NKT cell numbers were increased in groups A and C (which received Shiitake), compared with mice in group B and in control group D; B: The effect of Shiitake administration on the intrasplenic/ intrahepatic NKT cell ratio.

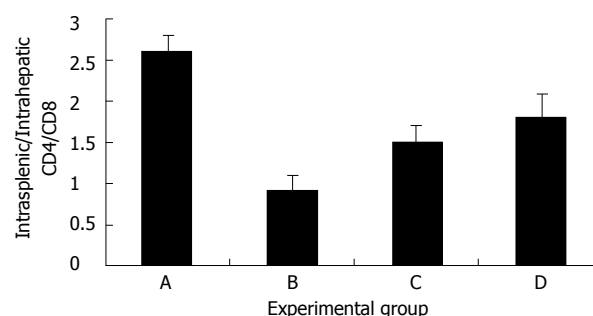
## DISCUSSION

Previous studies have shown that Lentinan, a (1-3)- $\beta$ -glucan obtained from *Lentinus edodes*, has anti-tumor activity, but the purported immunomodulatory effect has not yet been tested in immune-mediated disorders<sup>[19-21]</sup>. The present study indicates that a Shiitake-derived formulation has a beneficial effect in an animal model of TNBS-induced colitis. Administration of this formulation significantly improved the survival rate, and alleviated the macroscopic and microscopic evidence (scores) of colitis. These results were associated with a dramatic reduction in the number of antigen-specific IFN- $\gamma$ -producing colonies. The present study has shown that the liver has a role in mediating systemic induction of tolerance in the setting of our experimental model. This role comprises of sequestering and possibly destroying potentially harmful effector cells, resulting in disease amelioration. The systemic effect of Shiitake in reducing TNBS-induced colitis, was mediated by the trapping of CD8 T cells in the liver. Altered NKT lymphocyte distribution was also associated with the protective effect of Shiitake.

Shiitake and its active component, the polysaccharide lentinan, have previously been shown to be effective against different tumors<sup>[20,21]</sup>. Several studies have shown that these compounds are effective against gastrointestinal tumors, gynecologic tumors, as well as against leukemia and lymphoma<sup>[22,23]</sup>. In most of these studies Shiitake was used to augment the effect of other drugs<sup>[24]</sup>. It has been suggested that mushroom-derived factors do not attack the cancer cells directly, but rather produce their anti-tumor effects by activating different



**Figure 4** Effect of Shiitake administration on the intra-hepatic (open bars) and intrasplenic (black bars) CD4/CD8 ratio.



**Figure 5** The effect of Shiitake administration on the intrasplenic/intrahepatic CD4/CD8 ratio: The peripheral (spleen)/intrahepatic (liver) CD4/CD8 ratio was markedly increased in groups A, C, and D. By contrast, the ratio was markedly reduced in group B.

immune responses in the host<sup>[7,25]</sup>.

Mushroom derivatives can affect different parts of the immune system, including macrophage activation by induction of TNF- $\alpha$ , IL-6, and IL-1, dendritic cell activation, and various effects on T cells<sup>[5]</sup>. *Lentinus edodes* has been described as a T-cell adjuvant, skewing the Th1/Th2 balance towards Th1 through the specific induction of IL-12 from activated macrophages<sup>[26-28]</sup>.

In addition to the protective effects against cancer, Shiitake has also been shown to be hepatoprotective as well as an anti-fibrotic agent. In dimethylnitrosamine-induced hepatitis, Shiitake decreased the serum aminotransferase levels by inhibiting the over-accumulation of collagen fibrils and suppressing the over-expression of genes for smooth muscle  $\alpha$ -actin and heat shock protein-47<sup>[29]</sup>. Shiitake also inhibited, in a dose-dependent manner and without cytotoxicity, the morphologic changes and proliferation of isolated rat hepatic stellate cells (HSCs), which play a central role in liver fibrosis<sup>[29]</sup>. Interestingly, the hepatoprotective effects of Shiitake were also observed after oral administration<sup>[30]</sup>. In another model, the D-galactosamine (GalN)-induced liver injury in mice, oral administration of Shiitake decreased the release of aminotransferases and reduced the degree of histological injury<sup>[30]</sup>.

Inflammatory bowel disease (IBD) is a chronic, relapsing and remitting condition of unknown etiology that exhibits a variety of autoimmune features<sup>[31,32]</sup>. Studies in animal models and in humans implicate an abnormal intestinal epithelial cell barrier function, excessive production of Th1 or Th2 cytokines, and the



unrestrained activation of CD4<sup>+</sup> TCRαβ<sup>+</sup> T cells in the pathogenesis of this disorder<sup>[32]</sup>. Induction of systemic tolerance towards disease-associated antigens has been validated as a method to alter the immune response and alleviate colitis in both animals and humans<sup>[17,18]</sup>.

Rectal administration of TNBS in mice induces chronic intestinal inflammation similar to that seen in Crohn's disease in humans. Stimulated cells in the inflamed mucosa produce increased amounts of IFN-γ, IL-2, and IL-12, along with reduced IL-4 expression<sup>[33]</sup>. Administration of low dose colitis-extracted protein has been shown to inhibit the host colonic inflammatory response and to alleviate colitis in this model<sup>[17]</sup>. Tolerance induction led to an immunological shift from a pro-inflammatory Th1 type response to an anti-inflammatory Th2 type.

NKT regulatory lymphocytes differentiate through thymic and extrathymic pathways<sup>[34]</sup>. These cells are characterized as CD4<sup>+</sup> or CD4<sup>+</sup>CD8<sup>-</sup> and CD16<sup>-</sup>, express αβ TCRint, and share the surface molecules with NK cells, including NK1.1 and CD122<sup>[35]</sup>. NKT cells exist in low numbers in the peripheral blood as well as in most other tissues, but are abundant in the liver<sup>[35]</sup>. The expression of NK1.1-CD1 ligand in the liver is likely responsible for this phenomenon. Upon stimulation, these cells produce significant quantities of IL-4 and IFN-γ, and exhibit enhanced cytolytic activity<sup>[36]</sup>. Our group and others have recently shown that this subset of lymphocytes may play an important role in the induction of peripheral tolerance<sup>[33,37]</sup>. Induction of peripheral tolerance *via* oral administration of an antigen or FK506 treatment, have both been associated with a significant increase in NKT LAL production and cytotoxic activity<sup>[15]</sup>. Relevant to this study, NKT lymphocytes have also been shown to play an active role in the immune modulation of experimental colitis. Adoptive transfer of tolerized NKT cells mediate the transfer of tolerance to recipient mice and prevent the induction of disease<sup>[15]</sup>.

In the present study, the beneficial effect of Shiitake was associated with increased number of liver NKT cells irrespective of the induction of colitis. This change was accompanied by a systemic decrease in NKT cell number in treated animals. To determine whether the intra-hepatic NKT increase was part of a systemic increase in NKT cells or retention of these cells in the liver, the intrahepatic/intrasplenic NKT cell ratio was calculated. While no overall increase in the number of systemic NKT cells was found, this ratio was increased significantly in Shiitake treated mice. Thus, NKT residency in the liver was increased. Taken together, these data suggest that the Shiitake-derived formulation used in the present study has a substantial effect on NKT regulatory lymphocytes. As such, such formulations may carry the potential to alleviate similar NKT-dependent disorders.

In addition to the observed changes in NKT cells, the beneficial effect of Shiitake was associated with increased intrasplenic/intrahepatic ratio of CD4/CD8, suggesting a preferential trapping of CD8 lymphocytes in the liver during systemic tolerance induction. This

effect was similar to that observed in other previously described methods of tolerance induction, where the liver was shown to play an active role<sup>[9,10]</sup>. Similar to its effect on NKT regulatory cell distribution, the effect of Shiitake on intrahepatic lymphocyte sequestration was observed in treated animals whether or not colitis was induced. This finding suggests that Shiitake acts as a genuine immune modulator in both healthy and disease conditions, although a more pronounced effect was noted in disease states.

In summary, the Shiitake-derived formulation had a favorable effect on immune mediated colitis. This effect was associated with alterations in the NKT lymphocyte distribution, and on intra-hepatic CD8 lymphocyte trapping during tolerance induction. Further identification of the specific effects caused by several mushroom derived compounds may facilitate the development of oral administration of these products for the treatment of immune-mediated disorders, including IBD.

## COMMENTS

### Background

Natural killer T (NKT) lymphocytes play a regulatory role in various immune-mediated disorders. To determine the immunomodulatory impact of Shiitake we tested its effect on liver-mediated immune regulation in a model of immune-mediated colitis. Shiitake extract affected liver-mediated immune regulation by altering NKT lymphocyte distribution and increasing intra-hepatic CD8<sup>+</sup> T lymphocyte trapping, thereby leading to alleviation of immune-mediated colitis.

### Research frontiers

The liver is a site for lymphocyte clearance, and plays an important role in determining the CD4<sup>+</sup>/CD8<sup>+</sup> balance during tolerance induction.

### Innovations and breakthroughs

Shiitake extract altered NKT lymphocyte distribution and increased intra-hepatic CD8<sup>+</sup> T lymphocyte trapping.

### Applications

Shiitake extracts can serve as immune modulatory tools in various immune mediated disorders.

### Peer review

The present study looked at the role of mushroom extract on tolerance induction, using an established model of TNBS colitis. It was observed that there was alleviation of microscopic and macroscopic colitis and better survival with Shiitake treatment, accompanied with changes in intra-hepatic NKT, CD4, CD8 populations with reduced IFN secreting colonies in the peripheral blood. The authors concluded that Shiitake modulates the immune response in the liver to a more tolerogenic state, and results in improvement of the colitis.

## REFERENCES

- 1 Yin Y, Fu W, Fu M, He G, Traore L. The immune effects of edible fungus polysaccharides compounds in mice. *Asia Pac J Clin Nutr* 2007; **16** Suppl 1: 258-260
- 2 Lull C, Wichers HJ, Savelkoul HF. Antiinflammatory and immunomodulating properties of fungal metabolites. *Mediators Inflamm* 2005; **2005**: 63-80
- 3 Kodama N, Murata Y, Nanba H. Administration of a polysaccharide from *Grifola frondosa* stimulates immune function of normal mice. *J Med Food* 2004; **7**: 141-145
- 4 Inoue A, Kodama N, Nanba H. Effect of maitake (*Grifola frondosa*) D-fraction on the control of the T lymph node Th-1/Th-2 proportion. *Biol Pharm Bull* 2002; **25**: 536-540
- 5 Moradali MF, Mostafavi H, Ghods S, Hedjaroude GA. Immunomodulating and anticancer agents in the realm of macromycetes fungi (macrofungi). *Int Immunopharmacol*

- 2007; **7**: 701-724
- 6 **Kupfahl C**, Geginat G, Hof H. Lentinan has a stimulatory effect on innate and adaptive immunity against murine *Listeria monocytogenes* infection. *Int Immunopharmacol* 2006; **6**: 686-696
- 7 **Ng ML**, Yap AT. Inhibition of human colon carcinoma development by lentinan from shiitake mushrooms (*Lentinus edodes*). *J Altern Complement Med* 2002; **8**: 581-589
- 8 **Borchers AT**, Stern JS, Hackman RM, Keen CL, Gershwin ME. Mushrooms, tumors, and immunity. *Proc Soc Exp Biol Med* 1999; **221**: 281-293
- 9 **Crispe IN**, Giannandrea M, Klein I, John B, Sampson B, Wuensch S. Cellular and molecular mechanisms of liver tolerance. *Immunol Rev* 2006; **213**: 101-118
- 10 **Shibolet O**, Alper R, Zolotarov L, Trop S, Thalenfeld B, Engelhardt D, Rabbani E, Ilan Y. The role of intrahepatic CD8+ T cell trapping and NK1.1+ cells in liver-mediated immune regulation. *Clin Immunol* 2004; **111**: 82-92
- 11 **Polakos NK**, Klein I, Richter MV, Zaiss DM, Giannandrea M, Crispe IN, Topham DJ. Early intrahepatic accumulation of CD8+ T cells provides a source of effectors for nonhepatic immune responses. *J Immunol* 2007; **179**: 201-210
- 12 **Wuensch SA**, Pierce RH, Crispe IN. Local intrahepatic CD8+ T cell activation by a non-self-antigen results in full functional differentiation. *J Immunol* 2006; **177**: 1689-1697
- 13 **John B**, Crispe IN. Passive and active mechanisms trap activated CD8+ T cells in the liver. *J Immunol* 2004; **172**: 5222-5229
- 14 **Crispe IN**. Hepatic T cells and liver tolerance. *Nat Rev Immunol* 2003; **3**: 51-62
- 15 **Trop S**, Ilan Y. NK 1.1+ T cell: a two-faced lymphocyte in immune modulation of the IL-4/IFN-gamma paradigm. *J Clin Immunol* 2002; **22**: 270-280
- 16 **Shibolet O**, Alper R, Avraham Y, Berry EM, Ilan Y. Immunomodulation of experimental colitis via caloric restriction: role of Nk1.1+ T cells. *Clin Immunol* 2002; **105**: 48-56
- 17 **Ilan Y**, Weksler-Zangen S, Ben-Horin S, Diment J, Sauter B, Rabbani E, Engelhardt D, Chowdhury NR, Chowdhury JR, Goldin E. Treatment of experimental colitis by oral tolerance induction: a central role for suppressor lymphocytes. *Am J Gastroenterol* 2000; **95**: 966-973
- 18 **Margalit M**, Israeli E, Shibolet O, Zigmond E, Klein A, Hemed N, Donegan JJ, Rabbani E, Goldin E, Ilan Y. A double-blind clinical trial for treatment of Crohn's disease by oral administration of Alequel, a mixture of autologous colon-extracted proteins: a patient-tailored approach. *Am J Gastroenterol* 2006; **101**: 561-568
- 19 **Vetvicka V**, Vetvickova J, Frank J, Yvin JC. Enhancing effects of new biological response modifier beta-1,3 glucan sulfate PS3 on immune reactions. *Biomed Pharmacother* 2008; **62**: 283-288
- 20 **Mizuno M**. Anti-tumor polysaccharides from mushrooms during storage. *Biofactors* 2000; **12**: 275-281
- 21 **Kidd PM**. The use of mushroom glucans and proteoglycans in cancer treatment. *Altern Med Rev* 2000; **5**: 4-27
- 22 **Sullivan R**, Smith JE, Rowan NJ. Medicinal mushrooms and cancer therapy: translating a traditional practice into Western medicine. *Perspect Biol Med* 2006; **49**: 159-170
- 23 **Aoyagi K**, Koufuji K, Yano S, Murakami N, Miyagi M, Takeda J, Shirouzu K. Long-term survival after gastric cancer with liver metastasis: a report of two cases. *Kurume Med J* 2001; **48**: 335-338
- 24 **Zhang L**, Li X, Xu X, Zeng F. Correlation between antitumor activity, molecular weight, and conformation of lentinan. *Carbohydr Res* 2005; **340**: 1515-1521
- 25 **Vetvicka V**, Yvin JC. Effects of marine beta-1,3 glucan on immune reactions. *Int Immunopharmacol* 2004; **4**: 721-730
- 26 **Wasser SP**. Medicinal mushrooms as a source of antitumor and immunomodulating polysaccharides. *Appl Microbiol Biotechnol* 2002; **60**: 258-274
- 27 **Murata Y**, Shimamura T, Tagami T, Takatsuki F, Hamuro J. The skewing to Th1 induced by lentinan is directed through the distinctive cytokine production by macrophages with elevated intracellular glutathione content. *Int Immunopharmacol* 2002; **2**: 673-689
- 28 **Ooi VE**, Liu F. Immunomodulation and anti-cancer activity of polysaccharide-protein complexes. *Curr Med Chem* 2000; **7**: 715-729
- 29 **Akamatsu S**, Watanabe A, Tamesada M, Nakamura R, Hayashi S, Kodama D, Kawase M, Yagi K. Hepatoprotective effect of extracts from *Lentinus edodes* mycelia on dimethylnitrosamine-induced liver injury. *Biol Pharm Bull* 2004; **27**: 1957-1960
- 30 **Watanabe A**, Kobayashi M, Hayashi S, Kodama D, Isoda K, Kondoh M, Kawase M, Tamesada M, Yagi K. Protection against D-galactosamine-induced acute liver injury by oral administration of extracts from *Lentinus edodes* mycelia. *Biol Pharm Bull* 2006; **29**: 1651-1654
- 31 **Baumgart DC**, Carding SR. Inflammatory bowel disease: cause and immunobiology. *Lancet* 2007; **369**: 1627-1640
- 32 **Strober W**, Fuss I, Mannon P. The fundamental basis of inflammatory bowel disease. *J Clin Invest* 2007; **117**: 514-521
- 33 **Menachem Y**, Trop S, Kolker O, Shibolet O, Alper R, Nagler A, Ilan Y. Adoptive transfer of NK 1.1+ lymphocytes in immune-mediated colitis: a pro-inflammatory or a tolerizing subgroup of cells? *Microbes Infect* 2005; **7**: 825-835
- 34 **Nowak M**, Stein-Streilein J. Invariant NKT cells and tolerance. *Int Rev Immunol* 2007; **26**: 95-119
- 35 **Bendelac A**, Savage PB, Teyton L. The biology of NKT cells. *Annu Rev Immunol* 2007; **25**: 297-336
- 36 **Godfrey DI**, McConville MJ, Pellicci DG. Chewing the fat on natural killer T cell development. *J Exp Med* 2006; **203**: 2229-2232
- 37 **Yu KO**, Porcelli SA. The diverse functions of CD1d restricted NKT cells and their potential for immunotherapy. *Immunol Lett* 2005; **100**: 42-55

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## Measurement of circulating levels of VEGF-A, -C, and -D and their receptors, VEGFR-1 and -2 in gastric adenocarcinoma

Mansour S Al-Moundhri, A Al-Shukaili, M Al-Nabhani, B Al-Bahrani, IA Burney, A Rizivi, SS Ganguly

Mansour S Al-Moundhri, M Al-Nabhani, IA Burney, A Rizivi, Medical Oncology Unit, Department of Medicine, College of Medicine and Health Sciences, Sultan Qaboos University, Muscat 123, Oman

A Al-Shukaili, Department of Microbiology and Immunology (SQU), Muscat 123, Oman

B Al-Bahrani, National Cancer Institute, Royal Hospital, Muscat 123, Oman

SS Ganguly, Department of Epidemiology and Medical Statistics (SQU), Muscat 123, Oman

**Author contributions:** Al-Moundhri MS designed the research project and wrote the manuscript; Al-Moundhri MS, Al-Bahrani B, Burney IA, Rizivi A provided patient samples; Al-Shukaili A, Al-Nabhani M performed the research assay; Ganguly SS, Al-Moundhri MS performed data analysis and interpretation; Al-Bahrani B, Burney IA reviewed the manuscript.

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Correspondence to: Dr. Mansour S Al-Moundhri, Associate Professor and Consultant Oncologist, Department of Medicine, College of Medicine, Sultan Qaboos University, PO Box 35, Muscat 123, Oman. [mansours@squ.edu.om](mailto:mansours@squ.edu.om)

Telephone: +968-99437301 Fax: +968-24141198

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tumor differentiation and survival. VEGFR-2 levels were associated with poor tumor differentiation. There was no significant prognostic value for any of the VEGF family members or their receptors except for VEGFR-1 where high levels were associated with a poor overall survival.

**CONCLUSION:** Serum VEGF levels vary significantly in the same cohort of patients with variable clinico-pathological features and prognostic values. The simultaneous measurement of VEGF receptors levels in sera may overcome the limitations of a single biomarker assay.

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**Key words:** Gastric cancer; Serum; Vascular endothelial growth factor; Oman

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### Abstract

**AIM:** To analyze the serum levels and prognostic significance of vascular endothelial growth factor (VEGF) -A, -C, and -D, and their receptors, VEGFR-1 and -2 in gastric adenocarcinomas.

**METHODS:** The serum levels of VEGF family members were measured in 76 control subjects and 76 patients with gastric adenocarcinoma using an enzyme-linked immunosorbent assay (ELISA). These measurements were correlated with clinico-pathological features and survival rates.

**RESULTS:** The serum levels of VEGF-A and its receptor, VEGFR-1, were significantly higher in patients with gastric cancer than in healthy donors ( $t = 2.3$ ,  $P = 0.02$  and  $t = 4.2$ ,  $P < 0.0001$ , respectively). In contrast, the serum levels of VEGF-D were significantly higher in control subjects than in patients ( $t = 2.9$ ,  $P = 0.004$ ). There was no significant difference in serum levels of VEGF-C and VEGFR-2 between patients and controls. VEGF-C was associated with advanced tumor stage and presence of metastasis. VEGFR-1 was associated with metastasis, advanced overall stage,

### INTRODUCTION

Gastric cancer is the second most common cancer worldwide and remains a global health burden<sup>[1]</sup>. The prognosis of patients with gastric cancer has been shown to be influenced by established surgical-pathological features, such as pathological stage, location of the tumor, and histological type and grade of the tumor<sup>[2-4]</sup>. In comparison, the intense search for predictive molecular biomarkers has not yet translated into clinical use<sup>[5-7]</sup>.

There is growing recognition of the central role that the vascular endothelial growth factor (VEGF) family plays in angiogenesis in which the formation of new blood vessels is necessary for the growth and spread of tumors<sup>[8]</sup>. The VEGF family consists of seven members- VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E,

VEGF-F, and placental growth factor (PlGF)-which share eight cysteine residues in a VEGF homology domain<sup>[9,10]</sup>. The members act through specific tyrosine kinase receptors, VEGFR-1, -2 and -3. VEGF-A acts through VEGFR-1 and -2 receptors, VEGF-C and -D act through VEGFR-2 and -3<sup>[9,11]</sup>.

Studies have shown an association between intratumoral microvessel density and tumor aggressiveness in gastric cancer<sup>[12,13]</sup>. Among angiogenic stimulators, VEGF-A plays an essential role in both vasculogenesis and angiogenesis<sup>[14]</sup>. Serum concentrations of VEGF-A have been examined in patients with gastric cancer and a relationship between the serum concentration of VEGF and metastasis and/or poor outcome has been demonstrated<sup>[15,16]</sup>. However, the significance of other VEGF family members (VEGF-B, -C, and -D) in tumor angiogenesis and metastasis are not fully demonstrated<sup>[17]</sup>. Recently, the prognostic importance of serum concentrations of other VEGF family members (VEGF-C and -D and their receptors) in gastric cancer has been reported<sup>[18,19]</sup>. Overall, these studies were limited to one or two VEGF family members and their receptors and did not examine the full profile of expression of these proteins in the same patient<sup>[14,15,18]</sup>.

In the current study, we evaluated the serum levels of VEGF-A, -C, and -D, and their receptors, VEGFR-1 and -2 in both healthy controls and gastric cancer patients. We then correlated these serum levels with clinico-pathological features and patient survival rates.

## MATERIALS AND METHODS

### Study subjects

The study population consisted of a series of unrelated patients diagnosed with gastric cancer in three main hospitals in the Sultanate of Oman (Sultan Qaboos University Hospital, Royal Hospital, and Sohar Hospital). The control group was composed of subjects of the same ethnic and geographical origin as the patients. The Medical Research and Ethics Committee of the College of Medicine of Sultan Qaboos University approved the study design. The study subjects gave an informed consent prior to participation in the study.

### Serum VEGF family level assay

Peripheral venous blood samples were obtained prior to any treatment and allowed to clot at room temperature and centrifuged at 2000 g for 10 min. Sera were separated, aliquoted, and stored at -70°C until assay. Serum VEGF concentrations were determined using a commercially available enzyme-linked immunoassay (ELISA) designed to measure VEGF-A (VEGF 165), VEGF-C, VEGF-D, VEGFR-1, and VEGFR-2 levels (Quantikine, R&D Systems Europe, Abingdon, UK). Assays employed the quantitative sandwich enzyme immunoassay and were performed according to the manufacturer's procedure.

### Statistical analysis

The significance of differences in the levels of various

**Table 1** Serum concentrations (pg/mL) of VEGF-A, -C, and -D, and VEGFR-1 and -2 in patients with gastric adenocarcinoma and control subjects (*n* = 76)

	Patients	Control	<i>P</i>
VEGF-A	585.7 ± 408.4; 469.3 <sup>1</sup> (44.1-1927.7)	444.9 ± 328.9; 346.9 (31.1-630.6)	0.02
VEGF-C	6141.0 ± 2456.1; 5858.4 <sup>1</sup> (970.4-12158.9)	6067.8 ± 2219.3; 5872.3 (711.7-12158.9)	0.88
VEGF-D	483.0 ± 259.3; 428.9 <sup>1</sup> (205.7-1355.2)	671.1 ± 501.8; 605.9 (205.7-3859.7)	0.004
VEGFR-1	873.7 ± 360.8; 1026.4 <sup>1</sup> (279.4-1767.6)	645.3 ± 297.9; 475.4 (224.1-1333.6)	0.0001
VEGFR-2	9266.5 ± 2111.1; 9382.3 <sup>1</sup> (4726.1-15427.3)	9420.3 ± 1840.7; 9386.2 (5727.9-15433.3)	0.45

<sup>1</sup>Refers to mean ± SD; median (range).

VEGF molecules between patients and controls was studied using the unpaired *t* test and further confirmed with Mann-Whitney *U* test. The significance of the correlations between the expression of various VEGF proteins and clinico-pathological features was evaluated by the Spearman's correlation test.

Overall survival rates were determined from the time of biopsy-proven diagnosis until either the time of death or last known follow-up examination. The dates of death were obtained from either medical records or phone contact. The Kaplan-Meier method was used to estimate overall survival time, and the statistical significance was determined by log-rank test. Backward conditional Cox proportional hazards regression model was utilized for multivariate analyses where age ( $\leq 40$  years,  $> 40$  years), gender, proximal or distal location, histological classification (intestinal *vs* non-intestinal), T stage (1 + 2 *vs* 3 + 4), presence or absence of lymph node metastasis, overall all stage (I + II *vs* III + IV), tumor differentiation (well *vs* moderate + poor), all the VEGF factors were included. *P* values less than 0.05 were considered statistically significant. Analysis of data was performed using SPSS 10.0 software.

## RESULTS

A total of 76 gastric cancer patients and 76 unrelated controls were included. The age range for the participants included in the study was 21-82 years; the mean and standard deviation of ages for the patients and controls were 55.7 ± 11.3 and 38.5 ± 10.2 years, respectively. The proportion of males (*n* = 41) and females (*n* = 35) were equal in both groups. Fifty four patients received either chemotherapy alone or chemoradiotherapy.

### The serum values of VEGF-A, -C, -D, and VEGFR-1 and -2 in patients and controls

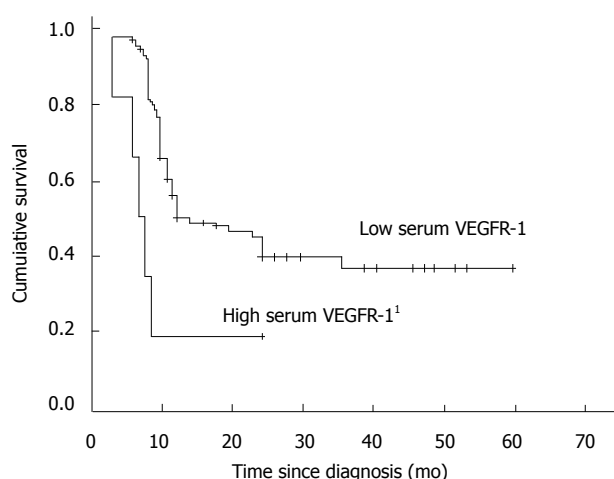
Table 1 shows the mean, standard deviation, median, and ranges of serum levels of VEGF-A, -C, and -D, and VEGFR-1 and -2 in patients with gastric cancer and controls. The serum VEGF-A and VEGFR-1 levels were significantly higher in patients with gastric cancer than



**Table 2** Associations between serum concentrations (pg/mL) of VEGF-A, -C, and -D, and VEGFR-1 and -2 and clinico-pathological features in 76 patients of gastric adenocarcinoma and 76 control subjects (mean  $\pm$  SD )

Pathological	n	VEGF-A	VEGF-C	VEGF-D	VEGFR-1	VEGFR-2
Site						
Distal	43	522.9 $\pm$ 371.6	6035.5 $\pm$ 2544.3	474.1 $\pm$ 276.5	863.0 $\pm$ 380.3	9058.9 $\pm$ 2140.2
Non-distal	33	667.5 $\pm$ 444.4	6278.5 $\pm$ 2368.0	494.6 $\pm$ 238.6	887.7 $\pm$ 341.7	9537.0 $\pm$ 2073.7
Classification						
Intestinal	40	586.0 $\pm$ 381.1	5895.3 $\pm$ 2315.7	472.3 $\pm$ 216.9	872.8 $\pm$ 361.7	8982.6 $\pm$ 2408.5
Diffuse or mixed	36	585.5 $\pm$ 436.4	6362.2 $\pm$ 2585.0	492.6 $\pm$ 367.2	874.6 $\pm$ 366.7	9522.0 $\pm$ 1795.4
T stage+						
T1 + T2	12	635.6 $\pm$ 291.3	4843.4 $\pm$ 1682.9	465.3 $\pm$ 248.0	661.4 $\pm$ 383.3	8144.9 $\pm$ 1804.8
T3 + T4	46	484.1 $\pm$ 377.1	6378.0 $\pm$ 2345.8 <sup>a</sup>	501.9 $\pm$ 286.4	872.3 $\pm$ 364.6	9344.9 $\pm$ 2254.5
Lymph node+						
Negative	8	597.4 $\pm$ 476.5	5450.7 $\pm$ 2381.5	397.3 $\pm$ 116.9	717.5 $\pm$ 364.9	8843.8 $\pm$ 2419.8
Positive	50	503.2 $\pm$ 346.2	6065.6 $\pm$ 2367.4	514.1 $\pm$ 291.8	828.8 $\pm$ 377.8	9065.2 $\pm$ 2076.8
Metastasis						
Absent	42	517.0 $\pm$ 331.5	5918.8 $\pm$ 2456.4	493.4 $\pm$ 283.9	779.1 $\pm$ 396.5	9007.8 $\pm$ 2297.8
Present	34	670.5 $\pm$ 478.8	6415.5 $\pm$ 2464.3	470.1 $\pm$ 228.8	990.7 $\pm$ 277.1 <sup>a</sup>	9586.1 $\pm$ 1838.1
Stage						
1 + 2	13	586.5 $\pm$ 400.1	4848.8 $\pm$ 1948.7	458.6 $\pm$ 225.1	633.1 $\pm$ 365.1	8785.6 $\pm$ 2082.9
3 + 4	63	585.5 $\pm$ 413.3	6407.7 $\pm$ 2477.9 <sup>a</sup>	488.1 $\pm$ 267.1	923.4 $\pm$ 343.4 <sup>b</sup>	9365.8 $\pm$ 2119.7
Differentiation						
Well	6	335.9 $\pm$ 310.5	4386.9 $\pm$ 2653.1	363.3 $\pm$ 102.0	595.5 $\pm$ 341.1	7265.1 $\pm$ 2188.0
Moderate + poor	70	607.1 $\pm$ 410.4	6291.4 $\pm$ 2399.2	493.3 $\pm$ 266.4	897.6 $\pm$ 355.8 <sup>a</sup>	9438.1 $\pm$ 2029.6 <sup>a</sup>

<sup>a</sup> $P < 0.05$  and <sup>b</sup> $P < 0.01$  vs determined in patients who had surgical resections ( $n = 58$ ).



**Figure 1** Kaplan-Meier survival curves in relation to serum VEGFR-1 levels in 76 patients with gastric adenocarcinoma. <sup>1</sup>Elevated serum VEGFR-1 levels were defined as greater than the 95th percentile in the healthy control group.

in healthy donors ( $P = 0.02$  and  $0.0001$ , respectively). Interestingly, the serum levels of VEGF-D were higher in controls than in patients with gastric cancer. There was no significant difference in serum levels of VEGF-C and VEGFR-2 between patients and controls.

#### **The association between serum values of VEGF-A, -C, -D, and VEGFR-1 and -2 and clinico-pathological features**

Table 2 shows the association between serum values of VEGF-A, -C, -D and VEGFR-1 and -2 and clinico-pathological features. VEGF-C was associated with advanced tumor stage and presence of metastasis. VEGFR-1 was associated with metastasis, advanced overall tumor stage, and tumor differentiation. VEGFR-2 levels were associated with poor tumor differentiation.

#### **Correlation between circulating VEGF levels and patient survival**

Elevated serum VEGF levels were defined as greater than the 95th percentile in the healthy control group as previously described<sup>[15]</sup>. There was no significant predictive value for any of the VEGFs or their receptors (data not shown) except for VEGFR-1 as shown in Figure 1. The median survival time for patients with high and low levels of VEGFR-1 was 5 mo (95% CI 2.6-7.4) and 13 mo (95% CI 1.4-24.6), respectively. The 5-year survival rates were 20% and 36%, respectively ( $P = 0.02$ ). Multivariate regression analysis showed that only advanced stage III and IV (Hazard ratio 6.7; 95% CI, 2.2-20.0;  $P = 0.001$ ) to be a significant independent factor.

## **DISCUSSION**

Studies on the role of the VEGF family in gastric cancer have focused on individual VEGF members and their respective receptors<sup>[14,15,18]</sup>. The evaluation of the simultaneous expression of VEGF family members and their receptors may provide more accurate prognostic information<sup>[17]</sup>. Therefore, we have investigated the serum levels of VEGF-A, -C, -D, and VEGFR-1 and -2 and studied their predictive and prognostic significance in the same group of patients.

In the present study, the VEGF-A and VEGFR-1 levels are higher in patients with gastric cancer than in healthy controls, which is consistent with results from other studies<sup>[15,20,21]</sup>. However, we found that serum VEGF-D levels were higher in the control group than in patients with gastric cancer. George *et al*<sup>[12]</sup> studied VEGF-A, -C, and -D mRNA tissue expression in colorectal cancer progression, demonstrating that VEGF-D mRNA remained at persistently low levels in carcinomas compared with adenomas, and that

both VEGFR-2 and VEGFR-3 mRNA expression levels remained constant. This result suggested that VEGF-D acts as a competitive agonist with VEGF-A and VEGF-C; a decrease in VEGF-D levels may allow increased access of VEGF-A and VEGF-C, which are more potent angiogenic cytokines than VEGF-D, to the two VEGF receptors<sup>[22]</sup>. It has been previously shown that tissue expression of certain VEGF family members correlates with their serum levels<sup>[18]</sup>. Therefore, the advanced tumor stage presentation that predominated in the current cohort of patients may have led to the differential expression of the VEGF family members, further emphasizing the importance of the simultaneous measurement of these factors.

The correlation of markers of angiogenesis and clinico-pathological features further highlights the complex interaction between various factors and their respective receptors<sup>[16,23]</sup>. Whereas VEGF-A and -D did not correlate with any of the clinico-pathological features studied, it is interesting to note the strong association between VEGFR-1 and advanced stage cancer, the presence of metastasis, and poor tumor differentiation. VEGF-C was a serum biomarker for advanced tumor stage and poor differentiation, whereas VEGFR-2 correlated with poor tumor differentiation. It is interesting that serum VEGF-C and -D levels did not correlate with lymph node metastasis, a result that has been shown previously<sup>[18]</sup>. This finding is most likely due to the fact that most patients included had lymph node metastasis and, therefore, such differences could not be found (Table 2).

The available data on the prognostic significance of serum VEGF levels in malignancies is controversial<sup>[24-26]</sup>. The prognostic significance of VEGF (in particular, of VEGF-A), as determined by ELISA, has been commonly studied in various tumors<sup>[24-27]</sup>. It has been shown that high serum VEGF levels correlate with poor prognosis in certain hematological malignancies<sup>[28,29]</sup>. In gastric cancer, it has been shown that serum VEGF-A levels correlated with local tumor extent, disease stage, and the presence of distant metastases, and is an independent prognostic factor for patient survival<sup>[14,15,20]</sup>. Recently, Wang *et al*<sup>[9]</sup> reported that serum VEGF-C levels might be a useful biomarker to determine the presence of lymph node metastasis in patients with gastric cancer as well as correlate with VEGF-C tissue expression<sup>[18]</sup>. Juttner *et al*<sup>[8]</sup> showed that both VEGF-D and VEGFR-3 are independent prognostic biomarkers to identify patients with poor prognosis after curative resection of gastric adenocarcinomas; when combined with an analysis of VEGF-C, patients with shorter survival times could be predicted<sup>[17]</sup>. In the current study, a significant correlation between VEGFR-1 and advanced tumor stage, presence of metastasis, and poor tumor differentiation in turn reflects poor survival outcome as shown in Figure 1. To our knowledge, this is the first study to report on the prognostic significance of serum VEGFR-1 levels in patients with gastric cancer. It has been suggested that the VEGFR-1/VEGF ratio in sera may be of greater prognostic value than VEGF

levels alone<sup>[28]</sup>, hence, overcoming the limitations of a single biomarker assay<sup>[27,30]</sup>.

In conclusion, we have demonstrated that there are significant variations in VEGF serum concentrations when measured simultaneously that correlate with important variations in clinico-pathological features and prognostic significance. Future work will address the application of this concept on a larger cohort of patients to generate a predictive profile that examines the simultaneous expression of these proteins at the tissue level.

## COMMENTS

### Background

The prognostic importance of serum concentrations of vascular endothelial growth factor (VEGF) family members in gastric cancer has been reported. Overall, these studies were limited to one or two VEGF family members and their receptors and did not examine the full profile of expression of these proteins in the same patient. In the current study, authors evaluated the serum levels of VEGF-A, -C, and -D, and their receptors, VEGFR-1 and -2 in both healthy controls and gastric cancer patients. They then correlated these serum levels with clinico-pathological features and patient survival rates.

### Research frontiers

To date, the prognostic value of simultaneous measurement of serum concentration several VEGF family proteins in gastric cancer remains unclear. The variation of serum concentration of various VEGF family members in the same patient may provide useful predictive and prognostic tool.

### Innovations and breakthroughs

In the present study, the authors measured the serum levels of VEGF-A, -C, and -D, and their receptors, VEGFR-1 and -2. This is the first study that measures serum level of VEGF family members simultaneously. The serum levels of VEGF-A and its receptor, VEGFR-1, were significantly higher in patients with gastric cancer than in healthy donors. In contrast, the serum levels of VEGF-D were significantly higher in control subjects than in patients. VEGFR-1 was associated with metastasis, advanced overall stage, tumor differentiation and survival. The result suggests that levels of VEGF family members may vary significantly and that simultaneous pre-operative measurement may provide a more accurate prognostic tool.

### Applications

The results of the present study demonstrate the variation in VEGF family members when measured simultaneously. The measurement of all these proteins may provide more accurate predictive and prognostic information.

### Terminology

Angiogenesis is a physiological process in the body that involves the formation of new blood vessels. VEGF family plays an important role in angiogenesis. The VEGF family consists of seven members-VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F, and placental growth factor. The members act through specific tyrosine kinase receptors-VEGFR-1, -2 and -3. VEGF-A acts through VEGFR-1 and -2 receptors; VEGF-C and -D act through VEGFR-2 and -3.

### Peer review

This is an interesting paper. Authors analyzed the serum levels and prognostic significance of VEGF-A, -C, and -D, and their receptors, VEGFR-1 and -2 in gastric adenocarcinomas.

## REFERENCES

- 1 Anderson C, Nijagal A, Kim J. Molecular markers for gastric adenocarcinoma: an update. *Mol Diagn Ther* 2006; **10**: 345-352
- 2 Al-Moundhri MS, Al-Bahrani B, Burney IA, Nirmala V, Al-Madhani A, Al-Mawaly K, Al-Nabhani M, Thomas V, Ganguly SS, Grant CS. The prognostic determinants of gastric cancer treatment outcome in Omani Arab patients. *Oncology* 2006; **70**: 90-96
- 3 Miyahara R, Niwa Y, Matsuura T, Maeda O, Ando T, Ohmiya N, Itoh A, Hirooka Y, Goto H. Prevalence and

- prognosis of gastric cancer detected by screening in a large Japanese population: data from a single institute over 30 years. *J Gastroenterol Hepatol* 2007; **22**: 1435-1442
- 4 **Barchielli A**, Amorosi A, Balzi D, Crocetti E, Nesi G. Long-term prognosis of gastric cancer in a European country: a population-based study in Florence (Italy). 10-year survival of cases diagnosed in 1985-1987. *Eur J Cancer* 2001; **37**: 1674-1680
  - 5 **Al-Moundhri MS**, Nirmala V, Al-Hadabi I, Al-Mawaly K, Burney I, Al-Nabhani M, Thomas V, Ganguly SS, Grant C. The prognostic significance of p53, p27 kip1, p21 waf1, HER-2/neu, and Ki67 proteins expression in gastric cancer: a clinicopathological and immunohistochemical study of 121 Arab patients. *J Surg Oncol* 2005; **91**: 243-252
  - 6 **Mihmanli M**, Dilege E, Demir U, Coskun H, Eroglu T, Uysalol MD. The use of tumor markers as predictors of prognosis in gastric cancer. *Hepatogastroenterology* 2004; **51**: 1544-1547
  - 7 **Boku N**, Ohtsu A, Yoshida S, Shirao K, Shimada Y, Hyodo I, Saito H, Miyata Y. Significance of biological markers for predicting prognosis and selecting chemotherapy regimens of advanced gastric cancer patients between continuous infusion of 5-FU and a combination of 5-FU and cisplatin. *Jpn J Clin Oncol* 2007; **37**: 275-281
  - 8 **Hormbrey E**, Gillespie P, Turner K, Han C, Roberts A, McGrouther D, Harris AL. A critical review of vascular endothelial growth factor (VEGF) analysis in peripheral blood: is the current literature meaningful? *Clin Exp Metastasis* 2002; **19**: 651-663
  - 9 **Otrock ZK**, Makarem JA, Shamseddine AI. Vascular endothelial growth factor family of ligands and receptors: review. *Blood Cells Mol Dis* 2007; **38**: 258-268
  - 10 **Moreira IS**, Fernandes PA, Ramos MJ. Vascular endothelial growth factor (VEGF) inhibition--a critical review. *Anticancer Agents Med Chem* 2007; **7**: 223-245
  - 11 **Simiantonaki N**, Taxeidis M, Jayasinghe C, Kirkpatrick CJ. Epithelial expression of VEGF receptors in colorectal carcinomas and their relationship to metastatic status. *Anticancer Res* 2007; **27**: 3245-3250
  - 12 **Zhao HC**, Qin R, Chen XX, Sheng X, Wu JF, Wang DB, Chen GH. Microvessel density is a prognostic marker of human gastric cancer. *World J Gastroenterol* 2006; **12**: 7598-7603
  - 13 **Zhou Y**, Ran J, Tang C, Wu J, Honghua L, Xingwen L, Ning C, Qiao L. Effect of celecoxib on E-cadherin, VEGF, Microvessel density and apoptosis in gastric cancer. *Cancer Biol Ther* 2007; **6**: 269-275
  - 14 **Aoyagi K**, Kouhiji K, Yano S, Miyagi M, Imaizumi T, Takeda J, Shirouzu K. VEGF significance in peritoneal recurrence from gastric cancer. *Gastric Cancer* 2005; **8**: 155-163
  - 15 **Karayiannakis AJ**, Syrigos KN, Polychronidis A, Zbar A, Kouraklis G, Simopoulos C, Karatzas G. Circulating VEGF levels in the serum of gastric cancer patients: correlation with pathological variables, patient survival, and tumor surgery. *Ann Surg* 2002; **236**: 37-42
  - 16 **Oh SY**, Kwon HC, Kim SH, Jang JS, Kim MC, Kim KH, Han JY, Kim CO, Kim SJ, Jeong JS, Kim HJ. Clinicopathologic significance of HIF-1 $\alpha$ , p53, and VEGF expression and preoperative serum VEGF level in gastric cancer. *BMC Cancer* 2008; **8**: 123
  - 17 **Juttner S**, Wissmann C, Jons T, Vieth M, Hertel J, Gretscher S, Schlag PM, Kemmner W, Hocker M. Vascular endothelial growth factor-D and its receptor VEGFR-3: two novel independent prognostic markers in gastric adenocarcinoma. *J Clin Oncol* 2006; **24**: 228-240
  - 18 **Wang TB**, Deng MH, Qiu WS, Dong WG. Association of serum vascular endothelial growth factor-C and lymphatic vessel density with lymph node metastasis and prognosis of patients with gastric cancer. *World J Gastroenterol* 2007; **13**: 1794-1797; discussion 1797-1798
  - 19 **Tas F**, Duranyildiz D, Oguz H, Camlica H, Yasasever V, Topuz E. Circulating serum levels of angiogenic factors and vascular endothelial growth factor receptors 1 and 2 in melanoma patients. *Melanoma Res* 2006; **16**: 405-411
  - 20 **Ding S**, Lin S, Dong X, Yang X, Qu H, Huang S, Liu W, Zhou L, Liu D. Potential prognostic value of circulating levels of vascular endothelial growth factor-A in patients with gastric cancer. *In Vivo* 2005; **19**: 793-795
  - 21 **Ohta M**, Konno H, Tanaka T, Baba M, Kamiya K, Syouji T, Kondoh K, Watanabe M, Terada H, Nakamura S. The significance of circulating vascular endothelial growth factor (VEGF) protein in gastric cancer. *Cancer Lett* 2003; **192**: 215-225
  - 22 **George ML**, Tutton MG, Janssen F, Arnaout A, Abulafi AM, Eccles SA, Swift RI. VEGF-A, VEGF-C, and VEGF-D in colorectal cancer progression. *Neoplasia* 2001; **3**: 420-427
  - 23 **Kondo K**, Kaneko T, Baba M, Konno H. VEGF-C and VEGF-A synergistically enhance lymph node metastasis of gastric cancer. *Biol Pharm Bull* 2007; **30**: 633-637
  - 24 **Tas F**, Duranyildiz D, Oguz H, Camlica H, Yasasever V, Topuz E. Serum vascular endothelial growth factor (VEGF) and bcl-2 levels in advanced stage non-small cell lung cancer. *Cancer Invest* 2006; **24**: 576-580
  - 25 **Byrne GJ**, McDowell G, Agarawal R, Sinha G, Kumar S, Bundred NJ. Serum vascular endothelial growth factor in breast cancer. *Anticancer Res* 2007; **27**: 3481-3487
  - 26 **Vincenzi B**, Santini D, Russo A, Gavasci M, Battistoni F, Dicuonzo G, Rocci L, Rosaria VM, Gebbia N, Tonini G. Circulating VEGF reduction, response and outcome in advanced colorectal cancer patients treated with cetuximab plus irinotecan. *Pharmacogenomics* 2007; **8**: 319-327
  - 27 **Poon RT**, Lau CP, Cheung ST, Yu WC, Fan ST. Quantitative correlation of serum levels and tumor expression of vascular endothelial growth factor in patients with hepatocellular carcinoma. *Cancer Res* 2003; **63**: 3121-3126
  - 28 **Wierzbowska A**, Robak T, Wrzesien-Kus A, Krawczynska A, Lech-Maranda E, Urbanska-Rys H. Circulating VEGF and its soluble receptors sVEGFR-1 and sVEGFR-2 in patients with acute leukemia. *Eur Cytokine Netw* 2003; **14**: 149-153
  - 29 **Poreba M**, Jazwiec B, Kulickowski K, Poreba R. [Circulating endothelial cells, endothelial precursors, VEGF and bFGF concentrations in patients with acute leukemias, lymphomas and myelomas] *Pol Arch Med Wewn* 2005; **113**: 27-34
  - 30 **Jacobsen J**, Grankvist K, Rasmuson T, Ljungberg B. Prognostic importance of serum vascular endothelial growth factor in relation to platelet and leukocyte counts in human renal cell carcinoma. *Eur J Cancer Prev* 2002; **11**: 245-252

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RAPID COMMUNICATION

## Increased intestinal macromolecular permeability and urine nitrite excretion associated with liver cirrhosis with ascites

Soong Lee, Seung-Cheol Son, Moon-Jong Han, Woo-Jin Kim, Soo-Hyun Kim, Hye-Ran Kim, Woo-Kyu Jeon, Ki-Hong Park, Myung-Geun Shin

Myung-Geun Shin, Soo-Hyun Kim, Department of Laboratory Medicine, Chonnam National University Medical School and Chonnam National University Hwasun Hospital, Hwasun 519-809, Korea

Soong Lee, Seung-Cheol Son, Moon-Jong Han, Woo-Jin Kim, Department of Internal Medicine, College of Medicine, Seonam University and Seonam University Hospital, Gwangju 502-157, Korea

Hye-Ran Kim, Brain Korea 21 Project, Center for Biomedical Human Resources at Chonnam National University Medical School, Gwangju 501-757, Korea

Woo-Kyu Jeon, Department of Internal Medicine, Kangbuk Samsung Hospital, Sungkyunkwan University School of Medicine, Seoul 110-746, Korea

Ki-Hong Park, Korea Polymer Testing & Research Institute Ltd., Seoul 136-120, Korea

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Correspondence to: Myung-Geun Shin, MD, PhD, Department of Laboratory Medicine, Chonnam National University Medical School and Chonnam National University Hwasun Hospital, Hwasun 519-809, Korea. [mgshin@chonnam.ac.kr](mailto:mgshin@chonnam.ac.kr)

Telephone: +82-61-3797950 Fax: +82-61-3797984

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significantly higher in patients with LC with ascites than in healthy control subjects or patients with LC without ascites ( $0.88 \pm 0.12$  vs  $0.52 \pm 0.05$  or  $0.53 \pm 0.03$ ,  $P < 0.05$ ) and correlated with urine nitrite excretion ( $r = 0.98$ ). Interestingly, the serum TNF- $\alpha$  concentration was significantly higher in LC without ascites than in control subjects or in LC with ascites ( $198.9 \pm 55.8$  pg/mL vs  $40.9 \pm 12.3$  pg/mL or  $32.1 \pm 13.3$  pg/mL,  $P < 0.05$ ). Urine nitrite excretion was significantly higher in LC with ascites than in the control subjects or in LC without ascites ( $1170.9 \pm 28.7$   $\mu$ mol/L vs  $903.1 \pm 55.1$   $\mu$ mol/L or  $956.7 \pm 47.7$   $\mu$ mol/L,  $P < 0.05$ ).

**CONCLUSION:** Increased intestinal macromolecular permeability and NO is probably of importance in the pathophysiology and progression of LC with ascites, but the serum TNF- $\alpha$  concentration was not related to LC with ascites.

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**Key words:** Intestinal permeability; Tumor necrosis factor- $\alpha$ ; Nitric oxide; Liver cirrhosis; Ascites

**Peer reviewer:** Dr. Soren Moller, Department of Clinical Physiology 239, Hvidovre Hospital, Kettegaard alle 30, DK-2650 Hvidovre DK-2650, Denmark

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### Abstract

**AIM:** To determine intestinal permeability, the serum tumor necrosis factor (TNF)- $\alpha$  level and urine nitric oxide (NO) metabolites are altered in liver cirrhosis (LC) with or without ascites.

**METHODS:** Fifty-three patients with LC and 26 healthy control subjects were enrolled in the study. The intestinal permeability value is expressed as the percentage of polyethylene glycol (PEG) 400 and 3350 retrieval in 8-h urine samples as determined by high performance liquid chromatography. Serum TNF- $\alpha$  concentrations and urine NO metabolites were determined using an enzyme-linked immunosorbent assay (ELISA) and Greiss reaction method, respectively.

**RESULTS:** The intestinal permeability index was

### INTRODUCTION

It has been shown that the gut, as a reservoir of enteric bacteria in the body, plays a protective role as mucosal barrier function, immunoglobulin secretion, and local and systemic macrophage system, but under liver cirrhosis (LC) with portal hypertension a correlative connection between liver damage and the functional activity of the intestine with mucosal abnormalities exist<sup>[1-3]</sup>. Increased intestinal permeability (IPI) with bacterial translocation and endotoxemia have been implicated



in the pathogenesis of chronic liver injury and as contributory factors in the development of dangerous complications, such as encephalopathy and bacterial infections in LC<sup>[4]</sup>. However, other investigators have suggested that intestinal permeability is probably of limited importance in the pathophysiology of bacterial infections in patients with LC<sup>[5]</sup>. Intestinal permeability in LC has been reported as being increased or normal<sup>[6-10]</sup>. The development of systemic endotoxemia may in turn act through the release of cytokines, to further increase intestinal permeability, impair host immunity and promote bacterial translocation from the gut, thus resulting in a vicious circle<sup>[11,12]</sup>. It has been proposed that some of these cytokines play a role in several known cirrhosis-related complications, such as hyperdynamic circulation, susceptibility to infection, and hepatic encephalopathy<sup>[13]</sup>. Tumor necrosis factor (TNF)- $\alpha$  is a 17 kDa cytotoxic protein produced by mononuclear cells on activation by bacterial endotoxin and tissue injury<sup>[13]</sup>. However, the TNF- $\alpha$  level in LC has been reported with controversial findings, as it may or may not correlate with an advanced stage of disease and a worse outcome<sup>[14-18]</sup>.

Nitric oxide (NO) has a role in cirrhosis. Endotoxemia, possibly from gut-derived bacterial translocation, causes induction of NO synthase leading to increase vascular NO production, which is the primary stimulus for the development of vasodilatation in cirrhosis and its accompanying clinical manifestations<sup>[19]</sup>. While NO is an unstable molecule, one means of investigating NO formation is to measure nitrite (NO<sub>2</sub>), which is one of two primary stable non-volatile breakdown products of NO. A dose dependent increase in nitrite has been demonstrated to occur when macrophages are activated with lipopolysaccharide (LPS) both *in vitro* and *in vivo*<sup>[20]</sup>. Therefore, we speculated that endotoxin mediated increases in the NO metabolite nitrite in urine are related to the magnitude of intestinal macromolecular permeability and hence to LC related complications.

Limited data exists on the state of intestinal macromolecular permeability using polyethylene glycol (PEG) (400 and 3350) in cirrhotic patients with or without ascites. To clarify the role of intestinal macromolecular permeability, the serum TNF- $\alpha$  level and nitrite level in urine to the development of LC with ascites, we investigated whether intestinal macromolecular permeability is altered in patients LC with or without ascites, and its relationship with the serum TNF- $\alpha$  level and NO metabolite level in urine.

## MATERIALS AND METHODS

### *Patients and healthy control subjects*

Participating patients and healthy control subjects were comprised of 26 patients with LC with ascites, 27 patients with LC without ascites and 26 age and sex-matched healthy individuals with a normal medical history, physical examination and blood chemistry.

Subjects with known infection, gastrointestinal or renal disease or diabetes mellitus were excluded from the study. Also excluded were patients that received substances known to affect intestinal permeability test results such as lactulose, non-steroidal anti-inflammatory drugs, or alcohol, in the previous 2 wk. The Institutional Review Board of the Seonam University Health Sciences Center (Namwon, Korea) approved the study. All subjects in this study gave informed consent. The diagnosis of LC was based on the typical findings of hepatic cirrhotic appearance, splenomegaly, esophageal varices, and ascites by ultrasonography and an upper gastrointestinal endoscopy, and laboratory results (prolonged prothrombin time, hypoalbuminemia with or without elevated liver enzymes). The severity of liver disease was determined according to Child-Pugh criteria.

### *Measurement of intestinal macromolecular permeability*

Urine samples used in this study were collected during 8 h from subjects that fasted overnight (last meal before 8 PM the day before). Subjects ingested a 100 mL test solution containing 1 g of PEG 400 and 10 g of PEG 3350 in water. Each subject ingested the PEG solution 1 h before a breakfast meal. Urine samples were been kept frozen (-20°C) until processing for analysis. In this study, we attempted to detect PEG 400 as a low molecular weight (MW) marker and 3350 as a higher MW marker simultaneously in urine samples by high performance liquid chromatography (HPLC) using evaporative light scattering detection. About 2 mL of urine was filtered through a 0.45  $\mu$ m syringe filter (Nylon membrane) and stored at 4°C until analysis. All of the 1 mL-vial urine samples for analysis were directly placed into a Waters 717+ autosampler with refrigerator (10°C). The HPLC column was a 5  $\mu$ m PLRP-S 100 A column (150 mm  $\times$  4.6 mm, Polymer Laboratories, Amherst, MA USA) packed with PS/DVB polymeric beads. To remove particles in the urine sample, a disposable Security Guard kit (Phenomenex, Torrance, CA USA) was used with the HPLC column. A gradient mobile phase (acetonitrile/H<sub>2</sub>O) for an elution of 40-60 min was used to separate efficiently all hydrophilic and hydrophobic compounds. As the HPLC eluents were controlled by a gradient controller program, we tried to set the program to allow the impurities elute first while the marker compounds (PEG 400 and PEG 3350) eluted later without peak overlap. The eluted components were analyzed by an evaporative light scattering detector (PL-ELSD 2100 under conditions of evaporation -85°C, nebulizer 85°C and gas flow 1.0; Polymer Laboratories). Calibration curves were obtained in the range of 200-1500 mg/L for PEG 400 and 10-200 mg/L for PEG 3350, respectively. The intestinal permeability was calculated by the concentration of the PEG marker compound and total urine volume. The calculated intestinal permeability index (IPI, in %), PEG retrieval ratio, is an expression of the PEG 3350 intestinal permeability, relative to PEG 400.

**Table 1** Demographics and characteristics of the subjects (mean  $\pm$  SE)

	Cirrhotics with ascites (n = 26)	Cirrhotics without ascites (n = 27)	Healthy controls (n = 26)
Age (yr)	54.7 $\pm$ 9.6	53.9 $\pm$ 9.7	50.3 $\pm$ 9.2
Sex (M/F)	23/3	21/6	17/9
Etiology			
Alcohol	16	15	
Viral <sup>1</sup>	9	12	
Alcohol/viral <sup>2</sup>	1	0	
Child class (A/B/C)	1/16/9	22/5/0	
Child-Pugh score	8.8 $\pm$ 0.44 <sup>b</sup>	6.3 $\pm$ 0.34	
Serum albumin (g/dL)	2.8 $\pm$ 0.11 <sup>b</sup>	3.5 $\pm$ 0.12	
Serum bilirubin (mg/dL)	5.1 $\pm$ 0.92 <sup>b</sup>	2.0 $\pm$ 0.43	
Prothrombin time (s)	15.7 $\pm$ 0.50 <sup>a</sup>	14.3 $\pm$ 0.40	
AST (IU/L)	78.3 $\pm$ 9.45	74.8 $\pm$ 11.4	
ALT (IU/L)	34.1 $\pm$ 4.8	51.5 $\pm$ 8.9	
Encephalopathy	9 <sup>a</sup>	2	
Esophageal varix	11	9	

<sup>1</sup>Viral etiology-cirrhosis with ascites (HBV-6, HCV-3) and cirrhosis without ascites (HBV-8, HCV-4); <sup>2</sup>Viral etiology-HBV-1. <sup>a</sup> $P < 0.05$ , <sup>b</sup> $P < 0.01$  vs cirrhosis without ascites.

### Measurement of serum TNF- $\alpha$

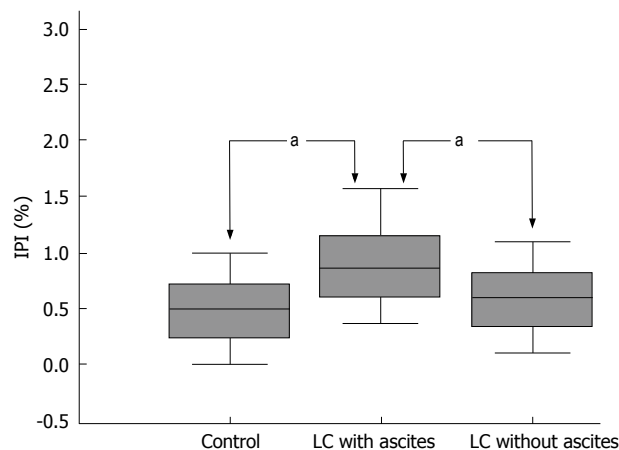
With in a 12 h period after oral administration of the PEG solution, 10 milliliters of a blood sample was taken from a forearm vein of each individual. All blood samples were anticoagulated with EDTA and then plasma was separated by centrifugation at 1600 *g* for 15 min. Plasma samples were stored at -70°C until analysis. The serum TNF- $\alpha$  concentration was determined by the enzyme-linked immunosorbent assay (ELISA) technique (Quantikine® human TNF- $\alpha$ , R & D Systems, Minneapolis, MN USA), according to the manufacturer instructions.

### Measurement of urinary nitrite excretion

About 2 mL of urine was filtered through a 10000 MW filter (Millipore Microcon YM-10) and was assayed for the NO metabolite nitrite by the Greiss reaction using Parameter TM Total NO/Nitrite/Nitrate kit (R & D Systems). The total concentration of nitrite was determined by absorbance at 540 nm after urine nitrate (NO<sub>3</sub><sup>-</sup>) was converted to nitrite (NO<sub>2</sub><sup>-</sup>) by the NADPH-dependent nitrate reductase.

### Statistical analysis

Data are reported as mean values and standard errors (mean  $\pm$  SE) or percentage according to variables. Differences among the three groups were analyzed by ANOVA. When a significant effect occurred, Scheffe post hoc comparisons were used to test differences among the means. An independent samples *t*-test was used to compare test results between two groups. A nonparametric test, the Mann-Whitney test, was used to compare independently two groups that had fewer than 10 samples. We calculated Pearson's correlation



**Figure 1** Polyethylene glycol retrieval ratio (PEG 3350/400) in the healthy control subjects and the cirrhotic patients. <sup>a</sup> $P < 0.05$ .

coefficient for associations between two variables. SPSS statistical software (version 11.0) was used for the statistical analysis. A two-tailed significant level of 5% was chosen as a type I error.

## RESULTS

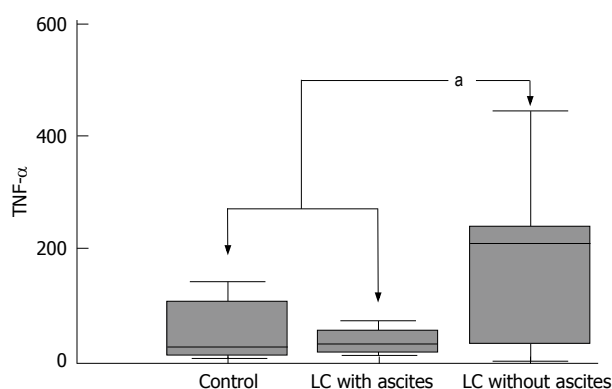
### Characteristics of the participating patients

There were no significant differences regarding age and gender between the cirrhotic patients with or without ascites and healthy control subjects. The distribution of causes of LC were alcohol ( $n = 31$ ), viral infection (HBV 14, HCV 7;  $n = 21$ ) and alcohol combined with HBV infection ( $n = 1$ ). Renal function as assessed based on blood urea and creatinine levels was normal in all patients and control subjects. Details of the demographics, etiology, severity, complications of the LC and concurrent infections are outlined in Table 1.

### Intestinal macromolecular permeability

Mean values for PEG 400 and 3350 retrieval were  $46.5 \pm 3.22$  and  $0.24 \pm 0.03$  in control subjects,  $44.1 \pm 5.17$  and  $0.21 \pm 0.02$  in patients with LC without ascites and  $37.4 \pm 3.55$  and  $0.31 \pm 0.04$  in patients with LC with ascites, respectively. The mean values for the IPI were different in patients from the healthy control subjects and patients with LC without ascites reflected the expected low diffusion of PEG 3350, being significantly higher in patients with LC with ascites ( $0.52 \pm 0.05$  and  $0.53 \pm 0.03$  vs  $0.88 \pm 0.12$ ,  $P < 0.05$ ) (Figure 1). However, there was no significant difference between the healthy control subjects and patients with LC without ascites (Table 2).

A sub-analysis relating intestinal permeability to the severity of LC for all patients as indicated by the Child-Pugh class showed significant differences between class A, B and C for PEG 3350 ( $0.20 \pm 0.02$ ,  $0.25 \pm 0.03$  vs  $0.42 \pm 0.08$ ,  $P < 0.05$ ) and IPI ( $0.52 \pm 0.04$ ,  $0.72 \pm 0.07$  vs  $1.12 \pm 0.27$ ,  $P < 0.05$ ). According to sub-analysis relating IPI to the presence of complications of LC for patients as indicated by encephalopathy and hypoalbuminemia, there were significant differences ( $P < 0.05$ ), but not for



**Figure 2** Tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) levels in the healthy control subjects and the cirrhotic patients. <sup>a</sup> $P < 0.05$ .

**Table 2** Intestinal permeability, serum TNF- $\alpha$  and urine nitrite levels in the healthy control subjects and cirrhotic patients (mean  $\pm$  SE)

	Cirrhotics with ascites (n = 26)	Cirrhotics without ascites (n = 27)	Healthy controls (n = 26)
PEG400	37.4 $\pm$ 3.55	44.1 $\pm$ 5.17	46.5 $\pm$ 3.22
PEG3350	0.31 $\pm$ 0.04	0.21 $\pm$ 0.02	0.24 $\pm$ 0.03
IPI	0.88 $\pm$ 0.12 <sup>a</sup>	0.53 $\pm$ 0.03	0.52 $\pm$ 0.05
Nitrite	1170.9 $\pm$ 28.7 <sup>a</sup>	956.7 $\pm$ 47.7	903.1 $\pm$ 55.1
TNF- $\alpha$	32.1 $\pm$ 13.3	198.9 $\pm$ 55.8 <sup>a</sup>	40.9 $\pm$ 12.3

PEG: Polyethylene glycol; IPI: Intestinal permeability index. <sup>a</sup> $P < 0.05$ .

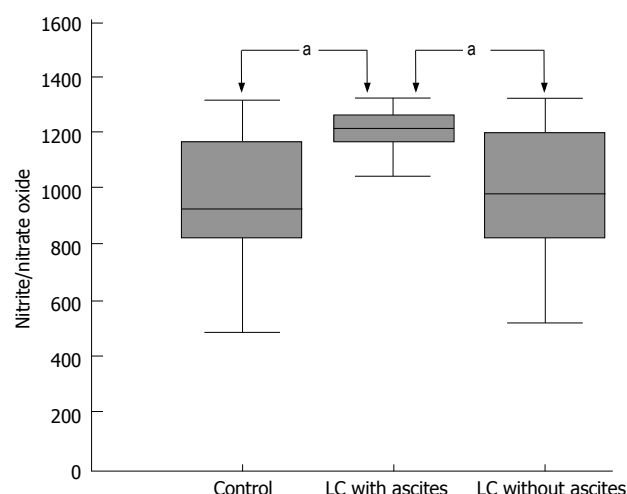
patients as indicated by a prolonged prothrombin time, esophageal varix or hyperbilirubinemia.

### Serum TNF- $\alpha$ level

The concentration of serum TNF- $\alpha$  was 198.9  $\pm$  55.8 pg/mL in patients with LC without ascites, 32.1  $\pm$  13.3 pg/mL in LC with ascites and 40.9  $\pm$  12.3 pg/mL in the control subjects (Figure 2). A group comparison by the Mann-Whitney test showed that the serum TNF- $\alpha$  level was significantly higher in patients with LC without ascites than in the control subjects and in patients with LC with ascites ( $P < 0.05$ ) (Table 2). According to the sub-analysis relating the TNF- $\alpha$  level to the severity of LC for all patients as indicated by the Child-Pugh class, class A was higher than class B and C (218.8  $\pm$  43.4 *vs* 78.9  $\pm$  26.3 and 17.7  $\pm$  3.1, respectively) and class B was higher than class C, and there were significant differences between them ( $P < 0.05$ ). According to the sub-analysis relating the TNF- $\alpha$  level to the presence of complications of LC for patients as indicated by the presence of hypoalbuminemia, there were significant differences ( $P < 0.05$ ), but not for patients as indicated by encephalopathy, prolonged prothrombin time, esophageal varix and hyperbilirubinemia (Table 3).

### Urinary nitrite excretion

Urinary nitrite excretion was significantly increased in patients with LC with ascites as compared to patients with LC without ascites or the healthy control subjects (1170.9  $\pm$  28.7  $\mu$ mol/L *vs* 956.7  $\pm$  47.7  $\mu$ mol/L or 903.1  $\pm$



**Figure 3** Urinary nitrite excretion in the healthy control subjects and the cirrhotic patients. <sup>a</sup> $P < 0.05$ .

55.1  $\mu$ mol/L,  $P < 0.05$ ) (Figure 3 and Table 2). According to a sub-analysis relating urinary nitrite excretion to the severity of LC as measured by the Child-Pugh class and to the presence of complications of LC for patients as indicated by the presence of encephalopathy, hypoalbuminemia, prolonged prothrombin time, esophageal varix and hyperbilirubinemia, there were no significant differences (Table 3).

### Correlation and statistical analysis

Patients with alcoholic *versus* non-alcoholic cirrhosis did not differ significantly ( $P > 0.05$ ) in the intestinal macromolecular permeability (PEG 400, 3350 retrieval and IPI), serum TNF- $\alpha$  level and urine nitrite level. There was a positive correlation between the Child-Pugh score and increasing intestinal macromolecular permeability ( $r = 0.494$  for PEG 3350 and  $r = 0.447$  for IPI,  $P < 0.01$ ), but no significant correlation between the Child-Pugh score and the TNF- $\alpha$  level or urinary nitrite level among patients with LC with or without ascites. No significant correlation was observed between PEG 400, 3350 percentage retrieval, IPI and the serum TNF- $\alpha$  level, between the TNF- $\alpha$  level, and urine nitrite level but there was a significant correlation between IPI and urine nitrite excretion ( $r = 0.98$ ,  $P < 0.05$ ) among patients with LC with or without ascites.

## DISCUSSION

The concept of altered intestinal permeability is important and has been implicated in a number of pathological situations, including celiac disease associated with antigen permeability, allergic intestinal diseases such as digestive hypersensitivity, inflammatory diseases such as Crohn's disease, ulcerative colitis, acute pancreatitis, alcoholic liver disease and LC associated with substance permeability during inflammation<sup>[5,21,22]</sup>.

The pathogenic mechanisms implicated in the failure of intestinal barrier in cirrhosis have not been fully elucidated as yet and remains to be investigated.

**Table 3** Analysis relating intestinal permeability, nitrite level and TNF- $\alpha$  level to the clinical and laboratory findings in the cirrhotic patients (mean  $\pm$  SE)

	<i>n</i>	PEG400	PEG3350	IPI (%)	Nitrite ( $\mu$ mol/L)	TNF- $\alpha$ (pg/mL)
Child-Pugh classification						
A	23	42.6 $\pm$ 5.7	0.20 $\pm$ 0.02	0.52 $\pm$ 0.04	1137.3 $\pm$ 45.4	218.8 $\pm$ 43.4 <sup>a</sup>
B	21	38.6 $\pm$ 4.4	0.25 $\pm$ 0.03	0.72 $\pm$ 0.07	967.7 $\pm$ 67.5	78.9 $\pm$ 26.3
C	9	41.5 $\pm$ 6.0	0.42 $\pm$ 0.08 <sup>a</sup>	1.12 $\pm$ 0.27 <sup>a</sup>	1086.3 $\pm$ 36.4	17.7 $\pm$ 3.1
Serum albumin (g/dL)						
> 3.4	16	41.4 $\pm$ 4.9	0.23 $\pm$ 0.03	0.52 $\pm$ 0.05	1013.2 $\pm$ 65.7	183.6 $\pm$ 43.5
< 3.4	37	40.6 $\pm$ 4.0	0.27 $\pm$ 0.02 <sup>a</sup>	0.78 $\pm$ 0.08 <sup>a</sup>	1114.7 $\pm$ 43.6	105.8 $\pm$ 28.4 <sup>a</sup>
Serum bilirubin (mg/dL)						
> 1.2	33	44.7 $\pm$ 4.5	0.29 $\pm$ 0.03	0.76 $\pm$ 0.09	1078.1 $\pm$ 45.7	76.6 $\pm$ 18.4
< 1.2	20	34.4 $\pm$ 3.7	0.20 $\pm$ 0.03	0.60 $\pm$ 0.07	1049.6 $\pm$ 61.2	216.3 $\pm$ 51.2
Prothrombin time (s)						
> 13	43	40.66 $\pm$ 3.6	0.27 $\pm$ 0.03	0.75 $\pm$ 0.07	1052.6 $\pm$ 40.4	121.2 $\pm$ 26.8
< 13	10	41.56 $\pm$ 7.0	0.21 $\pm$ 0.03	0.50 $\pm$ 0.07	1074.9 $\pm$ 88.8	163.8 $\pm$ 55.4
Encephalopathy						
No	42	41.58 $\pm$ 3.79	0.24 $\pm$ 0.02	0.67 $\pm$ 0.07	1097.6 $\pm$ 41.48	139.1 $\pm$ 27.52
Yes	11	37.98 $\pm$ 5.00	0.33 $\pm$ 0.04	0.82 $\pm$ 0.13 <sup>a</sup>	1030.0 $\pm$ 77.84	91.9 $\pm$ 49.89
Esophageal varix						
No	33	42.6 $\pm$ 4.54	0.24 $\pm$ 0.03	0.72 $\pm$ 0.08	1119.9 $\pm$ 39.7	145.6 $\pm$ 33.74
Yes	20	37.86 $\pm$ 3.16	0.29 $\pm$ 0.03	0.67 $\pm$ 0.10	1007.7 $\pm$ 70.1	102.4 $\pm$ 31.30

PEG: Polyethylene glycol; IPI: Intestinal permeability index. <sup>a</sup>*P* < 0.05.

Toxic metabolites of alcohol are known to induce alterations of enterocyte tight junctions, which may increase paracellular permeability<sup>[23]</sup>. However, other inflammatory conditions may alter barrier integrity, as measured by increased gut permeation. An alternative mechanism may be the proinflammatory cytokines, which can be produced locally by epithelial cells or may reach the intestinal mucosa from an inflammatory focus distant from the bowel<sup>[24]</sup>. Interestingly, *in vitro* studies in cell monolayers suggest that cytokines may mediate these permeation effects by changes in the production of NO<sup>[25]</sup>. The mechanism for this effect is not known, but may involve relaxation of the cytoskeleton or oxidation/nitration of cytoskeleton proteins<sup>[26]</sup>.

In the current study, PEG with different molecular masses was used to assess gut permeability, as it combines unique attributes in its chemical structure. It is non-toxic, water-soluble, as is endotoxin, and not metabolized either by the host or by intestinal bacteria<sup>[27]</sup>. After transmigration into the blood, the polar PEG-molecule is excreted with the urine. Because of its homogeneous chemical properties, its appropriately adaptable molecular mass and its linear, chain-like shape (mimicking the comparable structure of endotoxin)<sup>[28]</sup>, PEG seems to be an appropriate probe for the assessment of LPS translocation through the intestine. All of these demands cannot be met by other commonly used permeability marker compounds such as mono- or disaccharides, sugar alcohols, complexes with radioactive nuclides (51Cr-EDTA, 99mTc-DTPA), proteins, or even combinations of these compounds<sup>[29]</sup>.

The simultaneous use of two test marker compounds allows the expression of global intestinal permeability as an index reflecting the transfer value of the less permeable test marker (PEG 3350) relative to the most diffusible probe (PEG 400). Since pre-absorption factors

such as gastric emptying, dilution by digestive secretions and post-absorption factors such as systemic distribution and renal clearance are assumed to affect both molecules equally, the value of this index should then be directly comparable from one individual to another<sup>[30]</sup>.

It has been suggested that intestinal permeability markers pass through either a transcellular or a paracellular pathway. However, it is difficult to determine that endotoxins or other bacterial toxins from the gut lumen into the portal system pass paracellularly or transcellularly in cirrhotic patients, as there was no significant difference of PEG 400 and 3350 retrieval between the control subjects and cirrhotic patients with or without ascites in this study. To address this issue, further studies are needed for morphological or molecular changes of intestinal mucosa in LC during the PEG test. Distribution of PEG in ascites might have caused a lower urinary excretion rate and thus underestimated possible permeability changes in patients with ascites. However, Kalaitzakis *et al* assessing intestinal permeability with 51Cr-EDTA concluded that a loss of 51Cr-EDTA into the ascitic compartment was unlikely and paracentesis had no significant effect on the urinary 51Cr-EDTA excretion, which suggests that ascites in itself does not unduly affect the test results<sup>[5]</sup>. In the present study, ascitic fluid from three cirrhotic patients was tested for PEG and it was not detected; therefore, the possibility of lower urinary excretion rates due to distribution of PEG in ascites can be ruled out.

Previous studies have shown an association between IPI and severity of LC assessed according to the Child-Pugh classification<sup>[8,9]</sup>, but other studies have failed to reproduce these results<sup>[6]</sup>. In the present study, we observed significantly higher PEG 3350 retrieval and IPI in Child-Pugh class C patients as compared with that in class A and B patients. Methodological and/or patient selection differences should be taken into account when



interpreting the results of this study.

In this study, there were no concomitant infections and a significantly higher TNF- $\alpha$  level in cirrhotic patients without ascites than healthy control subjects or patients with LC with ascites was seen; thus, there was a tendency for a negative correlation between the TNF- $\alpha$  level and Child-Pugh class in the advanced stage of LC. In advanced cirrhosis, hepatic damage and inflammation are reduced due to a decreased liver reserve and marked fibrosis, and consequently, ALT levels decrease. Additionally, diminished amounts of cytokine-producing cells such as hepatocytes and Kupffer cells may lead to a decrease of TNF- $\alpha$  production<sup>[15]</sup>. In LC, several inflammatory states and commonly occurring infections may be another source of TNF- $\alpha$  production and could explain contradicting results. Increased production of TNF- $\alpha$  associated with inflammation and tissue necrosis is seen not only in hepatitis, but also in other inflammatory conditions. In the present study, IPI and urinary nitrite excretion were significantly higher in patients with LC with ascites as compared to patients with LC without ascites or healthy control subjects, with a significant correlation. Since NO is thought to have a wide range of biological functions other than vasodilatation, it is likely to affect both the progress and the clinical features of LC as well as the hemodynamics in cirrhotic patients. For example, NO is a potent inducer of increased membrane permeability in the vascular endothelium and intestinal mucosa, possibly contributing to the accumulation of ascites and to bacterial translocation<sup>[31]</sup>. In the current study, although TNF- $\alpha$  was thought to induce NO synthesis (NOS) through the inducible NOS and endothelial NOS<sup>[32,33]</sup>, there was no significant correlation between TNF- $\alpha$  level and NO level in LC. This is the same to some studies, where such a relation could not be observed<sup>[34,35]</sup>. It has been suggested that some other factors including TNF- $\alpha$  contribute to elevation of NO in LC.

A simple comparison of the published data with the findings of the present study is not easy to make. There were differences between the reported results of intestinal permeability, which means differences in the methods of assessment, including the composition of the probe solution and analytic techniques employed, as well as differences in the patient populations and in the causes and severity of LC. In conclusion, our results suggest that increased intestinal macromolecular permeability and NO are probably of importance in the pathophysiology and progression of LC with ascites, and furthermore, IPI may be a contributory factor in the development of encephalopathy in LC.

## ACKNOWLEDGMENTS

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## COMMENTS

### Background

Increased intestinal permeability (IPI) with bacterial translocation and endotoxemia have been implicated in the pathogenesis of chronic liver injury and as contributory factors in the development of dangerous complications, such as encephalopathy and bacterial infections in liver cirrhosis (LC). However, limited data exists on the state of intestinal macromolecular permeability using PEG (400 and 3350) in cirrhotic patients with or without ascites. To clarify the role of intestinal macromolecular permeability, the serum tumor necrosis factor (TNF)- $\alpha$  level and nitrite level in urine to the development of LC with ascites, the authors investigated whether intestinal macromolecular permeability is altered in patients LC with or without ascites, and its relationship with the serum TNF- $\alpha$  level and NO metabolite level in urine.

### Innovations and breakthroughs

The authors investigated the relation between intestinal permeability in compensated and decompensated cirrhosis in relation to TNF- $\alpha$  and urine nitrite oxide levels. Their results suggest that increased intestinal macromolecular permeability and NO are probably of importance in the pathophysiology and progression of LC with ascites, and furthermore, IPI may be a contributory factor in the development of encephalopathy in LC.

### Applications

All the findings of the current study will provide useful information for the understanding and the treatment of LC.

### Peer review

This is an interesting study on a relevant topic. The main results of the study are that the increased permeability and nitrite oxide is of importance in the pathophysiology of decompensated cirrhosis.

## REFERENCES

- 1 **Bjarnason I**, MacPherson A, Hollander D. Intestinal permeability: an overview. *Gastroenterology* 1995; **108**: 1566-1581
- 2 **DeMeo MT**, Mutlu EA, Keshavarzian A, Tobin MC. Intestinal permeation and gastrointestinal disease. *J Clin Gastroenterol* 2002; **34**: 385-396
- 3 **Budillon G**, Parrilli G, Pacella M, Cuomo R, Menzies IS. Investigation of intestine and liver function in cirrhosis using combined sugar oral loads. *J Hepatol* 1985; **1**: 513-524
- 4 **Farhadi A**, Banan A, Fields J, Keshavarzian A. Intestinal barrier: an interface between health and disease. *J Gastroenterol Hepatol* 2003; **18**: 479-497
- 5 **Kalaitzakis E**, Johansson JE, Bjarnason I, Bjornsson E. Intestinal permeability in cirrhotic patients with and without ascites. *Scand J Gastroenterol* 2006; **41**: 326-330
- 6 **Fujii T**, Seki T, Maruoka M, Tanaka J, Kawashima Y, Watanabe T, Sawamura T, Inoue K. Lactulose-L-rhamnose intestinal permeability test in patients with liver cirrhosis. *Hepatol Res* 2001; **19**: 158-169
- 7 **Huglo D**, De Botton S, Canva-Delcambre V, Colombel JF, Wallaert B, Steinling M, Marchandise X. Simultaneous determination of pulmonary and intestinal permeability in patients with alcoholic liver cirrhosis. *Eur J Nucl Med* 2001; **28**: 1505-1511
- 8 **Campillo B**, Pernet P, Bories PN, Richardet JP, Devanlay M, Aussel C. Intestinal permeability in liver cirrhosis: relationship with severe septic complications. *Eur J Gastroenterol Hepatol* 1999; **11**: 755-759
- 9 **Pascual S**, Such J, Esteban A, Zapater P, Casellas JA, Aparicio JR, Girona E, Gutierrez A, Carnices F, Palazon JM, Solà-Vera J, Perez-Mateo M. Intestinal permeability is increased in patients with advanced cirrhosis. *Hepatogastroenterology* 2003; **50**: 1482-1486
- 10 **Zuckerman MJ**, Menzies IS, Ho H, Gregory GG, Casner NA, Crane RS, Hernandez JA. Assessment of intestinal permeability and absorption in cirrhotic patients with ascites using combined sugar probes. *Dig Dis Sci* 2004; **49**:

- 621-626
- 11 **Michie HR**, Manogue KR, Spriggs DR, Revhaug A, O'Dwyer S, Dinarello CA, Cerami A, Wolff SM, Wilmore DW. Detection of circulating tumor necrosis factor after endotoxin administration. *N Engl J Med* 1988; **318**: 1481-1486
  - 12 **O'Dwyer ST**, Michie HR, Ziegler TR, Revhaug A, Smith RJ, Wilmore DW. A single dose of endotoxin increases intestinal permeability in healthy humans. *Arch Surg* 1988; **123**: 1459-1464
  - 13 **Odeh M**, Sabo E, Srugo I, Oliven A. Serum levels of tumor necrosis factor-alpha correlate with severity of hepatic encephalopathy due to chronic liver failure. *Liver Int* 2004; **24**: 110-116
  - 14 **Kiki I**, Yilmaz O, Erdem F, Gundogdu M, Demircan B, Bilici M. Tumour necrosis factor-alpha levels in hepatitis B virus-related chronic active hepatitis and liver cirrhosis and its relationship to Knodell and Child-Pugh scores. *Int J Clin Pract* 2006; **60**: 1075-1079
  - 15 **Zhang W**, Yue B, Wang GQ, Lu SL. Serum and ascites levels of macrophage migration inhibitory factor, TNF-alpha and IL-6 in patients with chronic virus hepatitis B and hepatitis cirrhosis. *Hepatobiliary Pancreat Dis Int* 2002; **1**: 577-580
  - 16 **Giron-Gonzalez JA**, Martinez-Sierra C, Rodriguez-Ramos C, Macias MA, Rendon P, Diaz F, Fernandez-Gutierrez C, Martin-Herrera L. Implication of inflammation-related cytokines in the natural history of liver cirrhosis. *Liver Int* 2004; **24**: 437-445
  - 17 **Lee FY**, Lu RH, Tsai YT, Lin HC, Hou MC, Li CP, Liao TM, Lin LF, Wang SS, Lee SD. Plasma interleukin-6 levels in patients with cirrhosis. Relationship to endotoxemia, tumor necrosis factor-alpha, and hyperdynamic circulation. *Scand J Gastroenterol* 1996; **31**: 500-505
  - 18 **Eriksson AS**, Gretzer C, Wallerstedt S. Elevation of cytokines in peritoneal fluid and blood in patients with liver cirrhosis. *Hepatogastroenterology* 2004; **51**: 505-509
  - 19 **Vallance P**, Moncada S. Hyperdynamic circulation in cirrhosis: a role for nitric oxide? *Lancet* 1991; **337**: 776-778
  - 20 **Oudenhoven IM**, Klaasen HL, Lapre JA, Weerkamp AH, Van der Meer R. Nitric oxide-derived urinary nitrate as a marker of intestinal bacterial translocation in rats. *Gastroenterology* 1994; **107**: 47-53
  - 21 **DeMeo MT**, Mutlu EA, Keshavarzian A, Tobin MC. Intestinal permeation and gastrointestinal disease. *J Clin Gastroenterol* 2002; **34**: 385-396
  - 22 **Rahman SH**, Ammori BJ, Larvin M, McMahon MJ. Increased nitric oxide excretion in patients with severe acute pancreatitis: evidence of an endotoxin mediated inflammatory response? *Gut* 2003; **52**: 270-274
  - 23 **Keshavarzian A**, Holmes EW, Patel M, Iber F, Fields JZ, Pethkar S. Leaky gut in alcoholic cirrhosis: a possible mechanism for alcohol-induced liver damage. *Am J Gastroenterol* 1999; **94**: 200-207
  - 24 **McKay DM**, Baird AW. Cytokine regulation of epithelial permeability and ion transport. *Gut* 1999; **44**: 283-289
  - 25 **Wallace JL**, Miller MJ. Nitric oxide in mucosal defense: a little goes a long way. *Gastroenterology* 2000; **119**: 512-520
  - 26 **Banan A**, Fields JZ, Zhang Y, Keshavarzian A. iNOS upregulation mediates oxidant-induced disruption of F-actin and barrier of intestinal monolayers. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G1234-G1246
  - 27 **Philipsen EK**, Batsberg W, Christensen AB. Gastrointestinal permeability to polyethylene glycol: an evaluation of urinary recovery of an oral load of polyethylene glycol as a parameter of intestinal permeability in man. *Eur J Clin Invest* 1988; **18**: 139-145
  - 28 **Parlesak A**, Bode JC, Bode C. Parallel determination of gut permeability in man with M(r) 400, M(r) 1500, M(r) 4000 and M(r) 10,000 polyethylene glycol. *Eur J Clin Chem Clin Biochem* 1994; **32**: 813-820
  - 29 **Parlesak A**, Schafer C, Schutz T, Bode JC, Bode C. Increased intestinal permeability to macromolecules and endotoxemia in patients with chronic alcohol abuse in different stages of alcohol-induced liver disease. *J Hepatol* 2000; **32**: 742-747
  - 30 **Loret S**, Nollevaux G, Remacle R, Klimek M, Barakat I, Deloyer P, Grandfils C, Dandrisosse G. Analysis of PEG 400 and 4000 in urine for gut permeability assessment using solid phase extraction and gel permeation chromatography with refractometric detection. *J Chromatogr B Analyt Technol Biomed Life Sci* 2004; **805**: 195-202
  - 31 **Guarner C**, Soriano G, Tomas A, Bulbena O, Novella MT, Balanzo J, Vilardell F, Mourelle M, Moncada S. Increased serum nitrite and nitrate levels in patients with cirrhosis: relationship to endotoxemia. *Hepatology* 1993; **18**: 1139-1143
  - 32 **Elsing C**, Harenberg S, Stremmel W, Herrmann T. Serum levels of soluble Fas, nitric oxide and cytokines in acute decompensated cirrhotic patients. *World J Gastroenterol* 2007; **13**: 421-425
  - 33 **Genesca J**, Gonzalez A, Segura R, Catalan R, Marti R, Varela E, Cadelina G, Martinez M, Lopez-Talavera JC, Esteban R, Groszmann RJ, Guardia J. Interleukin-6, nitric oxide, and the clinical and hemodynamic alterations of patients with liver cirrhosis. *Am J Gastroenterol* 1999; **94**: 169-177
  - 34 **Barsacchi R**, Perrotta C, Bulotta S, Moncada S, Borgese N, Clementi E. Activation of endothelial nitric-oxide synthase by tumor necrosis factor-alpha: a novel pathway involving sequential activation of neutral sphingomyelinase, phosphatidylinositol-3' kinase, and Akt. *Mol Pharmacol* 2003; **63**: 886-895
  - 35 **Wiest R**, Das S, Cadelina G, Garcia-Tsao G, Milstien S, Groszmann RJ. Bacterial translocation in cirrhotic rats stimulates eNOS-derived NO production and impairs mesenteric vascular contractility. *J Clin Invest* 1999; **104**: 1223-1233

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## Change of choline compounds in sodium selenite-induced apoptosis of rats used as quantitative analysis by *in vitro* 9.4T MR spectroscopy

Zhen Cao, Lin-Ping Wu, Yun-Xia Li, Yu-Bo Guo, Yao-Wen Chen, Ren-Hua Wu

Zhen Cao, Yun-Xia Li, Yu-Bo Guo, Yao-Wen Chen, Ren-Hua Wu, Department of Medical Imaging, The Second Affiliated Hospital, Shantou University Medical College, Shantou 515041, Guangdong Province, China

Lin-Ping Wu, Multidisciplinary Research Center, Shantou University, Shantou 515041, Guangdong Province, China

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Correspondence to: Dr. Ren-Hua Wu, Department of Medical Imaging, the 2nd Affiliated Hospital, Shantou University Medical College, Dongshan North Road, Shantou 515041, Guangdong Province, China. [rhwu@stu.edu.cn](mailto:rhwu@stu.edu.cn)

Telephone: +86-754-8915674 Fax: +86-754-8915674

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group were abnormal. Apoptosis of hepatic cells was confirmed by TUNEL assay.

**CONCLUSION:** High dose selenium compounds can cause the rat liver lesion and induce cell apoptosis *in vivo*. High resolution  $^1\text{H}$ -MRS *in vitro* can detect diversified metabolism. The changing trend for different ingredient of choline compounds is not completely the same at early period of apoptosis.

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**Key words:** Apoptosis of liver cell; Choline compounds; Sodium selenite; *In vitro*  $^1\text{H}$ -MRS; Quantitative analysis

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### Abstract

**AIM:** To study liver cell apoptosis caused by the toxicity of selenium and observe the alteration of choline compounds using *in vitro* 9.4T high resolution magnetic resonance spectroscopy.

**METHODS:** Twenty male Wistar rats were randomly divided into two groups. The rats in the treatment group were intraperitoneally injected with sodium selenite and the control group with distilled water. All rats were sacrificed and the livers were dissected.  $^1\text{H}$ -MRS data were collected using *in vitro* 9.4T high resolution magnetic resonance spectrometer. Spectra were processed using XWINNMR and MestRe-c 4.3. HE and TUNEL staining was employed to detect and confirm the change of liver cells.

**RESULTS:** Good  $^1\text{H}$ -MR spectra of perchloric acid extract from liver tissue of rats were obtained. The conventional metabolites were detected and assigned. Concentrations of different ingredient choline compounds in treatment group *vs* control group were as follows: total choline compounds,  $5.08 \pm 0.97$  mmol/L *vs*  $3.81 \pm 1.16$  mmol/L ( $P = 0.05$ ); and free choline,  $1.07 \pm 0.23$  mmol/L *vs*  $0.65 \pm 0.20$  mmol/L ( $P = 0.00$ ). However, there was no statistical significance between the two groups. The hepatic sinus and cellular structure of hepatic cells in treatment

Cao Z, Wu LP, Li YX, Guo YB, Chen YW, Wu RH. Change of choline compounds in sodium selenite-induced apoptosis of rats used as quantitative analysis by *in vitro* 9.4T MR spectroscopy. *World J Gastroenterol* 2008; 14(24): 3891-3896 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3891.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3891>

### INTRODUCTION

Apoptosis is a programmed, active, highly selective mechanism of cell death. Multicellular organisms' apoptosis is an essential component of cellular regulation. Abnormal regulation of apoptosis can lead to disorders such as cancer<sup>[1,2]</sup>.

The field of cell death research has undergone an explosion of new knowledge over the past decade. The methods to evaluate death of cells, especially in intact tissues, have led to the development of several techniques. However, the properties revealed in these assays are not always applicable to study of diversified metabolite of apoptosis at one time<sup>[3]</sup>.

Nuclear magnetic resonance spectroscopy is a non-destructive and non-invasive technique that can provide complete structural analysis of a wide range of organic molecules in complex mixtures. It generates

quantitative information, as the peak intensities can be proportional to analyze concentrations<sup>[4]</sup>. Because of their low sensitivity and small magnet gaps, the early spectrometers had limited applications, primarily to synthetic chemistry<sup>[5]</sup>. Over the past three decades, however, sensitivity has increased by orders of magnitude so that this technique can be used to detect the metabolite alteration of apoptosis<sup>[6-8]</sup>.

Choline compounds are one kind of biologically interesting metabolites that can be detected by <sup>1</sup>H-MRS. Declining of choline compounds is considered as a <sup>1</sup>H-NMR metabolite marker of advanced stage of apoptosis<sup>[9]</sup>. Lehtimäki *et al*<sup>[10]</sup> consider that choline compounds stay unchanged despite reduced cell density. However, how the intensity choline compounds change when apoptosis occurs is still confused.

Our hypothesis was that there is no reason for choline compounds to stay unchanged when apoptosis of liver cell occurs. The alteration of choline compounds could be observed through detailed quantitative analysis by high-resolution <sup>1</sup>H-MR spectroscopy. Therefore, the purpose of this study was to observe the liver cell apoptosis caused by the toxicity of selenium and the alteration of choline compounds using *in vitro* 9.4T high resolution magnetic resonance spectroscopy.

## MATERIALS AND METHODS

### Animal

Twenty male Wistar rats, weighing 280-320 g, were randomly divided into two groups (*n* = 10). The rats in the treatment group were intraperitoneally injected with sodium selenite liquor (Na<sub>2</sub>SeO<sub>3</sub>) at a dose of 20 μmol/kg and the control group with distilled water at a dose of 1 ml/kg. The rats of both groups were fasted for 12 h but with free access to water. All the rats were sacrificed after 24 h. The livers of all rats were immediately dissected. Parts of livers were frozen in liquid nitrogen and then stored at -70°C until measured. The rest parts of livers were fixed in formalin. All animal experiments were performed according to the guidelines approved by the Ethical Committee of the Medical College of Shantou University.

### *In vitro* <sup>1</sup>H-MR spectroscopy

Frozen liver tissue was pulverized with a pestle and mixed with a volume of 2 mmol/mL ice-cold perchloric acid. The mixture was transferred to a homogenizer and homogenized for 20 min at 4°C. The mixture was neutralized with ice-cold 3mmol/mL and 2 mmol/mL KOH and then centrifuged at 10 000 *g* for 30 min in order to eliminate perchlorate salts. The resulting supernatant was lyophilized and the precipitate discarded. The powder of extracts was transferred into a 5-mm NMR tube and redissolved in 500 μL D<sub>2</sub>O containing 1 mmol/L 2,2'-3,3'-tetra deuterio-trimethyl-silylpropionate (TMSP). D<sub>2</sub>O was added for locking signal. TMSP was used as an internal chemical shift reference at 0.00 ppm. Each sample was collected using *in vitro* 9.4T high resolution magnetic resonance spectrometer (Bruker

Avance 400 MHz). Spectra of extracts were acquired with a pulse sequence with water suppression from the Bruker zgpr pulse program. Data were obtained over a 5000 Hz sweep width and digitized with 4096 data points. The number of scans was 128. Total acquisition time was 6 min. Spectra were primarily processed in the frequency domain using XWINNMR (Bruker GmbH), including fourier-transformation, phased correction and baseline correction. The chemical shift was assigned according to the internal standard TMSP and advanced analysis was then performed using MestRe-c 4.3.

### Statistical analysis

The peak areas which were assigned as metabolites containing choline and TMSP and integral values were calculated separately by MestRe-c 4.3. The concentration of choline was calculated following the amended formula<sup>[11]</sup>.

$$(\text{metabolite}) = \frac{\text{square}(\text{metabolite})}{\text{square}(\text{TMSP})} \times \frac{\text{number of protons of metabolite}}{9} \times (\text{TMSP})$$

Square (metabolite) and square (TMSP) stand for the area of peaks of choline compounds and TMSP; 9 correspond to the number of protons giving rise to the TMSP peak; (TMSP) and (metabolite) represent the concentration of TMSP and metabolite.

The concentration data were put into computer and analyzed using SPSS 13.0 software. A two-sample *t* test was used for comparison of choline concentration of the samples in both groups. Significance level was set at *P* < 0.05.

### Histopathology

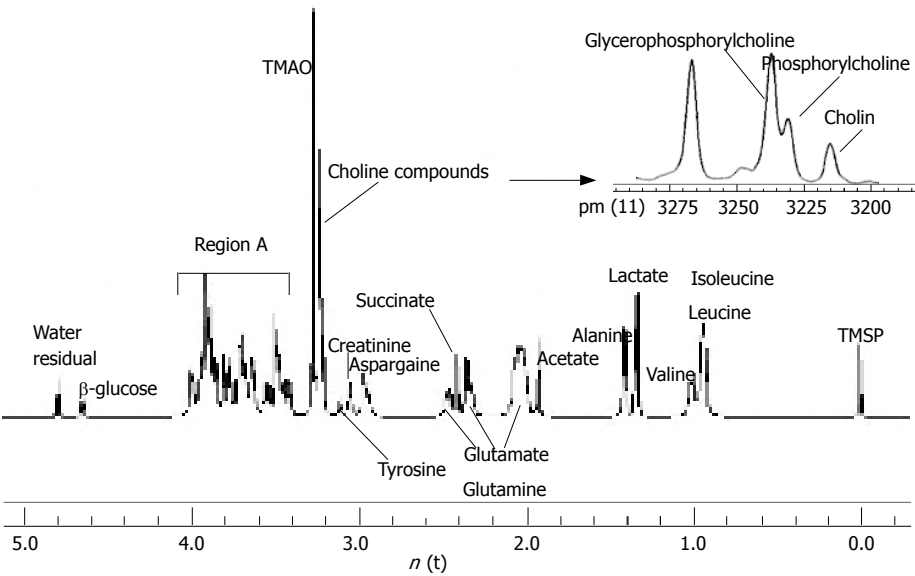
Parts of livers presenting no necrosis by visual inspection underwent histopathological examination during follow-up. Formalin fixed samples were embedded in paraffin and 4-mm sections were cut. Samples were stained with hematoxylin and eosin and examined under light microscopy.

The terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assay was performed using the *in situ* cell death detection kit according to Schrum LW<sup>[12]</sup>. Briefly, DNA ends were tagged with fluorescein-labeled dUTP using terminal deoxynucleotidyl transferase by incubating the samples at 37°C in a humidified chamber. Liver sections were then incubated with anti-fluorescein-alkaline phosphatase conjugate for 30 min in a humidified chamber. Slides were incubated with 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium for 20 min at room temperature and were counterstained with hematoxylin. The sections were observed under light microscopy.

## RESULTS

<sup>1</sup>H-MRS spectra of perchloric acid extract from rat liver tissues are shown in Figure 1. The conventional metabolites were detected and assigned. The assignments of spectra were performed following the





**Figure 1** High-resolution proton spectra of liver tissue of a rat in control group and internal standard (TMS). The field 0.0-5.0 ppm is shown.

**Table 1** Assignments of liver metabolites from male Wistar rats

Metabolite	Chemical shift (ppm)
TMS	0
Isoleucine and Leucine	0.87
Valine	0.96
Lactate	1.32
Lysine	1.47
Alanine	1.48
Acetate	1.92
Glutamate	2.07-2.34
Succinate	2.41
Glutamine	2.13-2.45
Asparagine	2.95
Creatinine	3.05
Tyrosine	3.11
Choline compounds	3.20-3.23
TMAO	3.27
Region A	3.41-4.0
β-glucose	4.67
Water (residual)	4.75

TMS: 2,2'-3,3'-tetra deuterio-trimethyl-silylpropionate; TMAO: Trimethylamine-N-oxide methyl; Region A: Glucose and amino acid CH resonances.

previous studies<sup>[13-16]</sup> and presented in Table 1. The resonances of partially megascopic region 3.20-3.27 ppm were well resolved, where N(CH<sub>3</sub>)<sub>3</sub> signals from the compounds choline, phosphorylcholine and glycerophosphorylcholine can be separated. Following the formula, the total choline compounds and free choline concentrations were calculated. The mean concentration of total choline compounds was 5.08 ± 0.97 mmol/L in control group and 3.81 ± 1.16 in treatment group and the mean concentration of free choline was 1.07 ± 0.23 mmol/L in control group and 0.65 ± 0.20 in treatment group. The differences of the two groups were statistically significant (*P* = 0.05 and *P* = 0.00, respectively). However, there were no statistical significances if we compared the concentrations of synthetical choline, including phosphorylcholine and glycerophosphorylcholin (3.71 ± 0.74 mmol/L *vs* 3.01 ±

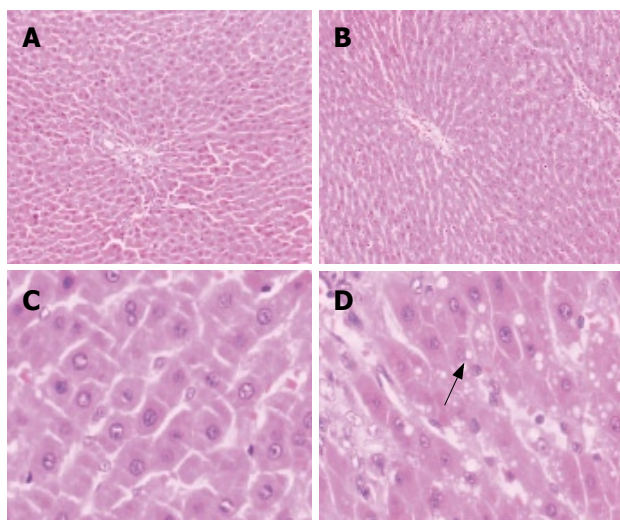
0.94 mmol/L, *P* = 0.46) between the two groups.

Although the structure of liver lobules was normal in both groups in the view of 100 times magnification, the hepatic sinus was wider in treatment group than in the control group. Cell shape was then inspected in 400 times magnification under light microscopy. The normal hepatic cells were like short shuttle while hepatic cells in treatment group were irregular and verge was not clear. Some of the cells were slightly bigger than the normal ones, but smaller ones were more often observed. We found that the cytoplasm was dyed redder than normal ones. Simultaneously, the condensed or diffuse chromatin remained randomly distributed, and the nuclear pores disappeared (Figure 2). No obviously inflamed cells could be detected under light microscope. Apoptosis of hepatic cells in both groups was confirmed by TUNEL assay. The brown ones in TUNEL assay were the apoptosis positive (Figure 3).

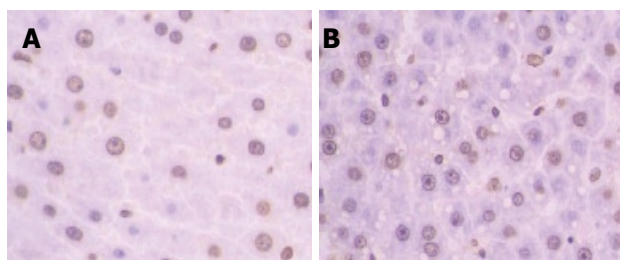
Although positive cells were detected in both groups, the number of positive cells was conspicuously more in treatment group than in control group. The positive cells were spread around in the control group but relatively assembled in the treatment group. Some of the apoptosis positive hepatocytes were vacuolated in treatment group. We found a very interesting phenomenon in our results: Few of apoptosis positive cells were detected in a rat of the treatment group. The <sup>1</sup>H-MRS data showed that choline concentrations were also much higher than the mean concentration of the treatment group, the concentration of total choline, free choline and synthetical choline of this rat was 5.67, 0.87 and 4.04 mmol/L, respectively.

**DISCUSSION**

Selenium is an essential trace element for human health. The cellular effects of selenite appear quite complex and are concentration-dependent. It is demonstrated that the serum level of selenite affects cell proliferation<sup>[17]</sup>. At intermediate concentrations, selenite appears to exert its chemopreventive activity. At higher concentrations,



**Figure 2** Light microscopy of HE stained livers. **A:** Specimen from control group (x 100); **B:** Specimen from treatment group (x 100); **C:** Specimen from control group (x 400); **D:** Specimen from treatment group (x 400). Arrowhead indicates vacuolated hepatocytes.



**Figure 3** The brown hepatocytes showing the cell apoptosis (TUNEL staining). **A:** Specimen from control group (x 400); **B:** Specimen from treatment group (x 400).

selenite induces oxidative stress and may become toxic<sup>[18]</sup>. Many researchers focused on the induction of apoptosis by toxic concentrations of selenite. Selenocompounds have been reported to induce apoptosis in non-malignant cell lines, such as the Chang liver cells<sup>[19]</sup>. The results of our experiments also intensively suggested that high-concentration selenium was able to cause lesions in rat livers and induce apoptosis *in vivo*. However, we also observed that one rat liver in treatment group did not present obvious cell apoptosis. We think that this phenomenon might be related to the individual diversity. Some of the rats are not sensitive towards this remedy. Bollard *et al* pointed out that various intrinsic physiological factors were known to affect the metabolic composition of biological samples from healthy experimental animals. These included well-being, genetic drift, strain, hormonal differences, rate of metabolism, age, and gender<sup>[20]</sup>. It is still unclear how selenocompounds might induce apoptosis. Many potential mechanisms have been proposed, including protection against oxidative damage, modulation of metabolism of carcinogens, cytotoxicity of selenium metabolites, induction of apoptosis secondary to production of ROS, regulation of the thioredoxin redox system, regulation of the cell cycle, and inhibition of angiogenesis<sup>[21]</sup>.

It has been reported that, with Se supplementation, the liver Se concentration increases disproportionately<sup>[22]</sup>. How to predict the liver lesion and inspect the efficiency and toxicity of selenocompounds is an important issue. <sup>1</sup>H-MRS is one of suitable, low-cost, and accurate methods for this study.

MR spectroscopy possesses the sensitivity required to measure subtle biochemical changes<sup>[23]</sup>. Although MR spectroscopy detects only a fairly small number of metabolites, it can still be used to monitor the activity of many cellular activities, because so many metabolic pathways are connected<sup>[24]</sup>. Choline compounds are one group of metabolites that can be detected by <sup>1</sup>H-MRS.

Choline is a nutrient essential for normal function of all cells<sup>[25]</sup>. It is a precursor not only for acetylcholine but also for phospholipids that are found in intracellular membranes and in the cell membrane<sup>[26]</sup>. The total choline peaks consist of glycerophosphorylcholine, phosphorylcholine and free choline, but the low resolution of *in vivo* spectroscopy can not identify the individual peaks from these compounds<sup>[27]</sup>. Because of the high resolution of the spectrometer which we used in this experiment, free choline is detached among the choline compounds so that we can further find out how free choline changes are when apoptosis of cells takes place separately. In our research, we found that the total choline declined when apoptosis occurred in the liver cells. This result is quite conformable with the result of Blankenberg FG and the conventional idea, but different from the results of Lehtimäki<sup>[10]</sup>. In our opinion, there are three reasons for this phenomenon: firstly, it is related to the inspected organ, which is liver but not brain. As it is known, all ingested choline and free choline generated by phospholipid metabolism enter the hepatic circulation, making the liver, where there are very active biochemical pathways for choline metabolism, a significant “sink” for choline<sup>[28]</sup>. But when a lesion arises in the liver, the liver loses the function of absorbing choline and causes choline declining. Secondly, the method used to induce liver cell apoptosis could cause this difference. One of the reasons why the selenocompounds cause cell apoptosis is that it is capable of inducing rapid superoxide generation and p53 phosphorylation<sup>[29]</sup>. This activation can initiate mitochondrial dysfunction and result in energy insufficiency. Ultimately, it may affect the exchange of the cell containing substance, including choline compounds. This is testified by Luck *et al*<sup>[30]</sup>. Finally, cell apoptosis period is also one of the influencing factors towards the result. In our study, the apoptosis was in its early phase. At the beginning of cell apoptosis, the total choline compounds declined because free choline decreased. This was supported by our spectroscopic and light microscopic data. It is also very interesting that the total concentration of synthetic choline, including phosphorylcholine and glycerophosphorylcholine did not have statistical difference between the two groups. Energy insufficiency and activity decline may originally cause the concentration decrease of synthetic choline when apoptosis takes place in liver cells. Because of the synthetic choline supplement by membrane dilatation

and release when the samples were mashed, the total concentration of synthetical choline finally remained fairly constant. This process of membrane perturbations is mainly the function of phospholipase A<sub>2</sub> activity *in vivo*<sup>[31]</sup>.

In summary, high dose selenium compounds can cause lesion of rat liver and induce cell apoptosis *in vivo*. *In vitro* high resolution <sup>1</sup>H-MRS can detect diversified metabolism that can resolve in water. The data of the spectroscopy include quantitative and qualitative information. Moreover, it can repetitively and accurately evaluate the lesion of the organ at early stage. Thus, this method has a potential role in oncology, including detection of malignancy, grading of tumor, predicting and monitoring the treatment response, and identifying persistent or recurrent diseases<sup>[32,33]</sup>. In our study, we found that the changing trend for different ingredient of choline compounds is not completely the same at early period of apoptosis. Further studies are needed to know how choline compounds change at the advanced and final stage of apoptosis.

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## COMMENTS

### Background

The field of cell death research has undergone an explosion of new knowledge over the past decade. The methods to evaluate death of cells have led to the development of several techniques. However, the properties revealed in these assays are not always applicable to study of diversified metabolite of apoptosis at one time. Magnetic resonance spectroscopy is a non-destructive and non-invasive technique that can provide complete structural analysis of a wide range of organic molecules in complex mixtures. This technique used to detect the metabolite alteration of apoptosis has been reported. Choline compounds are one kind of biologically interesting metabolites that can be detected by <sup>1</sup>H-MRS. However, how the intensity of choline compounds changes when apoptosis occurs is still confused.

### Research frontiers

The alteration of different ingredient of choline compounds could be quantitatively analyzed by *in vitro* 9.4T high-resolution <sup>1</sup>H-MR spectroscopy when apoptosis of liver cell takes place because of the toxicity of selenium. The results of present article will be helpful for further studies concerning metabolism of apoptosis.

### Innovations and breakthroughs

When apoptosis of liver cell takes place, the concentrations of total choline and free choline decline, whereas the total concentration of synthetical choline, including phosphorylcholine and glycerophosphorylcholine, stays unchanged.

### Applications

This study is useful to explain how apoptosis of liver cell occurs. It may also play an important role in guiding the clinical diagnosis and treatment of tumors.

### Peer review

This is an interesting study, where the advanced technique of <sup>1</sup>H-NMR to detect the effect of choline compounds is paralleled to TUNEL staining of liver tissues of rats injected with selenium.

## REFERENCES

- 1 Best PJ, Hasdai D, Sangiorgi G, Schwartz RS, Holmes DR Jr, Simari RD, Lerman A. Apoptosis. Basic concepts and

- implications in coronary artery disease. *Arterioscler Thromb Vasc Biol* 1999; **19**: 14-22
- 2 Fiedler N, Quant E, Fink L, Sun J, Schuster R, Gerlich WH, Schaefer S. Differential effects on apoptosis induction in hepatocyte lines by stable expression of hepatitis B virus X protein. *World J Gastroenterol* 2006; **12**: 4673-4682
- 3 Willingham MC. Cytochemical methods for the detection of apoptosis. *J Histochem Cytochem* 1999; **47**: 1101-1110
- 4 Neild GH, Foxall PJ, Lindon JC, Holmes EC, Nicholson JK. Uroscopy in the 21st century: high-field NMR spectroscopy. *Nephrol Dial Transplant* 1997; **12**: 404-417
- 5 Brown CE, Battocletti JH, Johnson LF. Nuclear magnetic resonance (NMR) in clinical pathology: current trends. *Clin Chem* 1984; **30**: 606-618
- 6 Shih CM, Ko WC, Yang LY, Lin CJ, Wu JS, Lo TY, Wang SH, Chen CT. Detection of apoptosis and necrosis in normal human lung cells using <sup>1</sup>H NMR spectroscopy. *Ann N Y Acad Sci* 2005; **1042**: 488-496
- 7 Griffin JL, Lehtimäki KK, Valonen PK, Grohn OH, Kettunen MI, Ylä-Herttuala S, Pitkanen A, Nicholson JK, Kauppinen RA. Assignment of <sup>1</sup>H nuclear magnetic resonance visible polyunsaturated fatty acids in BT4C gliomas undergoing ganciclovir-thymidine kinase gene therapy-induced programmed cell death. *Cancer Res* 2003; **63**: 3195-3201
- 8 Blankenberg FG, Katsikis PD, Storrs RW, Beaulieu C, Spielman D, Chen JY, Naumovski L, Tait JF. Quantitative analysis of apoptotic cell death using proton nuclear magnetic resonance spectroscopy. *Blood* 1997; **89**: 3778-3786
- 9 Blankenberg FG, Storrs RW, Naumovski L, Goralski T, Spielman D. Detection of apoptotic cell death by proton nuclear magnetic resonance spectroscopy. *Blood* 1996; **87**: 1951-1956
- 10 Lehtimäki KK, Valonen PK, Griffin JL, Vaisanen TH, Grohn OH, Kettunen MI, Vepsäläinen J, Ylä-Herttuala S, Nicholson J, Kauppinen RA. Metabolite changes in BT4C rat gliomas undergoing ganciclovir-thymidine kinase gene therapy-induced programmed cell death as studied by <sup>1</sup>H NMR spectroscopy *in vivo*, *ex vivo*, and *in vitro*. *J Biol Chem* 2003; **278**: 45915-45923
- 11 Serres S, Bezancon E, Franconi JM, Merle M. *Ex vivo* analysis of lactate and glucose metabolism in the rat brain under different states of depressed activity. *J Biol Chem* 2004; **279**: 47881-47889
- 12 Schrum LW, Bird MA, Salcher O, Burchardt ER, Grisham JW, Brenner DA, Rippe RA, Behrns KE. Autocrine expression of activated transforming growth factor-beta(1) induces apoptosis in normal rat liver. *Am J Physiol Gastrointest Liver Physiol* 2001; **280**: G139-G148
- 13 Sitter B, Sonnewald U, Spraul M, Fjosne HE, Gribbestad IS. High-resolution magic angle spinning MRS of breast cancer tissue. *NMR Biomed* 2002; **15**: 327-337
- 14 Martinez-Granados B, Monleon D, Martinez-Bisbal MC, Rodrigo JM, del Olmo J, Lluch P, Ferrandez A, Marti-Bonmati L, Celda B. Metabolite identification in human liver needle biopsies by high-resolution magic angle spinning <sup>1</sup>H NMR spectroscopy. *NMR Biomed* 2006; **19**: 90-100
- 15 Duarte IF, Stanley EG, Holmes E, Lindon JC, Gil AM, Tang H, Ferdinand R, McKee CG, Nicholson JK, Vilca-Melendez H, Heaton N, Murphy GM. Metabolic assessment of human liver transplants from biopsy samples at the donor and recipient stages using high-resolution magic angle spinning <sup>1</sup>H NMR spectroscopy. *Anal Chem* 2005; **77**: 5570-5578
- 16 Xiao YZ, Hui FW, Xiao JL, Feng KP, Jia ZN. NMR Studies on the Subacute Biochemical Effects of Aristolochic Acid on Rat Serum. *Chinese Chemical Letters* 2005; **16**: 1507-1510.
- 17 Yoon SO, Kim MM, Park SJ, Kim D, Chung J, Chung AS. Selenite suppresses hydrogen peroxide-induced cell apoptosis through inhibition of ASK1/JNK and activation of PI3-K/Akt pathways. *FASEB J* 2002; **16**: 111-113
- 18 Zhou N, Xiao H, Li TK, Nur-E-Kamal A, Liu LF. DNA damage-mediated apoptosis induced by selenium

- compounds. *J Biol Chem* 2003; **278**: 29532-29537
- 19 **Kim YS**, Jhon DY, Lee KY. Involvement of ROS and JNK1 in selenite-induced apoptosis in Chang liver cells. *Exp Mol Med* 2004; **36**: 157-164
  - 20 **Bollard ME**, Stanley EG, Lindon JC, Nicholson JK, Holmes E. NMR-based metabonomic approaches for evaluating physiological influences on biofluid composition. *NMR Biomed* 2005; **18**: 143-162
  - 21 **Zhong W**, Oberley TD. Redox-mediated effects of selenium on apoptosis and cell cycle in the LNCaP human prostate cancer cell line. *Cancer Res* 2001; **61**: 7071-7078
  - 22 **Tiwary AK**, Stegelmeier BL, Panter KE, James LF, Hall JO. Comparative toxicosis of sodium selenite and selenomethionine in lambs. *J Vet Diagn Invest* 2006; **18**: 61-70
  - 23 **Cheng LL**, Anthony DC, Comite AR, Black PM, Tzika AA, Gonzalez RG. Quantification of microheterogeneity in glioblastoma multiforme with ex vivo high-resolution magic-angle spinning (HRMAS) proton magnetic resonance spectroscopy. *Neuro Oncol* 2000; **2**: 87-95
  - 24 **Griffin JL**, Shockcor JP. Metabolic profiles of cancer cells. *Nat Rev Cancer* 2004; **4**: 551-561
  - 25 **Yen CL**, Mar MH, Meeker RB, Fernandes A, Zeisel SH. Choline deficiency induces apoptosis in primary cultures of fetal neurons. *FASEB J* 2001; **15**: 1704-1710
  - 26 **Martin K**. Concentrative accumulation of choline by human erythrocytes. *J Gen Physiol* 1968; **51**: 497-516
  - 27 **Ackerstaff E**, Pflug BR, Nelson JB, Bhujwalla ZM. Detection of increased choline compounds with proton nuclear magnetic resonance spectroscopy subsequent to malignant transformation of human prostatic epithelial cells. *Cancer Res* 2001; **61**: 3599-3603
  - 28 **Michel V**, Yuan Z, Ramsbair S, Bakovic M. Choline transport for phospholipid synthesis. *Exp Biol Med* (Maywood) 2006; **231**: 490-504
  - 29 **Hu H**, Jiang C, Schuster T, Li GX, Daniel PT, Lu J. Inorganic selenium sensitizes prostate cancer cells to TRAIL-induced apoptosis through superoxide/p53/Bax-mediated activation of mitochondrial pathway. *Mol Cancer Ther* 2006; **5**: 1873-1882
  - 30 **Luck DF**. Formation of mitochondria in neurospora crass. A Study Based on Mitochondrial Density Changes. *J Cell Biol* 1965; **24**: 461-470
  - 31 **Hakumaki JM**, Poptani H, Sandmair AM, Yla-Herttuala S, Kauppinen RA. <sup>1</sup>H MRS detects polyunsaturated fatty acid accumulation during gene therapy of glioma: implications for the in vivo detection of apoptosis. *Nat Med* 1999; **5**: 1323-1327
  - 32 **King AD**, Yeung DK, Ahuja AT, Leung SF, Tse GM, van Hasselt AC. In vivo proton MR spectroscopy of primary and nodal nasopharyngeal carcinoma. *AJNR Am J Neuroradiol* 2004; **25**: 484-490
  - 33 **Thomas EL**, Brynes AE, Hamilton G, Patel N, Spong A, Goldin RD, Frost G, Bell JD, Taylor-Robinson SD. Effect of nutritional counselling on hepatic, muscle and adipose tissue fat content and distribution in non-alcoholic fatty liver disease. *World J Gastroenterol* 2006; **12**: 5813-5819

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# 1,25-dihydroxyvitamin D<sub>3</sub> regulates LPS-induced cytokine production and reduces mortality in rats

Xiao-Ping Qi, Pei Li, Gang Li, Zhen Sun, Jie-Shou Li

Xiao-Ping Qi, Pei Li, Gang Li, Zheng Sun, Jie-Shou Li, School of Medicine, Nanjing University, Department of General Surgery, Jinling Hospital, 305 Zhongshandong Road, Nanjing 210002, Jiangsu Province, China  
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Author contributions: Qi XP designed the experiment and wrote the paper; Qi XP, Li P, Li G and Sun Z performed the experiment; Li P analysed the data; and Li JS revised the paper. Correspondence to: Xiao-Ping Qi, Department of General Surgery, Jinling Hospital, 305 Zhongshandong Road, Nanjing 210002, Jiangsu Province, China. [billc.cn@gmail.com](mailto:billc.cn@gmail.com)  
Telephone: +86-25-80860061 Fax: +86-25-84803956  
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## Abstract

**AIM:** To study the immunoregulatory effect of 1,25-dihydroxyvitamin-D<sub>3</sub> on dominant Th1 response in rats.

**METHODS:** Sixty adult Lewis rats were randomized into three groups. Rats in group 1 ( $n=25$ ) were treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> first and then challenged with LPS, rats in group 2 ( $n=25$ ) were treated with vehicle first and then challenged with LPS. Ten animals in groups 1 and 2 were preserved for mortality observation. The remaining animals were injected (i.p) with endotoxin, 24 h after the last administration of 1,25-(OH)<sub>2</sub>D<sub>3</sub> and vehicle. Rats in group 3 ( $n=10$ ) were treated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> only. Serum IL-12, IFN- $\gamma$ , IL-2 and IL-4 levels were measured and target gene of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on Th cells was studied after 6 h. Gene abundance was verified by real-time quantitative PCR.

**RESULTS:** No death occurred in rats pretreated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> after LPS injection. Death occurred 9 h after LPS injection in rats pretreated with the vehicle, and the number of deaths was 5 within 24 h, with a mortality rate of 50%. There was no change in the number of deaths within 96 h. Six hours after endotoxin stimulation, serum IL-12 and IFN- $\gamma$  levels decreased significantly in rats pretreated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> as compared with those in rats pretreated with the vehicle. The serum content of these two cytokines was very low in rats not challenged by endotoxin, and there was a significant difference as compared with the previous two groups.

**CONCLUSION:** 1,25-(OH)<sub>2</sub>D<sub>3</sub> attenuates injury

induced by the lethal dose of LPS, regulates Th1 and Th2 cells at the transcription level, and dominantly responds to cytokine production in rats.

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**Key words:** Endotoxin; Cytokine; 1,25-dihydroxyvitamin-D<sub>3</sub>; Immunoregulation; Mortality

**Peer reviewer:** Michael Kremer, MD, Skipper Bowles Center for Alcohol Studies, CB#7178, 3011 Thurston-Bowles Building, University of North Carolina, Chapel Hill, NC27599, United States

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## INTRODUCTION

1,25-dihydroxyvitamin D<sub>3</sub> [1,25-(OH)<sub>2</sub>D<sub>3</sub>] is an active form of vitamin D, which not only regulates the dynamic balance of calcium and phosphorus metabolism but also participates in differentiation and regulation of the immune system<sup>[1,2]</sup>. *In vitro* study<sup>[3]</sup> showed that both antigen-presenting cells (APCs) and activated lymphocytes express vitamin D receptor (VDR), and that 1,25-(OH)<sub>2</sub>D<sub>3</sub> acts on APCs (mainly dendritic cells) and helper T cells (Th) through VDR mediation<sup>[4]</sup>, inhibits proliferation and differentiation of Th1 and cytokine production, and induces differentiation of Th2. The status of Th1/Th2 differentiation determines the type of immune response and the final outcome of body response<sup>[5]</sup>. Cytokine environment is the key factor for initiating Th1/Th2 differentiation<sup>[6,7]</sup>.

Th1 immune response is not only associated with a variety of acute inflammatory responses but also plays a leading role in the development and progression of many autoimmune diseases and transplantation rejection<sup>[1,8-12]</sup>. Few *in vivo* studies reporting the influence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on Th1 immune response are available, and the experimental results about cytokine regulation are conflicting or completely different<sup>[5,13-17]</sup>. The target gene in Th cells

remains almost unknown<sup>[10,17]</sup>. *E.coli* endotoxin is a potent bacterial mitogen, able to promote maturity of immature dendritic cells (DC), directly activates T cells and induces Th1 immune response<sup>[18]</sup>. We established a Th1 dominant response animal model and pre-treated it with 1,25-(OH)<sub>2</sub>D<sub>3</sub>. The results of our study showed that 1,25-(OH)<sub>2</sub>D<sub>3</sub> was able to regulate the production of IL-12, IFN- $\gamma$  and IL-4 in dendritic, Th1 and Th2 cells. The effector target point of regulation was at the gene transcription level. It is the regulation of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on T cell polarization that attenuates injury induced by the lethal dose of LPS in rats and significantly reduces the mortality of rats.

## MATERIALS AND METHODS

### Animals

Inbred line Lewis rats (at the age of 3.5-4.5 mo, weighing 242  $\pm$  14 g) were provided by Experimental Animal Center of the Chinese Academy of Medical Sciences (Beijing, China) and fed with normal chow containing 1.6% calcium, 0.9% phosphorus and 0.3% vitamin D (Nanjing Animal Technology Co., Ltd, Nanjing, China) with free access to water. The experiment protocol followed the institutional regulations of the Ministry of Health of the People's Republic of China concerning animal experimentation.

### Experiment protocol

Sixty rats were randomized into three groups. Rats in group 1 ( $n = 25$ ) as the study group, were administered 1,25-(OH)<sub>2</sub>D<sub>3</sub> by gavage (GmbHcd&Go, Swiss) at 1  $\mu$ g/animal for 14 d<sup>[19]</sup>, rats in group 2 ( $n = 25$ ) as the positive control group were administered the same dose of the vehicle for 14 d by gavage. Animals in groups 1 and 2 were injected intraperitoneally with *E.coli* 0111, B4 (Sigma, USA). Rats in group 3 ( $n = 10$ ) as the negative control group were administered 1,25-(OH)<sub>2</sub>D<sub>3</sub> only by gavage at the dose of 1  $\mu$ g/animal for 14 d, and injected (i.p) with the same volume of normal saline (Sigma Chemical CO., St Louis, MO, USA).

Ten animals in groups 1 and 2 were preserved for mortality observation. The remaining animals were injected (i.p) with endotoxin (10 mg/kg), 24 h after the last administration of 1,25-(OH)<sub>2</sub>D<sub>3</sub> and vehicle. Six hours after the injection, they were anesthetized with 50 mg/kg (i.p) pentobarbital (Sigma-Aldrich, USA) and used for drawing 5mL blood from the abdominal major artery. The blood was centrifuged at 4°C for 15 min, and the serum was stored at -80°C for test. The spleen was removed aseptically, washed with PBS and stored in liquid nitrogen.

### Enzyme-linked immunosorbent assay (ELISA)

Serum IL-12, IL-2, IFN- $\gamma$  and IL-4 levels were measured with commercially available ELISA kits (Biosource CO., Camarillo, CA, USA) according to the manufacturer's instructions, and the quality control serum values were calculated.

### Ca<sup>2+</sup>/NF-AT signaling pathway gene array

Three spleen tissue samples were chosen randomly from rats in groups 1 and 2 for RNA extraction. UV absorption precipitation method and denaturing gel electrophoresis were used to test the quantity, quality and completion of RNA. The probe was synthesized by RT-PCR. Five  $\mu$ g RNA was used to prepare annealing solution and mixed with RT solution to undergo reverse transcription reaction under the action of reverse transcriptase (M1701, Promega, USA).

Chip hybridization was conducted by using Ca<sup>2+</sup>/NF-AT signaling pathway gene array chip (Super Array Bioscience CO., Cat.NO.HS-022 USA) and chemiluminescent assay kit (Super Array Bioscience CO., NO.D-01) according to the manufacturer's instructions. The chip was scanned with the ArtixScan 120tf scanner (Micro TEK CO., USA) and the original data were analyzed using the attached software GEArray analyzer. Each chip had 10 positive controls (2 for GAPDH, 4 for Ppia, 2 for RP113 and 2 for Actinb), three negative controls (PUC18DNA) and 3 blank controls. The original data were deduced by the background minimum value and then corrected by the content of home-keeping gene. The corrected data were analyzed for abundance of gene transcription between the two groups. The ratio  $\geq 2$  was considered up-regulation of the gene and  $\leq 0.5$  down-regulation<sup>[20]</sup>.

### Verification of IL-2 gene expression by RT-PCR

RNA extraction was done as previously described. The sample was RNA reverse transcribed to synthesized cDNA. The target gene and home-keeping gene of the sample were reacted by RT-PCR. A standard curve was plotted by measurement of the standard sample gradient to calculate the content of gene in the sample, which was corrected by the content of home-keeping gene to obtain the content of the related gene. All reagents used in the experiment were provided by Promega CO., USA. The sequences of  $\beta$ -actin (211 bp) and IL-2 (190 bp) are 5'-CCTGTACGCCAACACAGTGC-3' and 5'-ATACTCC TGCTTGCTGATCC-3', and 5'-CACTGACGCTTGTC CTCCTT-3' and 5'-TTCAATTCTGTGGCCTGCTT-3', respectively.

### Statistical analysis

Data were represented as mean  $\pm$  SD. SPSS 10.0 was used to perform *t*-test and *F*-test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Mortality of rats after LPS injection and protective effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub>

No death occurred in rats pretreated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> after LPS injection. Death occurred 9 h after LPS injection in rats pretreated with the vehicle, and the number of deaths was 5 within 24 h, with a mortality rate of 50%. There was no change in the number of deaths within 96 h.

**Table 1** 1,25-(OH)<sub>2</sub>D<sub>3</sub>-regulated LPS-induced cytokine production in rats (pg/mL, mean ± SD)

	1,25-(OH) <sub>2</sub> D <sub>3</sub> + LPS (n = 15)	Vehicle + LPS (n = 15)	1,25-(OH) <sub>2</sub> D <sub>3</sub> (n = 10)
IL-12	3986 ± 328 <sup>a</sup>	4160 ± 289	69.99 ± 3.99 <sup>b</sup>
IFN-γ	4840 ± 802 <sup>a</sup>	5264 ± 524	5.42 ± 0.12 <sup>b</sup>
IL-4	5.57 ± 1.75 <sup>a</sup>	3.72 ± 1.60	

<sup>a</sup>P < 0.05 *vs* vehicle + LPS; <sup>b</sup>P < 0.01 *vs* 1,25-(OH)<sub>2</sub>D<sub>3</sub> + LPS and vehicle + LPS.

### 1,25-(OH)<sub>2</sub>D<sub>3</sub> inhibited LPS-stimulated production of IL-12 and IFN-γ in rats

Six hours after endotoxin stimulation, serum IL-12 and IFN-γ levels decreased significantly in rats pretreated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> as compared with those in rats pretreated with the vehicle. The serum level of these two cytokines was very low in rats not challenged by endotoxin, and there was a significant difference as compared with the previous two groups. As the serum IL-2 was below the limit of measurement in most rats 3 and 6 h after LPS attack, measurement was not done.

### 1,25-(OH)<sub>2</sub>D<sub>3</sub> promoted IL-4 production in LPS-challenged rats

Six hours after endotoxin stimulation, serum IL-4 level elevated significantly in rats pretreated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> as compared with that in rats pre-treated with the vehicle. As the serum IL-4 was below the limit of measurement in most rats that are not attacked by LPS, measurement was not done (Table 1).

### Quality control of RNA extraction

Electrophoresis showed that RNA extracted from the rat spleen displayed two clear bands (18S and 28S), and the absorbance at 260 nm and 280 nm was between 1.8 and 2.0, indicating that no RNA degradation occurred and the extract outcome was good.

### 1,25-(OH)<sub>2</sub>D<sub>3</sub> regulated expression of Th1 and Th2 cytokines and related transcription factors

The gene chip used in the present experiment contains 95 target genes and other positive and negative controls. Expression difference was found in 39 genes between groups 1 and 2, accounting for 41% of the total number of the chip genes. These 39 genes include 10 up-regulated genes and 29 down-regulated genes (Table 2). The chip results showed that 1,25-(OH)<sub>2</sub>D<sub>3</sub> down-regulated gene expression of Th1 and up-regulated gene expression of Th2, and the gene expression level in related transcription factors (Table 3).

### Verification of down-regulation of IL-2 gene expression by RT-PCR

The results of the experiment showed that gene expression level in rats pretreated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> was significantly lower than that in rats pre-treated with the vehicle (0.476 ± 0.023 *vs* 0.678 ± 0.038, P < 0.01).

## DISCUSSION

The purpose of the present experiment was to clarify the immune regulatory effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on Th1 dominant response *in vivo*. The results showed that 1,25-(OH)<sub>2</sub>D<sub>3</sub> inhibited IFN-γ production of IL-12 and Th1 cytokines, suggesting that this inhibitory effect occurs at the transcription level. What implies in the results of the present experiment is the therapeutic effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on diseases mainly characterized by Th1 immune response (including autoimmune diseases) and transplantation rejection<sup>[16]</sup>. At the same time, as 1,25-(OH)<sub>2</sub>D<sub>3</sub> affects the secretary profile of Th1 and Th2 cytokines<sup>[21,22]</sup>, it inhibited the acute inflammatory reaction in the rats of group 1, indicating that 1,25-(OH)<sub>2</sub>D<sub>3</sub> attenuates LPS lethal dose-induced injury in rats. The fact that all rats survived in group 1 suggests that 1,25-(OH)<sub>2</sub>D<sub>3</sub> may also play a role in inhibiting the development and progression of acute inflammatory reaction.

IL-12 is a cytokine secreted by APCs and plays a central role in the growth of Th1 cells<sup>[23]</sup>. IL-12 has a potent biological function of inducing T cells to secrete IFN-γ<sup>[24]</sup>. IFN-γ is a pleiotropic cytokine, promoting inflammatory reaction and inducing expression of main tissue surface compatible complex of multiple cells<sup>[25]</sup>. Most recent studies found that this cytokine promotes vascular disease of the transplanted organ at the late stage of transplantation<sup>[26]</sup>. The present experiment confirmed that 1,25-(OH)<sub>2</sub>D<sub>3</sub> could inhibited IL-12 production in rats, suggesting that it is able to inhibit strong Th1 immune response *via* its action on APCs, thus reducing IFN-γ production. At the same time, 1,25-(OH)<sub>2</sub>D<sub>3</sub> may also directly inhibit the differentiation and proliferation of Th1 cells, as the cytokines mainly secreted by Th1 cells are reduced, especially transcription of NF-κB is inhibited. NF-κB is a key mediator of gene expression in immune and inflammatory responses. We also found that 1,25-(OH)<sub>2</sub>D<sub>3</sub> inhibited proliferation of splenic lymphocytes in rats challenged with LPS. We, therefore, think that the results of the above experiment suggest that differentiation and proliferation of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on Th1 may also have an inhibitory effect on proliferation of splenic lymphocytes and is able to selectively inhibit Th1 immune response.

IL-4 is a main factor influencing the development of T cells into Th2 cells<sup>[13,27]</sup>. Once IL-4 level is able to resist activation of IL-12 on Th cells and IFN-γ on IL-4, it promotes differentiation of juvenile T cells to Th2 cells<sup>[28]</sup>. It is controversial over the regulatory effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on IL-4. It was reported that the effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub> is mediated through IL-4<sup>[5]</sup>, and that it is the up-regulation of IL-4 and TGF-β by 1,25-(OH)<sub>2</sub>D<sub>3</sub> that inhibits the inflammatory reaction rather than by the reduction of Th1 cytokines IFN-γ and TNF-α<sup>[29]</sup>. It was also reported that 1,25-(OH)<sub>2</sub>D<sub>3</sub> has no influence on the production of IL-4, or down-regulates IL-4<sup>[16,17]</sup>. We detected serum IL-4 levels in four batches of rats pretreated with 1,25-(OH)<sub>2</sub>D<sub>3</sub> and

Table 2 Genes down-regulated by 1,25-(OH)<sub>2</sub>D<sub>3</sub> in rat spleens

GenBank	Description	Gene name	Gene expression	Abundance	(Exp/vehicle)
NM007595	Calcium/calmodulin-dependent protein kinase II, beta	CamK II	0.00E + 00	0.00E + 00	0.00E + 00
NM009793	Calcium/calmodulin-dependent protein kinase IV	CamK IV	0.00E + 00	0.00E + 00	0.00E + 00
NM009843	Cytotoxic T-lymphocyte-associated protein 4	Cd152	9.20E - 02	0.00E + 00	0.00E + 00
NM031162	CD <sub>3</sub> antigen, zeta polypeptide	CD3Z antigen	5.00E - 02	0.00E + 00	0.00E + 00
NM007726	Cannabinoid receptor 1	cb1	0.00E + 00	3.12E - 02	0.00E + 00
NM009969	Colony stimulating factor	GM-CSF	1.88E - 01	0.00E + 00	1.38E - 01
	Nuclear factor of activated				
NM022413	Epithelial calcium channel 2	Ecac 2	0.00E + 00	0.00E + 00	N/A
NM010118	Early growth response 2	Krox-20	2.99E - 02	0.00E + 00	N/A
NM010184	Fc receptor, IgE, high affinity 1, alpha polypeptide	Fcε1a, Fcr-5	1.36E - 01	0.00E + 00	0.00E + 00
NM016863	FK506 binding protein 1b	FKBP 1B/FKBP	0.00E + 00	0.00E + 00	0.00E + 00
NM019827	Glycogen synthase kinase3 beta	Gsk-3	2.31E - 01	4.74E - 02	1.05E + 00
NM008284	Sarcoma virus oncogene 1	H-ras	0.00E + 00	0.00E + 00	1.03E - 02
NM008337	Interferon gamma	IFN-γ	3.44E - 01	1.24E - 02	1.02E + 00
NM008366	Interleukin 2	IL-2	3.98E - 01	2.79E - 01	2.26E - 01
NM008367	Interleukin 2 receptor, alpha chain	CD25	1.99E - 01	3.47E - 01	2.11E - 01
NM010591	Jun oncogene	c-JUN	0.00E + 00	0.00E + 00	0.00E + 00
NM019686	Kinase interacting protein 2	KIP 2	1.84E - 01	0.00E + 00	8.28E - 03
NM007746	Mitogen activated protein kinase 8	Cot	1.50E - 02	0.00E + 00	0.00E + 00
NM011951	Mitogen activated protein Mus musculus Harvey rat	P38MAPK	3.65E - 03	0.00E + 00	1.88E - 02
NM016700	Mitogen activated protein	JNK1	4.21E - 01	4.87E - 01	3.59E - 01
NM008656	Myogenic factor 5	Myf 5	1.83E - 02	0.00E + 00	6.37E - 01
NM016791	Nuclear factor of activated T-cell, cytoplasmic 1	NF-ATc	3.59E - 01	0.00E + 00	1.38E - 01
NM008915	Protein phosphatase 3, catalytic subunit, gamma isoform	Calcineurin A gamma	6.12E - 02	1.06E - 02	3.54E - 01
NM013693	Colony stimulating factor	GM-CSF	1.88E - 01	0.00E + 00	0.00E + 00
NM010188	Fc receptor, IgG, Low affinity III	CD16	6.62E - 01	5.25E - 03	1.24E - 01
NM24684	Fos-like antigen 2	fra-2	5.59E - 01	0.00E + 00	0.00E + 00
NM41840	Protein phosphatase 3, regulatory subunit B, alpha isoform	Calcineurin B	2.23E + 00	4.01E - 01	4.73E - 01
NM010177	Tumor necrosis factor (ligand) superfamily, member 6	FasL	1.02E + 00	0.00E + 00	1.88E - 01
NM019408	Nuclear factor of kappa light polypeptide gene enhancer in B-cell	NF-κB	4.58E - 01	3.50E - 01	4.25E - 01

N/A: Gene expression level, 1,25-(OH)<sub>2</sub>D<sub>3</sub> = 0 and vehicle ≥ 2.

Table 3 Genes up-regulated by 1,25-(OH)<sub>2</sub>D<sub>3</sub> in rat spleens

GenBank	Description	Gene name	Gene expression	Abundance	(Exp/vehicle)
NM010548	Interleukin 10	IL-10	2.30E+00	8.25E-01	1.69E+00
NM010899	Nuclear factor of activated T-cell, cytoplasmic 2	NFAT1 (NFATP)	N/A	N/A	N/A
NM009192	Src-like adaptor	SLA	2.57E + 00	4.21E + 01	8.97E - 01
NM013672	Trans-acting transcription factor 1	Sp1	2.03E + 00	1.49E + 01	4.87E - 01
NM009505	Vascular endothelial growth factor A	VEGF/ VEGI	2.15E + 00	N/A	N/A
NM010234	FBJ osteosarcoma oncogene	c-fos	N/A	N/A	N/A
NM010510	Interferon beta, fibroblast	IFNb-1	N/A	N/A	N/A
NM010583	Mus musculus IL 2-inducible T-cell kinase	Tsk	6.77E - 01	N/A	5.29E + 00
NM021283	Interleukin 4	IL-4	N/A	N/A	N/A
NM010558	Interleukin 5	IL-5	N/A	N/A	N/A

N/A: Gene expression level, 1,25-(OH)<sub>2</sub>D<sub>3</sub> ≥ 2 and vehicle = 0.

those pretreated with the vehicle. Although we used inbred line Lewis rats with little individual variance in establishing the model, we still found a significant individual difference in serum IL-4 level of the same experiment group, where the IL-4 level was lower than the test baseline in some rats. Only when we expanded the sample capacity, were the statistically significant results obtained. The results of gene chip test also showed that there was a great difference in IL-4 expression level between the rats 6 h after LPS stimulation. Only in one of the three rats in the study group, was IL-4 mRNA expression up-regulated by more than two times. However, as the capacity of the samples tested by gene chips was relatively small, and

as there was still a tendency to up-regulate the gene expression of IL-4, IL-5 and IL-10 mainly secreted by Th2 cells, we performed another experiment, which confirmed again that 1,25-(OH)<sub>2</sub>D<sub>3</sub> was able to up-regulate serum IL-10 level in rats challenged with LPS suggesting that 1,25-(OH)<sub>2</sub>D<sub>3</sub> is able to promote the production of Th2 type cytokines<sup>[5,30]</sup>, and at the same time inhibit the extent and progression of Th1 type immune response, forming the so-called “immune deviation” phenomenon<sup>[16]</sup>, which is believed to help establish peripheral tolerance and is of significance in inhibiting transplantation rejection<sup>[4,31]</sup>.

IL-12 is an allodiploid consisting of two subunits (P<sub>35</sub> and P<sub>40</sub>) encoded by two genes independently<sup>[32]</sup>.



It is known that the P<sub>40</sub> gene initiator region contains a NF- $\kappa$ B combining site<sup>[33]</sup>. The finding in the present experiment that 1,25-(OH)<sub>2</sub>D<sub>3</sub> down-regulated the important transcription factor NF- $\kappa$ B, suggests that 1,25-(OH)<sub>2</sub>D<sub>3</sub> reduces the expression of IL-12P40 subunit by inhibiting NF- $\kappa$ B, thus down-regulating assembly and secretion of IL-12 protein<sup>[33]</sup>. After activation of T cells, VDR is induced within 6 h, where IL-2 is the first expression-producing gene<sup>[34]</sup>, and 1,25-(OH)<sub>2</sub>D<sub>3</sub> inhibits the expression of IL-2 and IFN- $\gamma$  mRNA, reaching the peak in 6-12 h<sup>[35]</sup>. It has been recognized that NF- $\kappa$ B and NF-ATp/c are specific transcription factors on IL-2 initiators<sup>[36,37]</sup>. It is also known that IFN- $\gamma$ , GM-CSF, TNF- $\alpha$ , IL-4 and IL-5 initiators contain NF-AT element<sup>[34]</sup> and IL-4 enhancer contains 5 independent NF-AT sites, of which NF-ATp is a combining site of high affinity<sup>[34,38]</sup>. In the present experiment, increased IL-4 secretion by 1,25-(OH)<sub>2</sub>D<sub>3</sub> might be related to up-regulation of NF-ATp. Although we were unable to identify the respective action of individual members of the NF-AT family on the expression of the cytokines in this study, we may still draw the conclusion that 1,25-(OH)<sub>2</sub>D<sub>3</sub> influences the activity of NF- $\kappa$ B and NF-AT, two important transcription factors associated with cytokine regulation, by up-regulating NF-ATp gene expression and down-regulating NF-ATc gene expression. Both MAPK P38 and TNF pathways are mitogen-activated protein kinase pathways, not only closely associated with inflammatory reaction but also with cell growth, differentiation and apoptosis. 1,25-(OH)<sub>2</sub>D<sub>3</sub> down regulates gene transcription of these important regulatory proteins in the MAPK pathways, suggesting that its influence on T help cell differentiation is the result of regulation on multiple signal pathways, and that the effector target of 1,25-(OH)<sub>2</sub>D<sub>3</sub> regulating cytokines is at the gene transcription level.

## COMMENTS

### Background

1,25-dihydroxyvitamin D<sub>3</sub> (1,25-(OH)<sub>2</sub>D<sub>3</sub>) has many effects on the production of cytokines, the gene expression maps of related cytokines and the mortality of rats. 1,25-(OH)<sub>2</sub>D<sub>3</sub>, the activated form of vitamin D, has, in addition to its central function in calcium and bone metabolism, important effects on the growth and differentiation of many cell types, and pronounced immunoregulatory properties. In the present study, the immunoregulatory effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in dominant Th1 response rats was investigated.

### Research frontiers

Th1-biased immune response is associated with acute inflammatory conditions, and also plays a major role in a variety of human autoimmune diseases and graft rejection. However, few studies on the selective immunosuppression *in vivo* of 1,25-(OH)<sub>2</sub>D<sub>3</sub> are available and the effect of 1,25-(OH)<sub>2</sub>D<sub>3</sub> on gene expression profiles in Th cells is still unclear.

### Innovations and breakthroughs

This is the first study to address the immunoregulatory effects of 1,25-(OH)<sub>2</sub>D<sub>3</sub> in a dominant Th1 response model. The results show that 1,25-(OH)<sub>2</sub>D<sub>3</sub> could regulate Th1-derived and Th2-derived cytokine production and protect rats from attacking of the LPS lethal dose.

### Applications

The immunoregulatory properties of 1,25-(OH)<sub>2</sub>D<sub>3</sub> were explored clinically for

the topical treatment of psoriasis, a Th1 cell-mediated autoimmune disease of the skin. Our findings suggest that 1,25-(OH)<sub>2</sub>D<sub>3</sub> may play an important role in Th1-inflammatory, autoimmune diseases and graft transplantation rejection.

### Peer review

The manuscript "1,25-(OH)<sub>2</sub>D<sub>3</sub> regulates LPS-induced cytokine production and reduces mortality in rats" by Qi XP *et al.* presents experimental data from rats. The authors claim by pretreating rats with 1,25-(OH)<sub>2</sub>D<sub>3</sub> that the LPS response is shifted towards a Th2-associated cytokine response with reduced Th1-associated cytokine response, so ensuring increased survival. The topic is of high interest.

## REFERENCES

- 1 Deluca HF, Cantorna MT. Vitamin D: its role and uses in immunology. *FASEB J* 2001; **15**: 2579-2585
- 2 Mathieu C, Adorini L. The coming of age of 1,25-dihydroxyvitamin D(3) analogs as immunomodulatory agents. *Trends Mol Med* 2002; **8**: 174-179
- 3 Marcinkowska E. A run for a membrane vitamin D receptor. *Biol Signals Recept* 2001; **10**: 341-349
- 4 Adorini L, Giarratana N, Penna G. Pharmacological induction of tolerogenic dendritic cells and regulatory T cells. *Semin Immunol* 2004; **16**: 127-134
- 5 Boonstra A, Barrat FJ, Crain C, Heath VL, Savelkoul HF, O'Garra A. 1 $\alpha$ ,25-Dihydroxyvitamin D<sub>3</sub> has a direct effect on naive CD4(+) T cells to enhance the development of Th2 cells. *J Immunol* 2001; **167**: 4974-4980
- 6 O'Garra A. Cytokines induce the development of functionally heterogeneous T helper cell subsets. *Immunity* 1998; **8**: 275-283
- 7 Pichler J, Gerstmayr M, Szepefalusi Z, Urbanek R, Peterlik M, Willheim M. 1  $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> inhibits not only Th1 but also Th2 differentiation in human cord blood T cells. *Pediatr Res* 2002; **52**: 12-18
- 8 Mattner F, Smirolto S, Galbiati F, Muller M, Di Lucia P, Poliani PL, Martino G, Panina-Bordignon P, Adorini L. Inhibition of Th1 development and treatment of chronic-relapsing experimental allergic encephalomyelitis by a non-hypercalcemic analogue of 1,25-dihydroxyvitamin D(3). *Eur J Immunol* 2000; **30**: 498-508
- 9 Cantorna MT, Hayes CE, DeLuca HF. 1,25-Dihydroxycholecalciferol inhibits the progression of arthritis in murine models of human arthritis. *J Nutr* 1998; **128**: 68-72
- 10 Cantorna MT, Munsick C, Bemiss C, Mahon BD. 1,25-Dihydroxycholecalciferol prevents and ameliorates symptoms of experimental murine inflammatory bowel disease. *J Nutr* 2000; **130**: 2648-2652
- 11 Pani MA, Knapp M, Donner H, Braun J, Baur MP, Usadel KH, Badenhop K. Vitamin D receptor allele combinations influence genetic susceptibility to type 1 diabetes in Germans. *Diabetes* 2000; **49**: 504-507
- 12 Stio M, Bonanomi AG, d'Albasio G, Treves C. Suppressive effect of 1,25-dihydroxyvitamin D<sub>3</sub> and its analogues EB 1089 and KH 1060 on T lymphocyte proliferation in active ulcerative colitis. *Biochem Pharmacol* 2001; **61**: 365-371
- 13 Staeva-Vieira TP, Freedman LP. 1,25-dihydroxyvitamin D<sub>3</sub> inhibits IFN- $\gamma$  and IL-4 levels during *in vitro* polarization of primary murine CD4+ T cells. *J Immunol* 2002; **168**: 1181-1189
- 14 Lemire JM, Archer DC, Beck L, Spiegelberg HL. Immunosuppressive actions of 1,25-dihydroxyvitamin D<sub>3</sub>: preferential inhibition of Th1 functions. *J Nutr* 1995; **125**: 1704S-1708S
- 15 Schulze-Koops H, Davis LS, Haverty TP, Wacholtz MC, Lipsky PE. Reduction of Th1 cell activity in the peripheral circulation of patients with rheumatoid arthritis after treatment with a non-depleting humanized monoclonal antibody to CD4. *J Rheumatol* 1998; **25**: 2065-2076
- 16 Adorini L. Immunomodulatory effects of vitamin D receptor ligands in autoimmune diseases. *Int Immunopharmacol* 2002; **2**: 1017-1028
- 17 Cantorna MT, Woodward WD, Hayes CE, DeLuca HF.

- 1,25-dihydroxyvitamin D3 is a positive regulator for the two anti-encephalitogenic cytokines TGF-beta 1 and IL-4. *J Immunol* 1998; **160**: 5314-5319
- 18 **Pulendran B**, Kumar P, Cutler CW, Mohamadzahe M, Van Dyke T, Banchereau J. Lipopolysaccharides from distinct pathogens induce different classes of immune responses in vivo. *J Immunol* 2001; **167**: 5067-5076
- 19 **Aschenbrenner JK**, Sollinger HW, Becker BN, Hullett DA. 1,25-(OH(2))D(3) alters the transforming growth factor beta signaling pathway in renal tissue. *J Surg Res* 2001; **100**: 171-175
- 20 **Mahon BD**, Wittke A, Weaver V, Cantorna MT. The targets of vitamin D depend on the differentiation and activation status of CD4 positive T cells. *J Cell Biochem* 2003; **89**: 922-932
- 21 **Nashold FE**, Hoag KA, Goverman J, Hayes CE. Rag-1-dependent cells are necessary for 1,25-dihydroxyvitamin D(3) prevention of experimental autoimmune encephalomyelitis. *J Neuroimmunol* 2001; **119**: 16-29
- 22 **Imazeki I**, Matsuzaki J, Tsuji K, Nishimura T. Immunomodulating effect of vitamin D3 derivatives on type-1 cellular immunity. *Biomed Res* 2006; **27**: 1-9
- 23 **Thierfelder WE**, van Deursen JM, Yamamoto K, Tripp RA, Sarawar SR, Carson RT, Sangster MY, Vignali DA, Doherty PC, Grosveld GC, Ihle JN. Requirement for Stat4 in interleukin-12-mediated responses of natural killer and T cells. *Nature* 1996; **382**: 171-174
- 24 **Muthian G**, Raikwar HP, Rajasingh J, Bright JJ. 1,25 Dihydroxyvitamin-D3 modulates JAK-STAT pathway in IL-12/IFN-gamma axis leading to Th1 response in experimental allergic encephalomyelitis. *J Neurosci Res* 2006; **83**: 1299-1309
- 25 **Hidalgo LG**, Halloran PF. Role of IFN-gamma in allograft rejection. *Crit Rev Immunol* 2002; **22**: 317-349
- 26 **Halloran PF**, Miller LW, Urmson J, Ramassar V, Zhu LF, Kneteman NM, Solez K, Afrouzian M. IFN-gamma alters the pathology of graft rejection: protection from early necrosis. *J Immunol* 2001; **166**: 7072-7081
- 27 **Wurster AL**, Tanaka T, Grusby MJ. The biology of Stat4 and Stat6. *Oncogene* 2000; **19**: 2577-2584
- 28 **Skapenko A**, Niedobitek GU, Kalden JR, Lipsky PE, Schulze-Koops H. Generation and regulation of human Th1-biased immune responses in vivo: a critical role for IL-4 and IL-10. *J Immunol* 2004; **172**: 6427-6434
- 29 **Hayes CE**. Vitamin D: a natural inhibitor of multiple sclerosis. *Proc Nutr Soc* 2000; **59**: 531-535
- 30 **Holick MF**. High prevalence of vitamin D inadequacy and implications for health. *Mayo Clin Proc* 2006; **81**: 353-373
- 31 **Adorini L**. 1,25-Dihydroxyvitamin D3 analogs as potential therapies in transplantation. *Curr Opin Investig Drugs* 2002; **3**: 1458-1463
- 32 **Yoshimoto T**, Kojima K, Funakoshi T, Endo Y, Fujita T, Nariuchi H. Molecular cloning and characterization of murine IL-12 genes. *J Immunol* 1996; **156**: 1082-1088
- 33 **D'Ambrosio D**, Cippitelli M, Cocciolo MG, Mazzeo D, Di Lucia P, Lang R, Sinigaglia F, Panina-Bordignon P. Inhibition of IL-12 production by 1,25-dihydroxyvitamin D3. Involvement of NF-kappaB downregulation in transcriptional repression of the p40 gene. *J Clin Invest* 1998; **101**: 252-262
- 34 **Alroy I**, Towers TL, Freedman LP. Transcriptional repression of the interleukin-2 gene by vitamin D3: direct inhibition of NFATp/AP-1 complex formation by a nuclear hormone receptor. *Mol Cell Biol* 1995; **15**: 5789-5799
- 35 **Rigby WF**, Denome S, Fanger MW. Regulation of lymphokine production and human T lymphocyte activation by 1,25-dihydroxyvitamin D3. Specific inhibition at the level of messenger RNA. *J Clin Invest* 1987; **79**: 1659-1664
- 36 **Takeuchi A**, Reddy GS, Kobayashi T, Okano T, Park J, Sharma S. Nuclear factor of activated T cells (NFAT) as a molecular target for 1alpha,25-dihydroxyvitamin D3-mediated effects. *J Immunol* 1998; **160**: 209-218
- 37 **Macian F**. NFAT proteins: key regulators of T-cell development and function. *Nat Rev Immunol* 2005; **5**: 472-484
- 38 **Monticelli S**, Rao A. NFAT1 and NFAT2 are positive regulators of IL-4 gene transcription. *Eur J Immunol* 2002; **32**: 2971-2978

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## Isolation and biological analysis of tumor stem cells from pancreatic adenocarcinoma

Peng Huang, Chun-You Wang, Shan-Miao Gou, He-Shui Wu, Tao Liu, Jiang-Xin Xiong

Peng Huang, Chun-You Wang, Shan-Miao Gou, He-Shui Wu, Tao Liu, Jiang-Xin Xiong, Department of Pancreatic Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China

**Author contributions:** Huang P and Wang CY contributed equally to this work; Huang P, Wang CY, Gou SM, Wu HS, Liu T and Xiong JX designed the research; Huang P and Gou SM performed the research; Wang CY provided new reagents/analytic tools; Huang P analyzed data; and Huang P and Wang CY wrote the paper.

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**Correspondence to:** Chun-You Wang, Department of Pancreatic Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, Hubei Province, China. [hpeng2003@sina.com](mailto:hpeng2003@sina.com)

Telephone: +86-27-65063409 Fax: +86-27-65063409

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### Abstract

**AIM:** To explore the method of isolation and biological analysis of tumor stem cells from pancreatic adenocarcinoma cell line PANC-1.

**METHODS:** The PANC-1 cells were cultured in Dulbecco modified eagle medium F12 (1:1 volume) (DMEM-F12) supplemented with 20% fetal bovine serum (FBS). Subpopulation cells with properties of tumor stem cells were isolated from pancreatic adenocarcinoma cell line PANC-1 according to the cell surface markers CD44 and CD24 by flow cytometry. The proliferative capability of these cells *in vitro* were estimated by 3-[4,5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT) method. And the tumor growth of different subpopulation cells which were injected into the hypodermis of right and left armpit of nude mice was studied, and expression of CD44 and CD24 of the CD44<sup>+</sup>CD24<sup>+</sup> cell-formed nodules and PANC-1 cells were detected by avidin-biotin-peroxidase complex (ABC) immunohistochemical staining.

**RESULTS:** The 5.1%-17.5% of sorted PANC-1 cells expressed the cell surface marker CD44, 57.8% -70.1% expressed CD24, only 2.1%-3.5% of cells were CD44<sup>+</sup>CD24<sup>+</sup>. Compared with CD44<sup>-</sup>CD24<sup>-</sup> cells, CD44<sup>+</sup>CD24<sup>+</sup> cells had a lower growth rate *in vitro*. Implantation of 10<sup>4</sup> CD44<sup>-</sup>CD24<sup>-</sup> cells in nude mice showed no evident

tumor growth at wk 12. In contrast, large tumors were found in nude mice implanted with 10<sup>3</sup> CD44<sup>+</sup>CD24<sup>+</sup> cells at wk 4 (2/8), a 20-fold increase in tumorigenic potential ( $P < 0.05$  or  $P < 0.01$ ). There was no obvious histological difference between the cells of the CD44<sup>+</sup>CD24<sup>+</sup> cell-formed nodules and PANC-1 cells.

**CONCLUSION:** CD44 and CD24 may be used as the cell surface markers for isolation of pancreatic cancer stem cells from pancreatic adenocarcinoma cell line PANC-1. Subpopulation cells CD44<sup>+</sup>CD24<sup>+</sup> have properties of tumor stem cells. Because cancer stem cells are thought to be responsible for tumor initiation and its recurrence after an initial response to chemotherapy, it may be a very promising target for new drug development.

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**Key words:** Pancreatic tumor; Stem cells; Tumor stem cells; Isolation; Identification

**Peer reviewers:** Minoti V Apte, Associate Professor, Pancreatic Research Group, South Western Sydney Clinical School, The University of New South Wales. Liverpool, NSW 2170, Australia

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### INTRODUCTION

Pancreatic carcinoma is an obstinate disease that is difficult to deal with. Though pancreatic cancer accounts for only 2%-3% of all cancers, it is the fourth most frequent cause of cancer death in industrialized countries<sup>[1]</sup>. It is estimated in the United States in 1998 that at least 29000 new cases of pancreatic cancer will be diagnosed<sup>[2]</sup>. Unfortunately, only 18% will survive one year after diagnosis, the five-year survival rate is 4%. This is because by the time a patient exhibits symptoms, and the cancer is diagnosed, it is no longer in its early stage<sup>[3-5]</sup>. The main conventional treatments for pancreatic cancer are surgery, radiation therapy and chemotherapy. Despite advances in surgical and

medical therapy, little effect has been made on the mortality rate of this disease. According to Bjerkvig *et al*<sup>[6]</sup>, the capacity of a tumor to grow and propagate is dependent on a small subset of cells (so-called tumor stem cells), tumor stem cells are immature cells that can replicate or self-renew, and are able to differentiate or grow into all the cells that an organism or particular organ system need. It has profound implications to understand how tumors evolve and how we treat tumors. If we can destroy these tumor stem cells, it will be possible to treat the patients successfully. However, it is difficult to purify tumor stem cells because of lack of specific cell surface markers in solid tumors. Recently, it was reported that cancer stem cells existed in some solid malignancies, including breast<sup>[7]</sup>, brain<sup>[8,9]</sup>, prostate<sup>[10]</sup>, and lung cancers<sup>[11]</sup>. Thus, we deduced that pancreatic cancer might contain its own stem cells responsible for its metastasis and recurrence. To prove this hypothesis, we isolated subpopulation cells that have characteristics of tumor stem cells according to markers CD44 and CD24 by flow cytometry from pancreatic adenocarcinoma cell line PANC-1, and explore their biological characteristics. This study was to identify the method of isolation of pancreatic tumor stem cells and the ability of propagation of the tumor stem cells *in vitro* and *in vivo*.

## MATERIALS AND METHODS

### Experimental materials

Male nude mice, aged 6-8 wk and weighing  $20 \pm 2$  g, were provided by the Experimental Animal Center, Hubei Center for Disease Control and Prevention, China. The nude mice were caged individually under specific pathogen free (SPF) conditions. Human pancreatic adenocarcinoma cell line PANC-1 was obtained from American Type Culture Collection, Manassas, Virginia, the Dulbecco modified eagle medium F12 (1:1 volume) (DMEM-F12) from Hyclone, Wuhan, China, the fetal bovine serum from Sijiqing, Hangzhou, China, trypsin from Sigma-Aldrich, Shanghai, China, the epidermal growth factor (EGF), basic fibroblast growth factor (b-FGF), insulin-transferrin-selenium solution (ITS) and trypsin from Sigma-Aldrich, Shanghai, China and PE anti-human CD44 and FITC anti-human CD24 were purchased from American Ancell.

### Cell culture

The cells were cultured in incubator filled with 5% CO<sub>2</sub> at 37°C. The PANC-1 cells were cultured in DMEM-F12 (1:1 volume) supplemented with 20% fetal bovine serum (FBS), penicillin ( $1 \times 10^5$  U/L) and streptomycin (100 mg/L).

### Flow cytometric analysis

Cells were dissociated by trypsin-EDTA solution (trypsin, 0.25%; EDTA, 0.02%) for 2-5 min at 37°C, transferred to a 5-mL tube, washed twice with PBS with 2% heat-inactivated calf serum (HICS; 5 min at 1000 r/min), resuspended in 100  $\mu$ L (per  $10^6$  cells) of PBS, then were counted. PE anti-human CD44 and (or) FITC anti-human CD24 (appropriate dilution per antibody) were added and incubated for 30 min

at 4°C, and then washed twice with PBS. Flow cytometry was performed on a FACS, and data were analyzed with the Cell Quest software (B.D., America). Using forward and side scatter profile, debris and dead cells were gated out. Cells were routinely sorted twice, and reanalyzed for purity. Then CD44<sup>+</sup>, CD44<sup>-</sup> cells, CD44<sup>+</sup>CD24<sup>+</sup> and CD44<sup>-</sup>CD24<sup>-</sup> and unsorted cells were obtained.

### Estimation of proliferative capability of cells in vitro

The CD44<sup>+</sup>CD24<sup>+</sup>, CD44<sup>-</sup>CD24<sup>-</sup> and unsorted cells were diluted to a density of about  $10^4$  cells/mL with serum-free medium (SFM), a mixture of DMEM-F12 containing 10 ng/mL fibroblast and 20 ng/mL epidermal growth factors, 5  $\mu$ g/mL insulin, 2.75 mg/mL transferrin, 2.75 ng/mL selenium (insulin-transferrin-selenium solution), penicillin ( $1 \times 10^5$  U/L) and streptomycin (100 mg/L). The 200- $\mu$ L/well diluted cell suspension was plated to 96-well culture dishes. The wells with  $2 \times 10^3$  cells were observed everyday under an Olympus CKX41 microscope; the images were captured using an Olympus C5050Z camera. Each group was set up with five duplicate holes. Their OD values were measured with spectrophotometer at 490 nm by 3-[4,5-dimethyl-2-thiazolyl]-2, 5-diphenyl-2H-tetrazolium bromide (MTT) method, and a 96-well plate was determined every 24h. The mean value was obtained and a growth curve was drawn.

### Transplantation of cells into nude mice

After resuspension, CD44<sup>+</sup>, CD44<sup>-</sup>, CD24<sup>+</sup>, CD24<sup>-</sup>, CD44<sup>+</sup>CD24<sup>+</sup>, CD44<sup>-</sup>CD24<sup>-</sup> and unsorted cells were diluted to a density of about  $5 \times 10^6$  to  $5 \times 10^3$  cells/mL with SCM. The cells (0.1 mL) were injected into the hypodermis of right and left armpit of nude mice. The mice were maintained in a specific pathogen-free room under constant temperature and humidity.

### Immunohistochemical staining of CD44 and CD24

All samples of the CD44<sup>+</sup>CD24<sup>+</sup> cell-formed nodules were placed into 10% formalin immediately, processed with routine histological procedures, and embedded in paraffin. Serial sections were cut 5  $\mu$ m thick, and parts of them were stained with hematoxylin and eosin for routine histological observation under light microscope. The others were used for immunohistochemical examination for the CD44 and CD24. After deparaffinization (hydration), sections were treated sequentially with normal goat serum, anti-human CD44 polyclonal antibody (1:200) or anti-human CD24 polyclonal antibody (1:200), biotin-labeled goat anti-mouse IgG, and avidin-biotin-peroxidase complex (ABC). The sites of peroxidase binding were demonstrated by the diaminobenzidine method. Sections were then counterstained with hematoxylin for microscopic examination. Similar procedures were done for the PANC-1 cells. The numbers and areas of CD44-positive and CD24-positive foci > 0.2 mm in diameter and the total areas of the examined sections were measured using a Olympus C5050Z digital camera, Adobe Photoshop version 7.0, and Image-Pro Plus version 6.0.



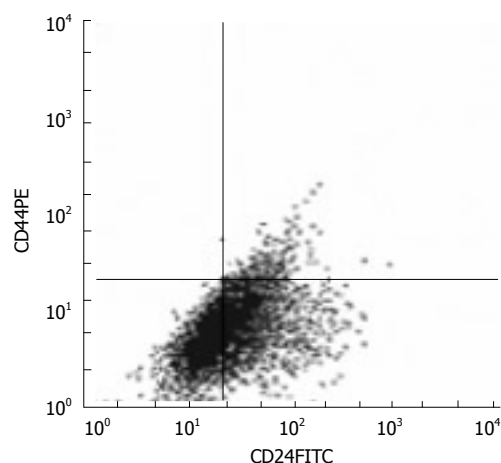


Figure 1 Analysis of Panc-1 pancreatic cancer cells by FACS.

### Statistical analysis

Data were expressed as means  $\pm$  SD, and were analyzed with SPSS 12.0,  $P < 0.05$  was considered significant in difference.

## RESULTS

### Presence of CD44 and CD24 on cell surface of pancreatic carcinoma cell lines

To determine the presence of CD44 and CD24 on the cell surface of the PANC-1 cells, flow cytometric analysis was made. The cell surface markers CD44 and CD24 were chosen as a starting point based on prior work on breast cancer stem cells, in which  $CD44^+CD24^{-/low}$  Lineage tumorigenic cells generated tumors histologically similar to primary breast tumors when as few as 100 cells were transplanted, whereas tens of thousands of bulk unsorted cancer cells were needed to form tumors in NOD/SCID mice<sup>[7]</sup>. CD44 and CD24 have been identified as the stem cell surface markers, which act as adhesive molecules with multiple signaling functions<sup>[12-14]</sup>. As shown in Figure 1, 5.1%-17.5% of sorted PANC-1 cells expressed the cell surface marker CD44, and 57.8%-70.1% expressed CD24. When expression of multiple surface markers was examined, only 2.1%-3.5% of cells were  $CD44^+CD24^+$  (Figure 1).

### Proliferation potential of cells *in vitro*

To evaluate the proliferation ability of cells *in vitro*, the  $CD44^+CD24^+$ ,  $CD44^+CD24^-$  and unsorted cells were cultured in SCM in 96-well culture dishes, their OD values were measured with spectrophotometer at 490nm by MTT method. Compared with  $CD44^+CD24^-$  cells,  $CD44^+CD24^+$  cells had a lower growth rate and longer doubling time *in vitro*. For the former, the index growth trend appeared at the 5th day, while the latter appeared at the day 7 (Figure 2).

### Establishment of xenografts

To test the capability of tumor initiation, we injected cells into the hypodermis of right and left armpit of nude mice. When unsorted PANC-1 cells ( $5 \times 10^3$ ) were injected, no tumor growth was found at wk 12 while  $10^4$  cells were

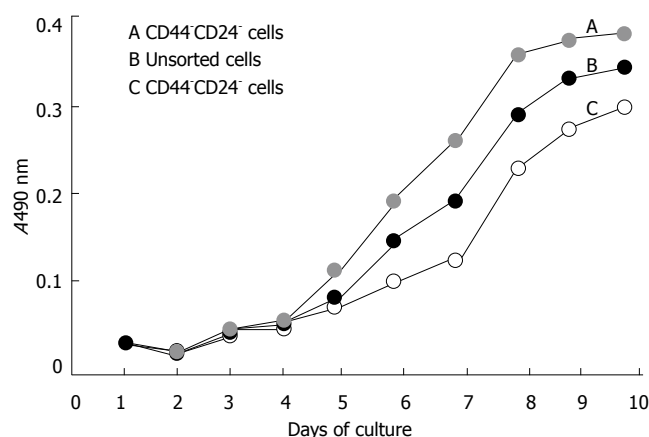


Figure 2 Growth curve of tumors cells *in vitro*.

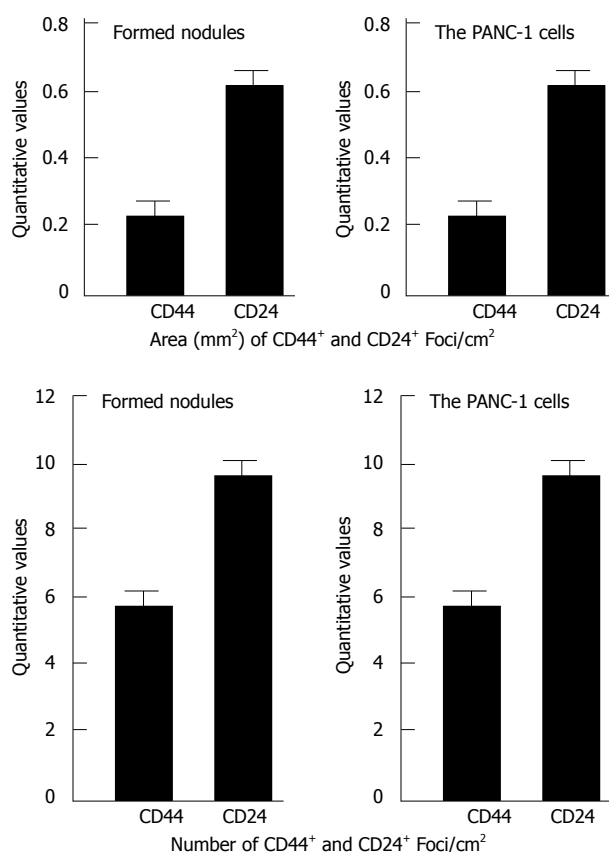
injected, one of six mice developed tumors. For cancer cells sorted for the markers CD44 and CD24, expression of individual markers identified cell populations with enhanced tumorigenic potential. For example, injection of  $5 \times 10^3$   $CD44^+$  cells would occasionally form a tumor (1 of 6 animals), whereas no tumor was observed with  $CD44^-$  cells until at least  $5 \times 10^4$  cells were injected (1 of 10 animals). Six of 10 animals developed tumors when injected with  $5 \times 10^4$   $CD44^+$  cells, representing a 10-fold increase in tumorigenic potential compared with marker negative cells ( $P = 0.029$ ). Similar results were obtained with  $CD24^+$ . Injection of  $CD44^+CD24^+$  cells resulted in an enhanced tumorigenic potential compared with single marker sorted cells. More tumors formed with injection of as few as  $10^3$  cells, and no tumor formed in marker-negative cells until at least  $5 \times 10^4$  cells were injected. The sorted cell population with the highest tumorigenic potential was those expressing CD44 and CD24. For example, injection of  $10^4$   $CD44^+CD24^-$  cells in nude mice found no tumor growth at wk 12. In contrast, nude mice injected with  $10^3$   $CD44^+CD24^+$  cells had large tumors at wk 4 (2 of 8), a 20-fold increase in tumorigenic potential ( $P < 0.05$  or  $P < 0.01$ ) (Table 1). There was no obvious histological difference between the  $CD44^+CD24^+$  cell-formed nodules and PANC-1 cells.

### CD44<sup>+</sup> and CD24<sup>+</sup> positive numbers and areas

For sections stained with hematoxylin and eosin, tumor cells with variable shape from polygon, spindle to irregular were seen under light microscope. The total  $CD44^+$  positive and  $CD24^+$  positive numbers and areas in the examined sections were measured using an Olympus C5050Z digital camera, Adobe Photoshop version 7.0, and Image-Pro Plus version 6.0. There was no significant difference in quantitative values of  $CD44^+$  and  $CD24^+$  cells between the formed nodules and the PANC-1 cells ( $P > 0.05$ ) (Figure 3).

## DISCUSSION

The theory of tumor stem cells<sup>[15,16]</sup> indicates that tumor cells have heterogeneity, i.e., the majority of cells in the



**Figure 3** Quantitative values of CD44<sup>+</sup> and CD24<sup>+</sup> cell foci in the formed nodules and PANC-1 cells. There was no significant difference between the formed nodules and PANC-1 cells.

tumor have lost the growth potential, only a small subset of cells have the capability of the infinite proliferation, the differentiation and the formation of cloning *in vitro*. The initial isolation and identification of tumor stem cells was first proved in hematological malignancies. The CD34<sup>+</sup>CD38<sup>-</sup> phenotype cells (5% of the cancer cells) with obvious proliferation, differentiation and self-renewal ability were purified from the blood of the patients with acute myeloid leukemia<sup>[17,18]</sup>. In 2003, researchers found that only a small subset of human breast cancer cells, with the phenotype CD44<sup>+</sup>CD24<sup>-</sup>, formed new tumors in NOD/SCID mice<sup>[7]</sup>. These breast cancer-initiating cells can be isolated and propagated *in vitro* as extensively proliferating, clonal, nonadherent spherical clusters are able to differentiate along different mammary epithelial lineages<sup>[19]</sup>. A small population of cancer-initiating cells (also called cancer stem cells) was later found in several malignancies, including brain<sup>[8]</sup>, prostate<sup>[10]</sup>, liver<sup>[20,21]</sup>, lung<sup>[22]</sup>, melanoma<sup>[23]</sup>, and colon tumors<sup>[24,25]</sup>.

Although there is increasing evidence that a rare population of undifferentiated cells is responsible for tumor formation and maintenance, little work has been done on the identification of pancreatic cancer special surface markers or on isolation of pancreatic tumor-initiating cells. Based on studies in breast cancer<sup>[7]</sup> and pancreatic adenocarcinoma<sup>[16]</sup>, we identified cells with the characteristics of tumor stem cells according to the cell surface markers CD44 and CD24 by flow cytometry from

**Table 1** Tumor formation ability of sorted pancreatic cancer cells using surface markers (number of tumors formed/number of injections)

Groups	$5 \times 10^5$	$10^5$	$5 \times 10^4$	$10^4$	$5 \times 10^3$	$10^3$	$10^2$
Unsorted	6/6	5/6	3/6	1/6	0/6	0/0	0/0
CD44 <sup>+</sup>	0/0	9/10	6/10	3/10	1/6	0/4	0/0
CD44	0/0	2/10	1/10	0/4	0/0	0/0	0/0
P		0.0027	0.0286	0.3297			
CD24 <sup>+</sup>	0/0	7/8	4/8	3/8	0/4	0/0	0/0
CD24	0/0	2/8	1/8	0/8	0/0	0/0	0/0
P		0.0203	0.1410	0.1000			
CD44 <sup>+</sup> CD24 <sup>+</sup>	0/0	8/8	7/8	6/8	4/8	2/8	0/4
CD44 <sup>+</sup> CD24 <sup>-</sup>	0/0	1/8	1/8	0/8	0/8	0/8	0/0
P		0.0007	0.0051	0.0035	0.0385	0.2333	

Compared with results from marker-negative cells.

pancreatic adenocarcinoma cell line PANC-1. Tumor stem cells have the capability to maintain themselves in culture in an undifferentiated state, initiate tumor growth after xenotransplantation in mice, and differentiate into cancers that are phenotypically indistinguishable from the original tumor. We found that 5.1%-17.5% of sorted PANC-1 cells expressed the cell surface marker CD44, 57.8%-70.1% expressed CD24, and only 2.1%-3.5% of cells were CD44<sup>+</sup>CD24<sup>+</sup>. To take a small subset of cells and put it in the organism and see if it regenerates the original tissues is the classic definition of a stem cell. We injected cells into the hypodermis of the right and left armpit of nude mice to test the capability of tumor initiation. When  $5 \times 10^3$  unsorted PANC-1 cells were injected into nude mice, no tumor grew at wk 12 unless at least  $10^4$  cells were injected. For cancer cells sorted for the markers CD44 and CD24, injection of  $5 \times 10^3$  CD44<sup>+</sup> cells would form a tumor, whereas no tumor was observed with CD44<sup>-</sup> cells until at least  $5 \times 10^4$  cells were injected. Similar results were obtained with CD24<sup>+</sup>. The sorted cell population with the highest tumorigenic potential was those cells expressing CD44 and CD24. For instance, injection of  $10^4$  CD44<sup>+</sup>CD24<sup>-</sup> cells into nude mice, no tumor growth was evident at wk 12. In contrast, nude mice injected with  $10^3$  CD44<sup>+</sup>CD24<sup>+</sup> cells had large tumors at wk 4. Moreover, the CD44<sup>+</sup>CD24<sup>+</sup> cells maintained the ability to engraft and reproduce the same histological and antigenic pattern of the PANC-1. In addition, compared with CD44<sup>+</sup>CD24<sup>-</sup> cells *in vitro*, CD44<sup>+</sup>CD24<sup>+</sup> cells had a lower growth rate. The reason is that tumor stem cells are similar to stem cells, which is in relatively static group of cells, and besides other primates, the stem cell pool proliferates once a year<sup>[26]</sup>. For the CD44<sup>+</sup>CD24<sup>+</sup> cells, there were biological behaviors of the lower proliferative index and the faster tumor growth rate *in vivo*. It is self-contradictory. The reason awaits further studies. In addition, there was no obvious histological difference between the CD44<sup>+</sup>CD24<sup>+</sup> cell-formed nodules and PANC-1 cells.

The above results showed that CD44 and CD24 may be used as markers for isolation of pancreatic cancer stem cells from pancreatic adenocarcinoma cell line PANC-1, subpopulation cells CD44<sup>+</sup>CD24<sup>+</sup> have the characteristics of tumor stem cells. The purification and

other biological behaviors of pancreatic adenocarcinoma stem cells need to be further studied in the future.

## COMMENTS

### Background

Pancreatic carcinoma is an obstinate disease that is difficult to deal with. Though pancreatic cancer accounts for only 2%-3% of all cancers, it is the fourth most frequent cause of cancer deaths in industrialized countries. Unfortunately, only 18% will survive one year after diagnosis, the five-year survival rate is only 4%. Conventional main treatments for pancreatic cancer are surgery, radiation therapy and chemotherapy. Despite advances in surgical and medical therapy, little effect has been achieved on the mortality rate of this disease.

### Research frontiers

The initial isolation and identification of tumor stem cells was first proved in hematological malignancies. The CD34<sup>+</sup>CD38<sup>-</sup> phenotype cells (5% of the cancer cells) with obvious proliferation, differentiation and self-renewal ability had been purified from the blood of the patients with acute myeloid leukemia. Researchers have discovered a small population of cancer-initiating cells (also called cancer stem cells) in several malignancies, including brain, prostate, liver, lung, melanoma, and colon tumors.

### Innovations and breakthroughs

The authors isolated pancreatic adenocarcinoma cell line PANC-1 according to the cell surface markers CD44 and CD24 by flow cytometry, obtained subpopulation cells which have properties of tumor stem cells, and identified the ability of propagation of the tumor stem cells *in vitro* and *in vivo*.

### Applications

Because cancer stem cells are thought to be responsible for tumor initiation and its recurrence after an initial response to chemotherapy, it may be a very promising target for new drug development.

### Peer review

This study corroborates a recent publication in the pancreas reporting that a subpopulation of Panc1 cells can propagate to form spheres and that these cells express stem cell markers such as CD44. The study is very interesting.

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## REFERENCES

- 1 Bardeesy N, DePinho RA. Pancreatic cancer biology and genetics. *Nat Rev Cancer* 2002; **2**: 897-909
- 2 Murphy SL. Deaths: final data for 1998. *Natl Vital Stat Rep* 2000; **48**: 1-105
- 3 Cameron JL, Crist DW, Sitzmann JV, Hruban RH, Boitnott JK, Seidler AJ, Coleman J. Factors influencing survival after pancreaticoduodenectomy for pancreatic cancer. *Am J Surg* 1991; **161**: 120-124; discussion 124-125
- 4 Niederhuber JE, Brennan MF, Menck HR. The National Cancer Data Base report on pancreatic cancer. *Cancer* 1995; **76**: 1671-1677
- 5 Jemal A, Thomas A, Murray T, Thun M. Cancer statistics, 2002. *CA Cancer J Clin* 2002; **52**: 23-47
- 6 Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJ. Opinion: the origin of the cancer stem cell: current controversies and new insights. *Nat Rev Cancer* 2005; **5**: 899-904
- 7 Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF. Prospective identification of tumorigenic breast cancer cells. *Proc Natl Acad Sci USA* 2003; **100**: 3983-3988
- 8 Singh SK, Clarke ID, Terasaki M, Bonn VE, Hawkins C, Squire J, Dirks PB. Identification of a cancer stem cell in human brain tumors. *Cancer Res* 2003; **63**: 5821-5828
- 9 Galli R, Binda E, Orfanelli U, Cipelletti B, Gritti A, De Vitis S, Fiocco R, Foroni C, Dimeco F, Vescovi A. Isolation and characterization of tumorigenic, stem-like neural precursors from human glioblastoma. *Cancer Res* 2004; **64**: 7011-7021
- 10 Collins AT, Berry PA, Hyde C, Stower MJ, Maitland NJ. Prospective identification of tumorigenic prostate cancer stem cells. *Cancer Res* 2005; **65**: 10946-10951
- 11 Kim CF, Jackson EL, Woolfenden AE, Lawrence S, Babar I, Vogel S, Crowley D, Bronson RT, Jacks T. Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* 2005; **121**: 823-835
- 12 Litvinov SV, Velders MP, Bakker HA, Fleuren GJ, Warnaar SO. Ep-CAM: a human epithelial antigen is a homophilic cell-cell adhesion molecule. *J Cell Biol* 1994; **125**: 437-446
- 13 Ponta H, Sherman L, Herrlich PA. CD44: from adhesion molecules to signalling regulators. *Nat Rev Mol Cell Biol* 2003; **4**: 33-45
- 14 Weichert W, Denkert C, Burkhardt M, Gansukh T, Bellach J, Altevoigt P, Dietel M, Kristiansen G. Cytoplasmic CD24 expression in colorectal cancer independently correlates with shortened patient survival. *Clin Cancer Res* 2005; **11**: 6574-6581
- 15 Bjerkvig R, Tysnes BB, Aboody KS, Najbauer J, Terzis AJ. Opinion: the origin of the cancer stem cell: current controversies and new insights. *Nat Rev Cancer* 2005; **5**: 899-904
- 16 Li C, Heidt DG, Dalerba P, Burant CF, Zhang L, Adsay V, Wicha M, Clarke MF, Simeone DM. Identification of pancreatic cancer stem cells. *Cancer Res* 2007; **67**: 1030-1037
- 17 Bonnet D, Dick JE. Human acute myeloid leukemia is organized as a hierarchy that originates from a primitive hematopoietic cell. *Nat Med* 1997; **3**: 730-737
- 18 Lapidot T, Sirard C, Vormoor J, Murdoch B, Hoang T, Caceres-Cortes J, Minden M, Paterson B, Caligiuri MA, Dick JE. A cell initiating human acute myeloid leukaemia after transplantation into SCID mice. *Nature* 1994; **367**: 645-648
- 19 Ponti D, Costa A, Zaffaroni N, Pratesi G, Petrangolini G, Coradini D, Pilotti S, Pierotti MA, Daidone MG. Isolation and *in vitro* propagation of tumorigenic breast cancer cells with stem/progenitor cell properties. *Cancer Res* 2005; **65**: 5506-5511
- 20 Chiba T, Kita K, Zheng YW, Yokosuka O, Saisho H, Iwama A, Nakauchi H, Taniguchi H. Side population purified from hepatocellular carcinoma cells harbors cancer stem cell-like properties. *Hepatology* 2006; **44**: 240-251
- 21 Suetsugu A, Nagaki M, Aoki H, Motohashi T, Kunisada T, Moriaki H. Characterization of CD133<sup>+</sup> hepatocellular carcinoma cells as cancer stem/progenitor cells. *Biochem Biophys Res Commun* 2006; **351**: 820-824
- 22 Dome B, Timar J, Dobos J, Meszaros L, Raso E, Paku S, Kenessey I, Ostoros G, Magyar M, Ladanyi A, Bogos K, Tovari J. Identification and clinical significance of circulating endothelial progenitor cells in human non-small cell lung cancer. *Cancer Res* 2006; **66**: 7341-7347
- 23 Grichnik JM, Burch JA, Schulteis RD, Shan S, Liu J, Darrow TL, Vervaeke CE, Seigler HF. Melanoma, a tumor based on a mutant stem cell? *J Invest Dermatol* 2006; **126**: 142-153
- 24 O'Brien CA, Pollett A, Gallinger S, Dick JE. A human colon cancer cell capable of initiating tumour growth in immunodeficient mice. *Nature* 2007; **445**: 106-110
- 25 Ricci-Vitiani L, Lombardi DG, Pilozzi E, Biffoni M, Todaro M, Peschle C, De Maria R. Identification and expansion of human colon-cancer-initiating cells. *Nature* 2007; **445**: 111-115
- 26 Dunnwald M, Chinnathambi S, Alexandrunas D, Bickenbach JR. Mouse epidermal stem cells proceed through the cell cycle. *J Cell Physiol* 2003; **195**: 194-201

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RAPID COMMUNICATION

## Assessment of hepatic VX<sub>2</sub> tumors with combined percutaneous transhepatic lymphosonography and contrast-enhanced ultrasonographic imaging

Cun Liu, Ping Liang, Yang Wang, Pei Zhou, Xin Li, Zhi-Yu Han, Shao-Ping Liu

Cun Liu, Shao-Ping Liu, Department of Ultrasound, Qilu Hospital, Shandong University, 107 Wenhua West Road, Jinan 250012, Shandong Province, China

Ping Liang, Yang Wang, Pei Zhou, Xin Li, Zhi-Yu Han, Department of Ultrasound, Chinese PLA General Hospital, 28 Fuxing Road, Beijing 100853, China

**Author contributions:** Liu C, Liang P and Liu SP designed the research; Liu C, Zhou P and Li X performed the research; Han ZY carried out the statistical analysis; Yang W helped write and correct the paper; Liang P and Liu SP supervised the organization process.

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**Correspondence to:** Shao-Ping Liu, Department of Ultrasound, Qilu Hospital, Shandong University, 107 Wenhua West Road, Jinan 250012, Shandong Province, China. [liu.sp3000@163.com](mailto:liu.sp3000@163.com)  
Telephone: +86-10-66939530 Fax: +86-10-88210006

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### Abstract

**AIM:** To evaluate the feasibility and efficacy of percutaneous transhepatic lymphosonography (PTL) as a novel method for the detection of tumor lymphangiogenesis in hepatic VX<sub>2</sub> of rabbits and to evaluate combined PTL and routine contrast-enhanced ultrasonographic imaging for the diagnosis of liver cancer.

**METHODS:** Ten rabbits with VX<sub>2</sub> tumor were included in this study. SonoVue (0.1 mL/kg) was injected into each rabbit *via* an ear vein for contrast-enhanced ultrasonographic imaging, and 0.5 mL SonoVue was injected into the normal liver parenchyma near the VX<sub>2</sub> tumor for PTL. Images and/or movie clips were stored for further analysis.

**RESULTS:** Ultrasonographic imaging showed VX<sub>2</sub> tumors ranging 5-19 mm in the liver of rabbits. The VX<sub>2</sub> tumor was hyperechoic and hypoechoic to liver parenchyma at the early and later phase, respectively. The hepatic lymph vessels were visualized immediately after injection of contrast medium and continuously visualized with SonoVue<sup>®</sup> during PTL. The boundaries of VX<sub>2</sub> tumors were hyperechoic to liver parenchyma and the tumors. There was a significant difference in the values for the boundaries of VX<sub>2</sub> tumors after injection compared with the liver normal parenchyma and the tumor parenchyma during PTL.

**CONCLUSION:** PTL is a novel method for the detection of tumor lymphangiogenesis in hepatic VX<sub>2</sub> of rabbits. Combined PTL and contrast-enhanced ultrasonographic imaging can improve the diagnosis of liver cancer.

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**Key words:** Percutaneous transhepatic lymphosonography; Ultrasound; Contrast-enhanced ultrasonographic imaging; Ultrasound contrast media; VX<sub>2</sub> tumor

**Peer reviewer:** Gianluigi Giannelli, MD, Dipartimento di Clinica Medica, Immunologia e Malattie Infettive, Sezione di Medicina Interna, Policlinico, Piazza G. Cesare 11, Bari 70124, Italy

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### INTRODUCTION

The liver is the largest organ in the abdominal cavity and the main region of primary tumor and distant metastasis of malignant tumors. Detection of tumor nodules in the liver is of major importance for formulating therapeutic strategies and predicting the prognosis in malignant tumors<sup>[1]</sup>.

Non-ionizing radiation, portable and noninvasive real-time imaging<sup>[2,3]</sup>, ultrasonography (US) are the most commonly used imaging techniques. Introduction of microbubbles as contrast agents for ultrasound has improved the image quality and diagnostic value<sup>[4-8]</sup>. Contrast-enhanced ultrasonographic imaging enables noninvasive measurements of microvascular perfusion in the heart, brain, kidney, skeletal muscle, skin grafts and solid tumors<sup>[9]</sup> and provides functional images of angiogenesis in animals and humans.

At present, contrast-enhanced ultrasonographic imaging research has mainly focused on angiogenic blood vessels, blood vessel function and efficacy of



angiogenesis inhibitors. Recently, lymphangiogenesis has become a new research frontier<sup>[10]</sup>. Tumor lymphangiogenesis is the process of forming new lymph vessels in tumors and closely related to tumor development and progression. It is necessary to find noninvasive methods for evaluating lymphangiogenesis *in situ*. However, little is known about the contrast-enhanced ultrasonographic imaging used to detect tumor lymphangiogenesis. Recently, lymphosonography after interstitial injection of microbubble-based contrast agents can trace the lymphatic channels from the injection site up to the draining sentinel lymph nodes<sup>[11-15]</sup>. However, no report is available on lymphosonography for tumor lymphangiogenesis.

The aim of the present study was to evaluate the feasibility and efficacy of PTL with a small volume of SonoVue<sup>®</sup> as a novel method for the detection of tumor lymphangiogenesis of hepatic VX<sub>2</sub> in rabbits and to evaluate the combined PTL and contrast-enhanced ultrasonographic imaging in the diagnosis of liver cancer.

## MATERIALS AND METHODS

### Animal model

Ten male health New Zealand rabbits, weighing 2.5-3.0 kg, were included in this study and housed in an approved facility with free access to water and standard diet throughout the study. The study, approved by the Institutional Review Board for Animal Research, was performed following the Guidelines for the Care and Use of Laboratory Animals<sup>[16]</sup>.

An undifferentiated VX<sub>2</sub> carcinoma growing rapidly in rabbits served as the experimental tumor. Two VX<sub>2</sub> tumors were implanted into the right and left lobes of liver, respectively. In brief, rabbits were anesthetized with ketamine hydrochloride (40 mg/kg) and xylazine hydrochloride (5 mg/kg) intramuscularly. The rabbits were intermittently given small supplementary doses of sodium pentobarbital (ranging from 3.1 to 6.5 mg/kg) during the experiment to maintain adequate sedation, fixed in a supine position on a rigid board of paper. Hair on the abdominal skin was shaved after the animals became stable. Diagnostic US was performed to assess the implantation site. Cryoconserved tumor material, implanted in the lower leg muscles of an additional animal and harvested after it reached a size of 1.5 cm, was placed into a saline solution and cut into sections measuring 1 mm × 1 mm × 1 mm.

The implantation method used has been described elsewhere<sup>[17]</sup>. Only part of tumor tissue showing no macroscopic signs of necrosis was used. A 16-gauge intravenous cannula was placed into the left and right liver lobe respectively under US guidance, and the prepared tumor tissue sections were pushed through the cannula and placed at the preselected position. The same procedure was performed on each animal. The rabbits were permitted to recover and followed up sonographically (Sequia 512, Siemens, Germany) weekly until a localized, avascular carcinoma-like mass developed at the injection site after 10-15 d.

### Equipment

Sequia 512 US image system was purchased from Siemens, Germany, with a L15-8 probe equipped for Cadence CPS software. Its acoustic output was carefully controlled by the operator. MI was set at 0.1-0.3 in order to avoid considerable bubble destruction and reduction of the contrast effect. Cadence CPS is a real-time, non-linear imaging technique specific for the second echo-contrast agent examination. Cadence CPS processing utilizes all non-linear responses, fundamental and higher order harmonics, to produce high sensitivity contrast agent images with excellent agent-to-tissue specificity at a very low MI. Images and/or movie clips were stored during PTL and contrast-enhanced ultrasonographic imaging.

### Contrast agent

Contrast agent used in this study was SonoVue<sup>®</sup> (Bracco, Milan, Italy). Microbubbles are sulfur hexafluoride stabilized in a phospholipid shell, 1-10 µm in diameter, averaging about 2.5 µm. The SonoVue<sup>®</sup> preparation was reconstituted just before administration by adding 5 mL sterile saline to the freeze-dried powder, so that sulfur hexafluoride had a concentration of 45 µg/mL in the suspension.

### SonoVue<sup>®</sup> injection

SonoVue (0.1 mL/kg) was injected *via* an ear vein as a rapidly injected bolus, followed by a 1.5 mL saline flush for routinely contrast-enhanced ultrasonographic imaging.

SonoVue (0.5 mL) was injected into the normal liver parenchyma near the VX<sub>2</sub> tumors as a rapidly injected bolus using a tuberculin syringe and a 26-gauge needle for PTL. The absorption of the contrast agent and its flow were observed in lymphatic channels of the VX<sub>2</sub> tumors.

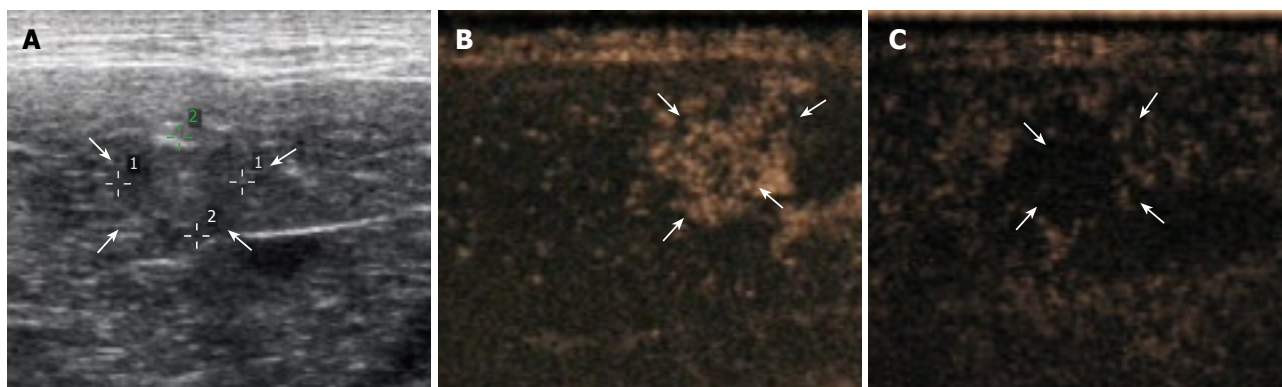
### Statistical analysis

For quantitative analysis, videodensities of the appropriate regions of interest (ROI), including perineoplastic liver parenchyma, boundaries of the tumor and tumor parenchyma were recorded during PTL. Respective evaluations were made for PTL. Data analysis was carried out using SPSS 16 statistical software. All videodensity data were expressed as mean ± SD. Parameters were tested using paired *t* test. Statistical analysis was performed using one-way analysis of variance and Dunnett's multiple comparison tests. *P* < 0.05 was considered statistically significant.

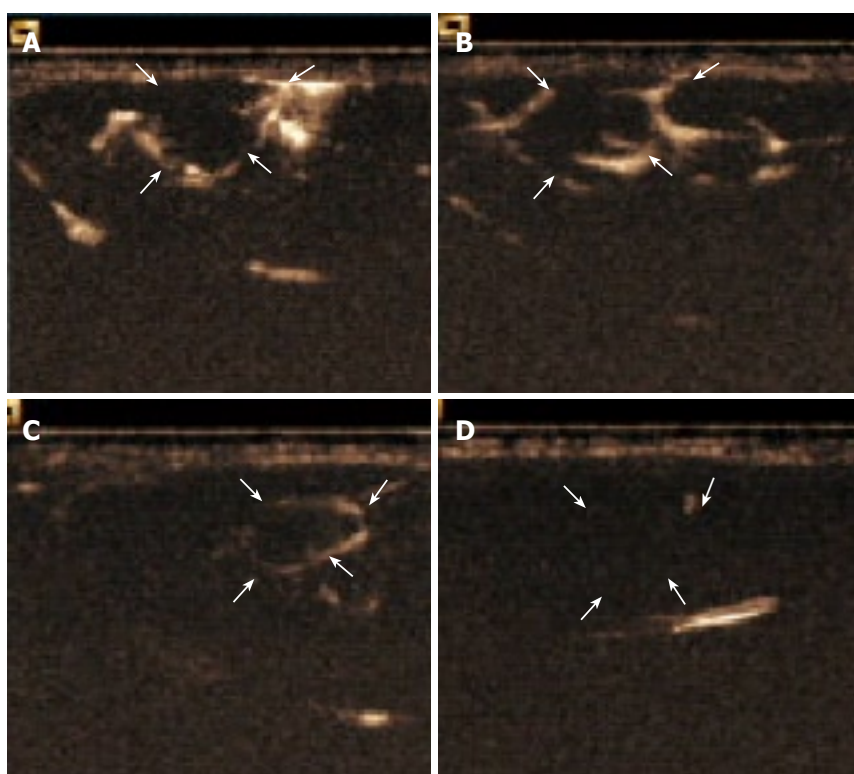
## RESULTS

The VX<sub>2</sub> tumor in liver of rabbits ranging 5-19 mm was found to be a low echoic mass. However, because the VX<sub>2</sub> tumor was almost isoechoic with the normal tissue and boundaries of the masses were unclear, detection and delineation of the lesion were difficult before SonoVue<sup>®</sup> injection (Figure 1A).

Since the typical enhancement pattern of VX<sub>2</sub> tumor detected by routine contrast-enhanced ultrasonographic imaging was hyperechoic and hypoechoic to liver



**Figure 1** Liver of a VX<sub>2</sub> tumor-bearing rabbit imaged in the conventional mode before (A), immediately after 18 s (B) and 96 s (C) of injection of 0.1 mL sonazoid microbubbles/kg. Arrows indicate VX<sub>2</sub> tumor.



**Figure 2** Hepatic lymph vessels visualized 36 s (A), 4 min (B), 7 min (C) 18 min (D) after injection of contrast agent and continuously visualized with SonoVue® during PTL with hyperechoic boundaries of VX<sub>2</sub> tumors to liver parenchyma and the tumor.

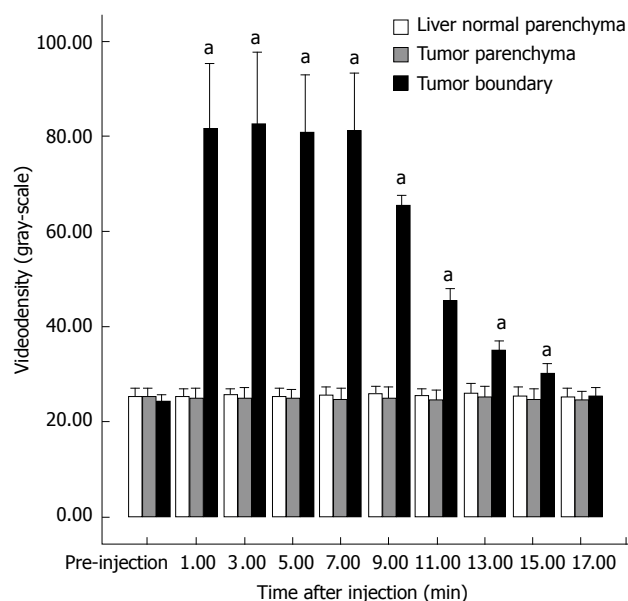
parenchyma during the early and later phase, respectively, a much more rapid wash-in and -out of ultrasonographic contrast agent was observed compared to the normal liver parenchyma (Figure 1B and C).

The enhancement pattern of VX<sub>2</sub> tumors detected by PTL was significantly different from the typical enhancement pattern of VX<sub>2</sub> tumors detected by routine contrast-enhanced ultrasonographic imaging. The hepatic lymph vessels were visualized immediately and continuously during PTL. SonoVue® was deposited in the parenchyma relatively quickly in winding channels. At the same time, the boundaries of VX<sub>2</sub> tumors were hyperechoic to liver parenchyma and the tumors. The hyperechoic boundaries clearly delineated VX<sub>2</sub> tumors compared with the normal liver and tumor parenchyma (Figure 2A-C). The difference in the videodensitometric measurements of the boundaries of VX<sub>2</sub> tumors was significantly higher than the baseline (Figure 3).

Conversely, videodensity in the normal liver and tumor parenchyma had no signal enhancement compared with the baseline (Figure 3). There was a significant difference in the boundaries of VX<sub>2</sub> tumors compared with the baseline as well as the normal liver and tumor parenchyma (Figure 3).

## DISCUSSION

Ultrasound is an important and useful imaging method for the detection of tumors. Ultrasound contrast agents containing encapsulated microbubbles are mainly used to increase the diagnostic imaging of tumors. McCarville *et al*<sup>[18]</sup> showed that gray-scale US measurements of microbubble contrast agent flow can be used to detect the functional consequences of antiangiogenic therapy for tumors and to assess angiogenesis inhibitors that act through different mechanisms<sup>[19-23]</sup>.



**Figure 3** Videodensitometric measurements of liver normal parenchyma (white), tumor parenchyma (gray) and tumor boundary (black) before and after percutaneous transhepatic injection of contrast agent SonoVue<sup>®</sup> into the normal liver parenchyma near the VX<sub>2</sub> tumors during PTL. \**P* < 0.05 vs respective pre-injection values (Dunnett).

Recently, lymphangiogenesis has become a new research frontier<sup>[10]</sup>. The important functions of the lymphatic system are to remove damaged cells from the body and to prevent the spread of infection and cancer for the maintenance of normal tissue fluid balance and immune surveillance. In spite of its important functions in physiological and pathological conditions, including tumor metastasis, lymphoedema and inflammation, lymphatic vessels have not received as much attention as blood vessels, and the mechanisms regulating their development and growth have been poorly understood<sup>[24]</sup>. Lymphangiogenesis is associated with increased tumor cells in lymphatics and lymph nodes, served as an independent prognostic factor and a potential target in the development of new therapies for hilar cholangiocarcinoma<sup>[25]</sup>. At present, neovessel formation, including lymphangiogenesis, represents the key event in tumor progression. Inhibition of metastatic spread may be achieved by restriction of lymphatic vessel growth with novel therapeutic strategies for anti-lymphangiogenic therapies<sup>[26]</sup>.

Currently, histologic determination of the mean intratumoral or peritumoral lymphatic vessels is the most commonly used method for assessing lymphangiogenesis. However, obtaining tissue for histologic evaluation may require an invasive procedure that cannot be normally accepted by patients. Furthermore, determination of the lymphatic microvessel density does not provide an accurate assessment of the functionality of tumor lymphatic vessels because many poorly functioning or collapsed lymphatic vessels have endothelial cells that are stained and counted. Therefore, the lymphatic microvessel density *in vivo* may be a potentially useful marker for assessing lymphangiogenesis in tumors at diagnosis, and accurately reflects the effectiveness of antitumor therapy.

Ultrasound lymphography with subcutaneous injection of ultrasound contrast material enables direct visualization of the lymphatic drainage pathways and sentinel lymph nodes of breast diseases, melanoma, *etc*<sup>[11-15]</sup>.

In the present study, the traditional percutaneous hepatic injection method was used to deliver SonoVue<sup>®</sup> microbubbles into the liver under US guidance to investigate tumor lymphangiogenesis. To the best of our knowledge, lymphosonography for the detection of tumor lymphangiogenesis has not been reported before. Hepatic lymph vessels were visualized immediately after injection of contrast agent and opacified with SonoVue<sup>®</sup> during PTL, whereas liver parenchyma was not enhanced by SonoVue<sup>®</sup>. SonoVue<sup>®</sup> was deposited in the parenchyma relatively quickly in winding lymph vessels. At the same time, the boundaries of VX<sub>2</sub> tumors were hyperechoic to liver parenchyma and the tumors, indicating that hyperechoic boundaries clearly delineate the peritumoral lymphatic vessels of VX<sub>2</sub> tumors. Compared with the hyperechoic boundaries of VX<sub>2</sub> tumors, the videodensity in the tumor parenchyma had no signal enhancement compared with the baseline. This is consistent with the findings in a previous study<sup>[27]</sup>. It was reported that three-dimensional changes of lymphatic architecture in rabbit VX<sub>2</sub> tongue cancer, dynamics of its adjacent lymphatic architecture, especially the increased number of capillaries in preexisting lymphatic vessels outside the tumor margin, are associated with lymph node metastasis<sup>[28,29]</sup>. The morphological features of lymphatic vessels during PTL may be important predictive markers for evaluating lymphatic metastasis and prognosis of tumors. The lymphatic drainage paths and lymphatic distribution pattern in hepatic tissue have been found to be very constant, showing that angiogenesis is a critical factor for tumor growth and metastasis<sup>[23]</sup>. In this study, the typical enhancement pattern of VX<sub>2</sub> tumors detected by routine contrast-enhanced ultrasonographic imaging was hyperechoic and hypoechoic to the liver parenchyma at the early and later phases, respectively, confirming that routine contrast-enhanced ultrasonographic imaging can assess tumor vascularity and reveal the microvascular perfusion and function<sup>[23,30,31]</sup>.

The specific mechanism by which the contrast agents used in this study enter the lymphatic system is unclear. SonoVue<sup>®</sup> microbubbles have a mean diameter of 2.5  $\mu\text{m}$  with 99% smaller than 11  $\mu\text{m}$ , allowing a free passage of capillaries, but keeping within the vascular lumen. This means that SonoVue<sup>®</sup> microbubbles in the hepatic inter-space cannot come into blood vessels. Although the optimal particle diameter for lymphatic uptake is 10-50 nm, particles up to hundreds of nanometers in diameter appear to be able to cross the lymphatic endothelium<sup>[32-34]</sup>. Due to the flexibility of microbubbles, phospholipidic shell and poor solubility and diffusivity of SF<sub>6</sub>, SonoVue<sup>®</sup> is highly resistant to pressure. This means the microbubbles may more easily distort and traverse lymphatic wall fenestrations into lymph capillaries.

Due to the different membranes, 99% of Sonazoid and Optison are phagocytosed by Kupffer cells, whereas only 7.3% of SonoVue<sup>®</sup> is phagocytosed by Kupffer cells<sup>[35]</sup>. This means that the SonoVue<sup>®</sup> microbubbles are



not easily phagocytosed by macrophages. Tracing the SonoVue<sup>®</sup> microbubble flowing in the lymph vessels can improve the pathologic staging of the disease and its treatment.

At the same time, microbubbles are used not only for contrast enhancement of ultrasound images and improvement of diagnosis, but also for delivery of drugs and genes<sup>[36-40]</sup>. The ability to localize lymphatic vessels in tumors may be of value for a new route to the administration of drugs, gene and immunotherapy, etc. Drugs/genes containing vesicles may be injected simultaneously with microbubbles or microbubbles in combination with microbubble-forming vesicle aggregates. Using microbubbles oscillation and cavitation under US guidance might assist in delivering drugs/genes from vesicles to the interstitial tissue, which may be an effective treatment for some diseases.

Since few studies about hepatic lymphography are available at present, it is difficult to find microbubbles in lymphatic vessels. Due to this reason, the study only limited to the ultrasound characteristic aspects of PTL, which were not compared with the histopathologically aspects of rabbit VX<sub>2</sub> tumors.

In conclusion, PTL with a small volume of SonoVue microbubbles is a novel method for the detection of tumor lymphangiogenesis of hepatic VX<sub>2</sub> in rabbits. Combined PTL and contrast-enhanced ultrasonographic imaging can improve the diagnosis of liver cancer. Additional research is needed to determine the potential advantages of PTL and to determine if PTL can be used in clinical practice.

## COMMENTS

### Background

Ultrasonography (US) is one of the most commonly used imaging techniques. Lymphangiogenesis has become a new research frontier. Tumor lymphangiogenesis is the process of generating new lymph vessels within and surrounding tumors, which is closely related to tumor development and progression. It is necessary to develop noninvasive methods for evaluating lymphangiogenesis *in situ*. However, to the best of our knowledge, lymphosonography showing tumor lymphangiogenesis with percutaneous hepatic injection of ultrasound contrast material has not been reported before.

### Research frontiers

This study investigated tumor angiogenesis and lymphangiogenesis with combined percutaneous transhepatic lymphosonography (PTL) and contrast-enhanced ultrasonographic imaging for hepatic VX<sub>2</sub> in rabbit liver.

### Innovations and breakthroughs

Contrast-enhanced ultrasonographic imaging enables noninvasive measurements of microvascular perfusion in the heart, brain, kidney, skeletal muscle, skin grafts and solid tumors in animals and humans. It was recently reported that lymphosonography after interstitial injection of microbubble-based contrast agents can trace lymphatic channels from the injection site up to the draining sentinel lymph nodes. This is the first study to evaluate the feasibility and efficacy of PTL with a small volume of SonoVue<sup>®</sup> as a novel method for the detection of tumor lymphangiogenesis of hepatic VX<sub>2</sub> in rabbits and to evaluate the role of combined PTL and contrast-enhanced ultrasonographic imaging in improving the diagnosis of liver cancer.

### Applications

PTL with a small volume of SonoVue microbubbles is a novel method for the detection of tumor lymph angiogenesis of hepatic VX<sub>2</sub> in rabbits. Combined PTL and contrast-enhanced ultrasonographic imaging can improve the diagnosis of liver cancer. Additional research is needed to determine the potential advantages of PTL and to determine if PTL can be used in clinical practice.

### Peer review

PTL is a new tool for the diagnosis of liver cancer. The study is well designed and interesting.

## REFERENCES

- 1 Maruyama H, Matsutani S, Saisho H, Mine Y, Kamiyama N, Hirata T, Sasamata M. Real-time blood-pool images of contrast enhanced ultrasound with Definity in the detection of tumour nodules in the liver. *Br J Radiol* 2005; **78**: 512-518
- 2 McDonald DM, Choyke PL. Imaging of angiogenesis: from microscope to clinic. *Nat Med* 2003; **9**: 713-725
- 3 Stewart VR, Sidhu PS. New directions in ultrasound: microbubble contrast. *Br J Radiol* 2006; **79**: 188-194
- 4 Bloch SH, Dayton PA, Ferrara KW. Targeted imaging using ultrasound contrast agents. Progress and opportunities for clinical and research applications. *IEEE Eng Med Biol Mag* 2004; **23**: 18-29
- 5 Nicolau C, Catala V, Vilana R, Gilabert R, Bianchi L, Sole M, Pages M, Bru C. Evaluation of hepatocellular carcinoma using SonoVue, a second generation ultrasound contrast agent: correlation with cellular differentiation. *Eur Radiol* 2004; **14**: 1092-1099
- 6 Cosgrove D. Future prospects for SonoVue and CPS. *Eur Radiol* 2004; **14** Suppl 8: P116-P124
- 7 Hettiarachchi K, Talu E, Longo ML, Dayton PA, Lee AP. On-chip generation of microbubbles as a practical technology for manufacturing contrast agents for ultrasonic imaging. *Lab Chip* 2007; **7**: 463-468
- 8 Zhao S, Kruse DE, Ferrara KW, Dayton PA. Selective imaging of adherent targeted ultrasound contrast agents. *Phys Med Biol* 2007; **52**: 2055-2072
- 9 Lindner JR. Microbubbles in medical imaging: current applications and future directions. *Nat Rev Drug Discov* 2004; **3**: 527-532
- 10 Zhang XH, Huang DP, Guo GL, Chen GR, Zhang HX, Wan L, Chen SY. Coexpression of VEGF-C and COX-2 and its association with lymphangiogenesis in human breast cancer. *BMC Cancer* 2008; **8**: 4
- 11 Choi SH, Kono Y, Corbeil J, Lucidarme O, Mattrey RF. Model to quantify lymph node enhancement on indirect sonographic lymphography. *AJR Am J Roentgenol* 2004; **183**: 513-517
- 12 Mattrey RF, Kono Y, Baker K, Peterson T. Sentinel lymph node imaging with microbubble ultrasound contrast material. *Acad Radiol* 2002; **9** Suppl 1: S231-S235
- 13 Omoto K, Mizunuma H, Ogura S, Hozumi Y, Nagai H, Taniguchi N, Itoh K. New method of sentinel node identification with ultrasonography using albumin as contrast agent: a study in pigs. *Ultrasound Med Biol* 2002; **28**: 1115-1122
- 14 Goldberg BB, Merton DA, Liu JB, Thakur M, Murphy GF, Needleman L, Tornos A, Forsberg F. Sentinel lymph nodes in a swine model with melanoma: contrast-enhanced lymphatic US. *Radiology* 2004; **230**: 727-734
- 15 Omoto K, Hozumi Y, Omoto Y, Taniguchi N, Itoh K, Fujii Y, Mizunuma H, Nagai H. Sentinel node detection in breast cancer using contrast-enhanced sonography with 25% albumin--Initial clinical experience. *J Clin Ultrasound* 2006; **34**: 317-326
- 16 National Research Council. Guide for the care and use of laboratory animals. 7th ed. Washington, DC: National Academy Press; 1996: 321. Available from: URL: <http://www.nap.edu/readingroom/books/labrats/>
- 17 Hauff P, Fritzsche T, Reinhardt M, Weitschies W, Luders F, Uhlendorf V, Heldmann D. Delineation of experimental liver tumors in rabbits by a new ultrasound contrast agent and stimulated acoustic emission. *Invest Radiol* 1997; **32**: 94-99
- 18 McCarville MB, Streck CJ, Dickson PV, Li CS, Nathwani AC, Davidoff AM. Angiogenesis inhibitors in a murine neuroblastoma model: quantitative assessment of



- intratumoral blood flow with contrast-enhanced gray-scale US. *Radiology* 2006; **240**: 73-81
- 19 **Palmowski M**, Morgenstern B, Hauff P, Reinhardt M, Huppert J, Maurer M, Woenne EC, Doerk S, Ladewig G, Jenne JW, Delorme S, Grenacher L, Hallscheidt P, Kauffmann GW, Semmler W, Kiessling F. Pharmacodynamics of streptavidin-coated cyanoacrylate microbubbles designed for molecular ultrasound imaging. *Invest Radiol* 2008; **43**: 162-169
  - 20 **Willmann JK**, Paulmurugan R, Chen K, Gheysens O, Rodriguez-Porcel M, Lutz AM, Chen IY, Chen X, Gambhir SS. US imaging of tumor angiogenesis with microbubbles targeted to vascular endothelial growth factor receptor type 2 in mice. *Radiology* 2008; **246**: 508-518
  - 21 **Rychak JJ**, Graba J, Cheung AM, Mystry BS, Lindner JR, Kerbel RS, Foster FS. Microultrasound molecular imaging of vascular endothelial growth factor receptor 2 in a mouse model of tumor angiogenesis. *Mol Imaging* 2007; **6**: 289-296
  - 22 **Lyshchik A**, Fleischer AC, Huamani J, Hallahan DE, Brissova M, Gore JC. Molecular imaging of vascular endothelial growth factor receptor 2 expression using targeted contrast-enhanced high-frequency ultrasonography. *J Ultrasound Med* 2007; **26**: 1575-1586
  - 23 **Wang Z**, Tang J, An L, Wang W, Luo Y, Li J, Xu J. Contrast-enhanced ultrasonography for assessment of tumor vascularity in hepatocellular carcinoma. *J Ultrasound Med* 2007; **26**: 757-762
  - 24 **Makinen T**, Alitalo K. Lymphangiogenesis in development and disease. *Novartis Found Symp* 2007; **283**: 87-98; discussion 98-105, 238-241
  - 25 **Thelen A**, Scholz A, Benckert C, Weichert W, Dietz E, Wiedenmann B, Neuhaus P, Jonas S. Tumor-associated lymphangiogenesis correlates with lymph node metastases and prognosis in hilar cholangiocarcinoma. *Ann Surg Oncol* 2008; **15**: 791-799
  - 26 **Sundlisaeter E**, Dicko A, Sakariassen PO, Sondenaa K, Enger PO, Bjerkvig R. Lymphangiogenesis in colorectal cancer--prognostic and therapeutic aspects. *Int J Cancer* 2007; **121**: 1401-1409
  - 27 **Schneider M**, Buchler P, Giese N, Giese T, Wilting J, Buchler MW, Friess H. Role of lymphangiogenesis and lymphangiogenic factors during pancreatic cancer progression and lymphatic spread. *Int J Oncol* 2006; **28**: 883-890
  - 28 **Seki S**, Fujimura A. Three-dimensional changes in lymphatic architecture around VX2 tongue cancer--dynamic changes after administration of antiangiogenic agent. *Lymphology* 2003; **36**: 199-208
  - 29 **Seki S**, Fujimura A. Three-dimensional changes in lymphatic architecture around VX2 tongue cancer--dynamics of growth of cancer. *Lymphology* 2003; **36**: 128-139
  - 30 **Lassau N**, Roche A. [Imaging and angiogenesis: DCE-US (dynamic contrast enhanced-ultrasonography)] *Bull Cancer* 2007; **94** Spec No: S247-S253
  - 31 **Pollard RE**, Broumas AR, Wisner ER, Vekich SV, Ferrara KW. Quantitative contrast enhanced ultrasound and CT assessment of tumor response to antiangiogenic therapy in rats. *Ultrasound Med Biol* 2007; **33**: 235-245
  - 32 **Ikomi F**, Hanna GK, Schmid-Schonbein GW. Mechanism of colloidal particle uptake into the lymphatic system: basic study with percutaneous lymphography. *Radiology* 1995; **196**: 107-113
  - 33 **Bergqvist L**, Strand SE, Persson BR. Particle sizing and biokinetics of interstitial lymphoscintigraphic agents. *Semin Nucl Med* 1983; **13**: 9-19
  - 34 **Wolf G**. Specific imaging agents for lymph nodes. In: Torchilin, VP, ed. *Handbook of Targeted Delivery of Imaging Agents*. Boca Raton, FL: CRC Press; 1995: 365-384. Available from: URL: <http://www.amazon.com/Handbook-Targeted-Delivery-Pharmacology-Toxicology/dp/0849383080>
  - 35 **Yanagisawa K**, Moriyasu F, Miyahara T, Yuki M, Iijima H. Phagocytosis of ultrasound contrast agent microbubbles by Kupffer cells. *Ultrasound Med Biol* 2007; **33**: 318-325
  - 36 **Taylor SL**, Rahim AA, Bush NL, Bamber JC, Porter CD. Targeted retroviral gene delivery using ultrasound. *J Gene Med* 2007; **9**: 77-87
  - 37 **Dijkmans PA**, Juffermans LJ, Musters RJ, van Wamel A, ten Cate FJ, van Gilst W, Visser CA, de Jong N, Kamp O. Microbubbles and ultrasound: from diagnosis to therapy. *Eur J Echocardiogr* 2004; **5**: 245-256
  - 38 **Feinstein SB**. The powerful microbubble: from bench to bedside, from intravascular indicator to therapeutic delivery system, and beyond. *Am J Physiol Heart Circ Physiol* 2004; **287**: H450-H457
  - 39 **Rapoport N**, Gao Z, Kennedy A. Multifunctional nanoparticles for combining ultrasonic tumor imaging and targeted chemotherapy. *J Natl Cancer Inst* 2007; **99**: 1095-1106
  - 40 **Borden MA**, Caskey CF, Little E, Gillies RJ, Ferrara KW. DNA and polylysine adsorption and multilayer construction onto cationic lipid-coated microbubbles. *Langmuir* 2007; **23**: 9401-9408

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RAPID COMMUNICATION

## Tuberculosis *versus* non-Hodgkin's lymphomas involving small bowel mesentery: Evaluation with contrast-enhanced computed tomography

Peng Dong, Bin Wang, Quan-Ye Sun, Hui Cui

Peng Dong, Bin Wang, Quan-Ye Sun, Hui Cui, Department of Medical Imaging, Medical Imaging Centre of the Affiliated Hospital, Weifang Medical University, Weifang 261042, Shandong Province, China

Author contributions: Dong P, Wang B, Sun YQ and Cui H contributed equally to this work; Dong P, Wang B, Sun YQ and Cui H wrote the paper.

Correspondence to: Dong Peng, Department of Medical Imaging, Weifang Medical University, Weifang 261042, Shandong Province, China. [dongpeng98021@sina.com](mailto:dongpeng98021@sina.com)

Telephone: +86-536-8068959 Fax: +86-536-8238243

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### Abstract

**AIM:** To evaluate the specific computed tomography (CT) imaging criteria for differentiating tuberculosis involving the small bowel mesenteric lymph nodes from lymphomas.

**METHODS:** We retrospectively reviewed the anatomic distribution, CT enhancement patterns of lymphoma in 18 patients with mesenteric tuberculosis and 22 with untreated non-Hodgkin's lymphomas (NHL) involving small bowel mesentery (SBM). Of the 18 patients with tuberculosis, 9 had purely mesenteric tuberculous lymphadenopathy (TL), and 9 had mesenteric TL accompanied with tuberculous mesenteritis (TLM).

**RESULTS:** CT showed that tuberculosis and NHL mainly affected lymph nodes in the body and root of SBM. Homogeneously enhanced lymph nodes in the body and root of SBM were found more often in the NHL ( $P < 0.05$ ). Homogeneously mixed peripheral enhanced lymph nodes in the body of SBM were found more often in mesenteric TL and TLM ( $P < 0.05$ ). Peripheral enhanced lymph nodes in the root of SBM were found more often in mesenteric TL and TLM ( $P < 0.01$ ). "Sandwich sign" in the root of SBM was observed more often in NHL ( $P < 0.05$ ).

**CONCLUSION:** Anatomic lymph node distribution, sandwich sign and specific enhancement patterns of lymphadenopathy in SBM on CT images can be used in differentiating between tuberculosis and untreated NHL involving SBM.

### INTRODUCTION

The incidence of tuberculosis is increasing<sup>[1-4]</sup>. Abdominal tuberculosis can affect the gastrointestinal tract, peritoneum and lymph nodes. When the prevalence of abdominal tuberculosis is high, it is difficult to establish its diagnosis<sup>[5-7]</sup>. Lymphadenopathy is the most common manifestation of abdominal tuberculosis and may be easily confused with lymphomas involving abdominal lymph nodes in up to 55% of cases without other evidence of abdominal involvement<sup>[8]</sup>. Clinical and radiologic differentiation between the two can be challenging<sup>[6,8,9]</sup>. To our knowledge, a comparison of computed tomography (CT) findings in tuberculosis and lymphoma of the mesenteric lymph nodes has not been reported<sup>[10]</sup>. Lymphoma<sup>[11]</sup> is the most common malignant neoplasm affecting the mesentery and Hodgkin's Lymphoma can rarely involve the mesentery<sup>[10]</sup>, so we conducted a comparison of CT findings in tuberculosis and non-Hodgkin's lymphoma (NHL) involving the small bowel mesentery (SBM) to improve the physicians' ability to distinguish between these entities.

### MATERIALS AND METHODS

We retrospectively reviewed the medical records of 40 consecutive patients with documented tuberculosis [18 (45%) with mesenteric tuberculosis and 22 (55%) with untreated non-Hodgkin's lymphomas (NHL) involving small bowel mesentery (SBM)] who underwent contrast-

Table 1 Anatomic distribution and enhancement patterns in mesenteric lymph nodes

	TL ( <i>n</i> = 9)			TLM ( <i>n</i> = 9)			NHL ( <i>n</i> = 22)		
	Margin of SBM	Body of SBM	Root of SBM	Margin of SBM	Body of SBM	Root of SBM	Margin of SBM	Body of SBM	Root of SBM
Homogeneous	0	2	3	0	5	5	5	20	18
Peripheral	1	2	6	0	0	4	0	0	0
Homogeneously mixed peripheral	0	4	0	0	4	0	0	2	4

TL: Tuberculous lymphadenopathy; TLM: Tuberculous lymphadenopathy accompanied with mesenteritis; NHL: Non-Hodgkin's lymphoma.

enhanced CT from October 1998 to May 2007 in our hospital. The patients with tuberculosis included 12 men and 6 women at the age ranging from 19 to 56 years (mean, 29 years) with no evidence of HIV infection, neoplastic disease, or opportunistic infection. Of the 18 patients with tuberculosis, 9 (50%) had purely mesenteric tuberculous lymphadenopathy (TL) [presented with a small quantity of ascites (*n* = 3), thickened peritoneum (*n* = 2), thickened small bowel wall (*n* = 1), "dirty" great omentum (*n* = 1), renal tuberculosis (*n* = 1), hepato-duodenum ligament tuberculosis (*n* = 1), omental bursa tuberculosis (*n* = 1), pleuritis (*n* = 2)], and 9 (50%) had mesenteric TL accompanied with tuberculous mesenteritis (TLM) [presented with ascites (*n* = 8), thickened peritoneum (*n* = 8), thickened small bowel wall (*n* = 5), "dirty" great omentum (*n* = 6), "caked" great omentum (*n* = 3), infra-bowel abscess (*n* = 1), renal tuberculosis (*n* = 1), pleuritis (*n* = 1)]. All the patients with tuberculosis had constitutional symptoms, such as weight loss, easy fatigability, night sweats, and obscure abdominal pains. Five of them had clinically palpable abdominal masses. Tuberculosis was diagnosed if lymphadenopathy was found through pathologic examination of specimens (*n* = 8) taken at laparotomy or microbiologic examination of abdominal tissues (*n* = 3). Tuberculosis was also diagnosed in patients for whom CT showed improvement in documented tuberculosis at extra-abdominal sites after anti-tuberculous chemotherapy (*n* = 7).

No evidence of HIV infection was found in the 22 patients (including 10 men and 12 women at the age ranging from 20 to 74 years, mean 49 years) with newly diagnosed and previously untreated NHL. The diagnosis was made by histologic examination of biopsy specimens of enlarged lymph nodes. Involvement of the left major psoas muscle in 2 patients and the adrenal gland in 1 patient was observed. Three patients had splenomegaly.

All patients giving their written informed consent were examined with a spiral CT scanner (Elscent HeliCAT Flash). Before undergoing CT, the patients drank 500 mL of a 1.5% diatrizoate solution. An 80-100 mL bolus of Ultravist (Schering Germany, 300 mgI/mL) at a rate of 2.5-3.0 mL/s was administered through veins. Contiguous axial images (5-8 mm thick) were obtained at 5-8 mm intervals from the dome of diaphragm to the symphysis pubis (120-140 KV, 212-250 Ma, pitch 1-1.5). Sixty seconds after injection of the contrast material, contrast-enhanced CT scan was performed.

Two observers unaware of the final diagnosis

independently reviewed each CT image and recorded a number of characteristics of enlarged lymph nodes in SBM, including anatomic location, enhancement patterns and "sandwich sign". Discrepancies in interpretation between observers were solved by consensus. Small bowel mesenteric lymph nodes were grouped anatomically into the following three sites: the root, margin (area including mesenteric marginal vessels and vasa rectas) and body of SBM (the area between the root and margin of SBM). The short-axis diameter of each node was measured. The CT images of enlarged lymph nodes were compared with those of normal lymph nodes as previously described<sup>[12]</sup>. The enhancement patterns of enlarged lymph nodes in the 40 patients were described as homogeneous, peripheral, and homogeneously mixed peripheral enhancement. Enhancement was considered peripheral when thick, irregular or thin rim was seen, and homogeneously mixed peripheral when some enlarged nodes showed homogeneous enhancement and other nodes at the same site showed peripheral enhancement. Additionally, we observed the extranodal sites of tuberculosis and NHL, including spleen and abdominal wall. Differences in anatomic distribution, enhancement patterns and presence of "sandwich sign" between the two groups were compared by statistical analysis. Because of the small number of cases, Fisher's exact test was used to compare tuberculosis with NHL involving SBM.

## RESULTS

The anatomic distribution and enhancement pattern findings are listed in Table 1 and the findings of "sandwich sign" are shown in Table 2.

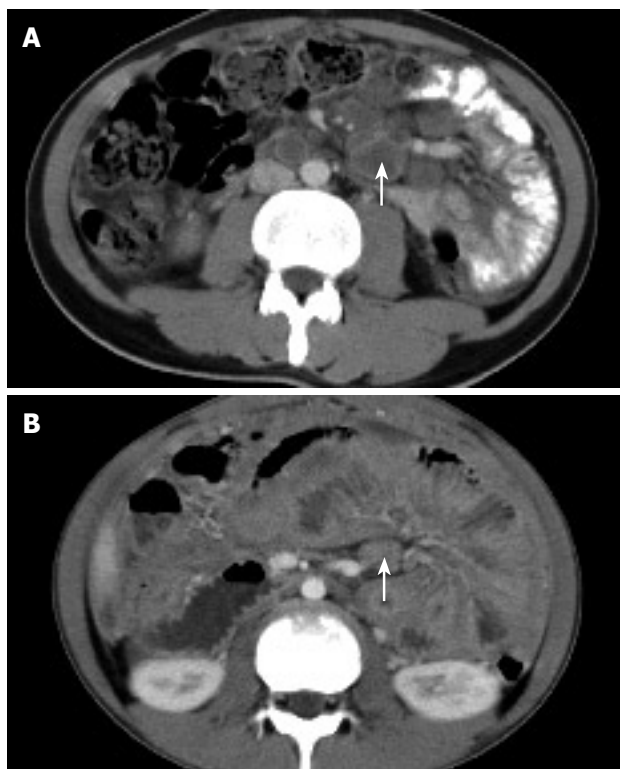
CT revealed that TL and NHL affected mainly lymph nodes in the body and root of SBM (Figures 1 and 2). The margin of SBM was involved in NHL [5 patients (23%)], TL [1 patients (11%)], and TLM [0 patient (0%)].

Homogeneous enhancement (in the body of SBM) was found more often in NHL than in mesenteric TL and TLM (*P* < 0.01, *P* < 0.05). Homogeneously mixed peripheral enhancement (in the body of SBM) was observed more often in mesenteric TL and TLM than in NHL (*P* < 0.05). Homogeneous enhancement (in the root of SBM) was demonstrated more often in NHL than in TL (*P* < 0.05). Peripheral enhancement (in the root of SBM) was revealed more often in mesenteric TL and TLM than in NHL (*P* < 0.01). Enlarged lymph nodes

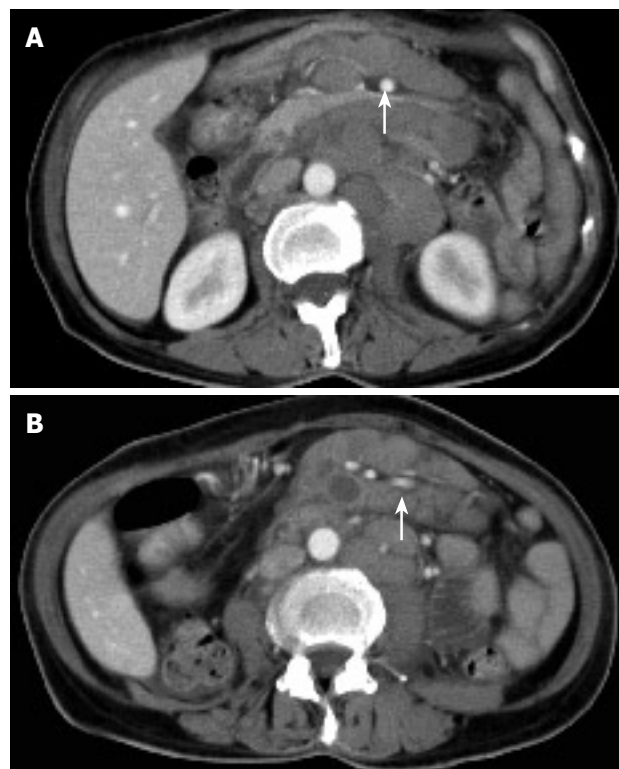
Table 2 Distribution of TL, TLM and NHL in mesenteric lymph nodes

	TL ( <i>n</i> = 9)			TLM ( <i>n</i> = 9)			NHL ( <i>n</i> = 22)		
	Margin of SBM	Body of SBM	Root of SBM	Margin of SBM	Body of SBM	Root of SBM	Margin of SBM	Body of SBM	Root of SBM
Disperse	1	6	7	0	9	9	5	14	10
Confluence	0	2	2	0	0	0	0	8	12
Sandwich sign	0	1	1	0	0	0	0	6	12

TL: Tuberculous lymphadenopathy; TLM: Tuberculous lymphadenopathy accompanied with mesenteritis; NHL: Non-Hodgkin's lymphoma.



**Figure 1** Contrast enhanced CT scan for a 25-year-old man with mesenteric TL showing enlarged lymph nodes in the body and root of SBM with peripheral enhancement (arrow) (A) and in the body of SBM with homogeneous enhancement (arrow) (B). The SBM was contracted and the wall of the small bowel was thickened.



**Figure 2** Contrast enhanced CT scan for a 56-year-old woman with NHL involving SBM showing enlarged lymph nodes in the root of SBM encasing the superior mesenteric artery (arrow), producing the "sandwich sign" (A) and homogeneously mixed peripheral enhancement of lymph nodes in the body of SBM encasing the small bowel mesenteric vessels (arrow), producing the "sandwich sign" (B).

(in the root of SBM) were dispersed in TLM, whereas confluence was found in NHL ( $P < 0.01$ ). "Sandwich sign" (in the root of SBM) was displayed more often in NHL than in mesenteric TL and TLM ( $P < 0.05$ ,  $P < 0.01$ ).

## DISCUSSION

TL is the most common manifestation of abdominal tuberculosis, and tuberculous infection may result in mesenteric lymphadenopathy<sup>[10,13]</sup>. It may be transmitted by three major routes. The first route is ingestion of materials infected with tubercle bacilli which are carried from a lesion in the intestinal submucosal layer to the lymph nodes draining the bowel segment. Drainage is usually from the lymphatics of the ileocecum, jejunum, ileum, and right side of colon to the peripancreatic and superior mesenteric lymph nodes. The second route is

hematogenous spread. Bacteria are disseminated from a distant site of infection, usually the lungs, to the abdominal lymphatic system. Because this process is systemic, it may cause infection of mesenteric lymph nodes. The third route is infection spreading directly to the abdominal lymph nodes from the serosa of adjacent infected structures. Literature *et al*<sup>[14]</sup> reported that most patients with a past history of TB come from areas with a high prevalence of active tuberculosis and have epigastric pain, fever and weight loss, and enlarged nodules with focal calcification sometimes.

SBM, in a series of fan-like ruffles, suspends the jejunum and ileum to the posterior abdominal wall consisting of two posterior peritoneal layers. It is composed of fatty, extraperitoneal connective tissue, blood vessels, nerves, lymph nodes, and peritoneal



investment. The attached border of SBM root extends obliquely from the distal duodenum at the lower border of pancreas on the left side of L2 to the cecum in the right iliac fossa. The line of attachment passes from the duodenojejunal junction over the third portion of the duodenum, then obliquely across the aorta, inferior vena cava, right ureter and psoas major muscle, to the right ilica region<sup>[15-17]</sup>.

The mesenteric lymph nodes can be divided into three subgroups: some lie close to the wall of small intestine, others occur in relation to primary branches of mesenteric vessels, and some consisting of central nodes along the main trunk of the superior mesenteric artery<sup>[18]</sup>.

In this study, 18 patients with tuberculosis and 22 patients with NHL mainly involved mesenteric lymph nodes in the body and root of SBM. Hence, distribution of enlarged lymph nodes in the diseases closely paralleled to the anatomic distribution.

Pathologic findings from surgical specimens of TL indicated that caseation or liquefactive substances at the center of enlarged lymph nodes had a low attenuation presumably resulting from insufficient blood supply, whereas peripheral inflammatory lymphatic tissue had a higher attenuation on enhanced CT resulting from the preserved blood supply<sup>[19]</sup>.

In most patients with untreated NHL, lymph nodes in the SBM increased homogeneously. In 9% of patients with NHL involving the body of SBM and 18% of patients with NHL involving the root of SBM, lymph nodes had a homogeneous and peripheral enhancement. Our findings on the morphology of lymph nodes are similar to those of previous reports, in which the enhancement patterns of untreated NHL are homogeneous or less frequently necrotic with central hypodensity in the neck and mediastinum<sup>[20-22]</sup>.

In this study, 25% of patients had mesenteric TL in the body of SBM, lymph nodes had peripheral enhancement. In 50% of patients with mesenteric TL and 44% of patients with TLM in the root of SBM, lymph nodes had a homogeneous and peripheral enhancement, more than that in NHL.

The focus of this study was to differentiate mesenteric tuberculosis from untreated NHL involving the SBM using contrast-enhanced CT. Anatomic distribution in patients with NHL involving SBM was similar to that in patients with mesenteric TL and TLM. Oliver *et al*<sup>[23]</sup> reported that enlarged lymph nodes have a decreased density and mesenteric stranding in 20% of patients with lymphoma after treatment. However, if relapse of the disease occurs, the lymph nodes appear homogeneous, suggesting that it is important to know if patients with NHL have undergone therapy that may have caused central low attenuation within nodes or mesenteric stranding in SBM, simulating TL involving the SBM.

Neoplastic involvement of the mesentery can be diagnosed in lymphoma on the basis of a characteristic appearance of “sandwich-sign” encasement of the superior mesenteric artery<sup>[18,24]</sup>. In our study, one patient with TL had “sandwich-sign” in the body and root of

SBM. However, it was detected in 27.3% of patients with NHL involving the body of SBM and in 54.5% of patients with NHL involving the root of SBM. When “sandwich-sign” is considered, mesenteric TL and TLM are rarely confused with NHL involving SBM in clinical practice.

One limitation of this study is the relatively small number of cases of tuberculosis involving SBM. Enlarged lymph nodes with a peripheral enhancement in SBM can also be seen in metastatic malignancy and other diseases. In general, if primary malignancy is known, most metastatic malignancies are easily diagnosed. Other causes for mesenteric lymphadenopathy that characteristically demonstrates central low attenuation on CT are Whipple disease<sup>[25]</sup> and cavitating mesenteric lymph node syndrome of celiac disease<sup>[26,27]</sup>. Mesenteric lymphadenopathy has also been reported in patients with familial Mediterranean fever during an acute abdominal attack, Castleman disease and Crohn's disease<sup>[28-31]</sup>.

In conclusion, contrast-enhanced CT can be used in differentiating mesenteric TL and TLM from NHL involving SBM on the basis of enhancement patterns of enlarged lymph nodes and presence of “sandwich-sign”. Mesenteric tuberculosis involves predominantly lymph nodes in the root and body of SBM. Lymph nodes at the margin of SBM are involved in only 5.5% of patients with mesenteric tuberculosis. In contrast, lymph nodes at the margin of SBM are involved in 22.7% of patients with NHL involving SBM. The presence of “sandwich sign” can be more frequently observed in NHL involving SBM than in mesenteric TL and TLM. A distinct difference in characteristic nodal enhancement patterns can also be observed.

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## COMMENTS

### Background

The incidence of tuberculosis is increasing. When the prevalence of abdominal tuberculosis is high, it is difficult to establish its diagnosis. Lymphadenopathy is the most common manifestation of abdominal tuberculosis and may be easily confused with lymphoma involving abdominal lymph nodes. Lymphoma is the most common malignant neoplasm affecting mesentery, and Hodgkin's Lymphoma rarely involves mesentery. We conducted a comparison of CT findings in tuberculosis and non-Hodgkin's lymphoma (NHL) involving small bowel mesentery (SBM) to improve physicians' ability to distinguish between these entities.

### Research frontiers

The incidence of tuberculosis is increasing. Lymphadenopathy is the most common manifestation of abdominal tuberculosis. A comparison of CT findings in tuberculosis and lymphomas of retroperitoneal lymph nodes has been reported.

### Innovations and breakthroughs

SBM, in a series of fan-like ruffles, suspends the jejunum and ileum to the posterior abdominal wall consisting of two posterior peritoneal layers. NHL and tuberculous lymphadenopathy may involve SBM, and the correct diagnosis and

differential diagnosis are important for their clinical treatment. We compared CT findings in tuberculosis and NHL involving SBM to improve the physicians' ability to distinguish between these entities.

### Applications

This study may improve the physicians' ability to distinguish tuberculosis from NHL involving SBM, and specific CT imaging criteria may be used in the differential diagnosis of other malignant tumors involving SBM.

### Peer review

This study evaluated the specific CT imaging criteria for differentiating tuberculosis involving small bowel mesenteric lymph nodes from lymphomas, showing that distribution of anatomic lymph nodes, sandwich sign and specific enhancement patterns of lymphadenopathy in SBM on CT images can be used in differentiating tuberculosis from NHL involving SBM. The study is well designed and interesting.

## REFERENCES

- 1 Goodman PC. Tuberculosis and AIDS. *Radiol Clin North Am* 1995; **33**: 707-717
- 2 Collins FM. Tuberculosis: the return of an old enemy. *Crit Rev Microbiol* 1993; **19**: 1-16
- 3 Cantwell MF, Snider DE Jr, Cauthen GM, Onorato IM. Epidemiology of tuberculosis in the United States, 1985 through 1992. *JAMA* 1994; **272**: 535-539
- 4 Raviglione MC, Snider DE Jr, Kochi A. Global epidemiology of tuberculosis. Morbidity and mortality of a worldwide epidemic. *JAMA* 1995; **273**: 220-226
- 5 Leder RA, Low VH. Tuberculosis of the abdomen. *Radiol Clin North Am* 1995; **33**: 691-705
- 6 Jadvar H, Mindelzun RE, Olcott EW, Levitt DB. Still the great mimicker: abdominal tuberculosis. *AJR Am J Roentgenol* 1997; **168**: 1455-1460
- 7 Dong P, Wang B, Sun YQ. Tuberculous abscess in hepatoduodenal ligament: Evaluation with contrast-enhanced computed tomography. *World J Gastroenterol* 2008; **14**: 2284-2287
- 8 Hulnick DH, Megibow AJ, Naidich DP, Hilton S, Cho KC, Balthazar EJ. Abdominal tuberculosis: CT evaluation. *Radiology* 1985; **157**: 199-204
- 9 Epstein BM, Mann JH. CT of abdominal tuberculosis. *AJR Am J Roentgenol* 1982; **139**: 861-866
- 10 Yang ZG, Min PQ, Sone S, He ZY, Liao ZY, Zhou XP, Yang GQ, Silverman PM. Tuberculosis versus lymphomas in the abdominal lymph nodes: evaluation with contrast-enhanced CT. *AJR Am J Roentgenol* 1999; **172**: 619-623
- 11 Whitley NO, Bohlman ME, Baker LP. CT patterns of mesenteric disease. *J Comput Assist Tomogr* 1982; **6**: 490-496
- 12 Lucey BC, Stuhlfaut JW, Soto JA. Mesenteric lymph nodes: detection and significance on MDCT. *AJR Am J Roentgenol* 2005; **184**: 41-44
- 13 Lucey BC, Stuhlfaut JW, Soto JA. Mesenteric lymph nodes seen at imaging: causes and significance. *Radiographics* 2005; **25**: 351-365
- 14 Xia F, Poon RT, Wang SG, Bie P, Huang XQ, Dong JH. Tuberculosis of pancreas and peripancreatic lymph nodes in immunocompetent patients: experience from China. *World J Gastroenterol* 2003; **9**: 1361-1364
- 15 Okino Y, Kiyosue H, Mori H, Komatsu E, Matsumoto S, Yamada Y, Suzuki K, Tomonari K. Root of the small-bowel mesentery: correlative anatomy and CT features of pathologic conditions. *Radiographics* 2001; **21**: 1475-1490
- 16 Oliphant M, Berne AS. Computed tomography of the subperitoneal space: demonstration of direct spread of intraabdominal disease. *J Comput Assist Tomogr* 1982; **6**: 1127-1137
- 17 Oliphant M, Berne AS, Meyers MA. Spread of disease via the subperitoneal space: the small bowel mesentery. *Abdom Imaging* 1993; **18**: 109-116
- 18 Mueller PR, Ferrucci JT Jr, Harbin WP, Kirkpatrick RH, Simeone JF, Wittenberg J. Appearance of lymphomatous involvement of the mesentery by ultrasonography and body computed tomography: the "sandwich sign". *Radiology* 1980; **134**: 467-473
- 19 Griffith RC, Janney CG. Lymph nodes. In: Kissance JM, editor. *Anderson's pathology*, 9th ed. St. Louis: Mosby, 1990: 1429-1492
- 20 Pombo F, Rodriguez E, Caruncho MV, Villalva C, Crespo C. CT attenuation values and enhancing characteristics of thoracoabdominal lymphomatous adenopathies. *J Comput Assist Tomogr* 1994; **18**: 59-62
- 21 Lee YY, Van Tassel P, Nauert C, North LB, Jing BS. Lymphomas of the head and neck: CT findings at initial presentation. *AJR Am J Roentgenol* 1987; **149**: 575-581
- 22 Hopper KD, Diehl LF, Cole BA, Lynch JC, Meilstrup JW, McCauslin MA. The significance of necrotic mediastinal lymph nodes on CT in patients with newly diagnosed Hodgkin disease. *AJR Am J Roentgenol* 1990; **155**: 267-270
- 23 Oliver TW Jr, Bernardino ME, Sones PJ Jr. Monitoring the response of lymphoma patients to therapy: correlation of abdominal CT findings with clinical course and histologic cell type. *Radiology* 1983; **149**: 219-224
- 24 Sheth S, Horton KM, Garland MR, Fishman EK. Mesenteric neoplasms: CT appearances of primary and secondary tumors and differential diagnosis. *Radiographics* 2003; **23**: 457-473; quiz 535-536
- 25 Friedman HD, Hadfield TL, Lamy Y, Fritzing D, Bonaventura M, Cynamon MT. Whipple's disease presenting as chronic wastage and abdominal lymphadenopathy. *Diagn Microbiol Infect Dis* 1995; **23**: 111-113
- 26 Schmitz F, Herzig KH, Stuber E, Tiemann M, Reinecke-Luthge A, Nitsche R, Folsch UR. On the pathogenesis and clinical course of mesenteric lymph node cavitation and hyposplenism in coeliac disease. *Int J Colorectal Dis* 2002; **17**: 192-198
- 27 Al-Kawas FH, Murgo A, Foshag L, Shiels W. Lymphadenopathy in celiac disease: not always a sign of lymphoma. *Am J Gastroenterol* 1988; **83**: 301-303
- 28 Zissin R, Rathaus V, Gayer G, Shapiro-Feinberg M, Hertz M. CT findings in patients with familial Mediterranean fever during an acute abdominal attack. *Br J Radiol* 2003; **76**: 22-25
- 29 Ferreira J, Gomez Leon N, Mata ML, Casanova R, Pedrosa CS, Cuevas A. Computed tomography in abdominal Castleman's disease. *J Comput Assist Tomogr* 1989; **13**: 433-436
- 30 Avila NA, Ling A, Worobec AS, Mican JM, Metcalfe DD. Systemic mastocytosis: CT and US features of abdominal manifestations. *Radiology* 1997; **202**: 367-372
- 31 Healy JC, Reznick RH. The peritoneum, mesenteries and omenta: normal anatomy and pathological processes. *Eur Radiol* 1998; **8**: 886-900

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# A new approach to endoscopic treatment of tumors of the esophagogastric junction with individually designed self-expanding metal stents

Serhat Aymaz, Arno J Dormann

Serhat Aymaz, Arno J Dormann, Department of Medicine, Cologne City Hospital, Holweide, Neufelder Strasse 32, Cologne D-51067, Germany

Author contributions: Aymaz S and Dormann AJ contributed equally to this work.

Correspondence to: Serhat Aymaz, MD, MSc, Department of Medicine, Cologne City Hospital, Holweide, Neufelder Strasse 32, Cologne D-51067, Germany. [AymazS@kliniken-koeln.de](mailto:AymazS@kliniken-koeln.de)  
Telephone: +49-221-89072527 Fax: +49-221-89072388

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## Abstract

The incidence of adenocarcinoma of the esophagogastric junction is constantly increasing. Curative treatment is no longer possible at the time of diagnosis in more than 50% of patients with esophageal carcinoma, and palliative treatment focusing on eliminating dysphagia is required. Endoscopic therapy with stent implantation is an established method of achieving this. It can be carried out quickly, with a low rate of early complications, and leads to fast symptomatic improvement, assessed using the dysphagia score. The relatively high rate of late complications such as stent migration, hemorrhage, and gastroesophageal mucosal prolapse has led to recent debate on the role of metal stents in palliative therapy. We present here a new type of stent design for transcatheter application, which is intended to prevent bleeding due to mechanical mucosal lesions caused by the distal end of the stent extending into the stomach. The further intention of this case report is to force the discussion on individually designed nitinol stents in special anatomic conditions.

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**Key words:** Esophagus cancer; Treatment; Palliative therapy; Endoscopic therapy; Stent; Cardiac cancer

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## INTRODUCTION

The incidence of adenocarcinoma of the esophagogastric junction is constantly increasing<sup>[1,2]</sup>. Curative treatment is no longer possible at the time of diagnosis in more than 50% of patients with esophageal carcinoma, and palliative treatment<sup>[3]</sup> focusing on eliminating dysphagia is required. Endoscopic therapy with stent implantation is an established method of achieving this. It can be carried out quickly, with a low rate of early complications, and leads to fast symptomatic improvement, assessed using the dysphagia score<sup>[4-7]</sup>. The relatively high rate of late complications such as stent migration, hemorrhage, and gastroesophageal mucosal prolapse has led to recent debate on the role of metal stents in palliative therapy<sup>[8]</sup>. We present here a new type of stent design for transcatheter application, which is intended to prevent bleeding due to mechanical mucosal lesions caused by the distal end of the stent extending into the stomach. The further intention of this case report is to force the discussion on individually designed nitinol stents in special anatomic conditions.

## CASE REPORT

An 82-year-old patient presented at our department with 2-wk dysphagia, followed most recently by intermittent vomiting. He had a history of progressive prostate carcinoma (T3b Nx M0), which was treated with orchiectomy and antiandrogen therapy (bicalutamide). As there was local progression with infiltration of the urinary bladder, radiotherapy was planned. In addition, the patient had type 2 diabetes mellitus and arterial hypertension.

Gastroscopy showed a dilated esophagus, corresponding to the radiographic findings, with a high-grade stenosis 38 cm from the incisors, cranial to a 3-cm





**Figure 1** Radiographic image of the esophagus stenosis.



**Figure 4** Radiographic image of the stent.



**Figure 2** Endoscopic view of the hiatus hernia after passage through the tumor stenosis.



**Figure 5** Endoscopic view of the distal end of the stent in inversion, 2 mo after treatment.



**Figure 3** New stent design (Micro-Tech [Nanjing] Co. Ltd., Nanjing, China; distributed by Leufen Medizintechnik, Aachen, Germany).

long sliding hernia (Figures 1 and 2). The stenosis was diagnosed histologically as an adenocarcinoma of the esophagogastric junction, which already developed a hepatic metastasis. Reviewing the findings, we decided to carry out palliative stent implantation to treat the stenosis. As transcatheter positioned stents are associated with higher complication rates<sup>[9]</sup> and experience shows that bleeding often occurs due to stent-related mucosal lesions in the stomach, we requested individual production of an unusually shaped self-expanding nitinol stent (SEMS) (Figure 3). Decisive factors in developing this new designed SEMS included the special anatomic conditions in this patient, with a medium-sized esophageal hernia. The stent was woven from nitinol in accordance with our specifications and was 140 mm long and 24 mm wide. A 30-mm wide bulb was formed at the cranial end of the stent to prevent stent migration. The distal end of the stent was to bend cranially to ensure that the stent would

fit the cardia and not extend freely into the stomach. In addition, a circular ring-like widening was added 2 cm above the distal end of the stent to prevent migration. With the exception of the uncovered proximal bulb, the stent was completely covered.

After the patient's informed consent was obtained, the stent was placed on a carrier system with a diameter of 8 mm and released in the conventional way from the distal end by withdrawing an over tube. After the applicator with the stent was positioned fluoroscopically at the tumor level over a guide wire (Boston Scientific, Super Stiff Guidewire, 0.035 inch), the distal end of the stent was partially released. The proximally bent end of the stent was then pulled until the distal end fitted the cardia tightly. Finally, the stent was fully released by pulling and positioned without any special difficulties. The patient was able to take solid food 24 h later. A radiographic check showed that the stent was correctly positioned (Figure 4). No incidents of bleeding, vomiting as evidence of mucosal prolapse, or recurrent dysphagia occurred during a 4-mo follow-up period. An endoscopic check-up 2 mo after introduction of the stent showed that it was still correctly positioned, with no evidence of mucosal lesions (Figure 5).

## DISCUSSION

Although the use of self-expanding metal stents is now an established part of palliative treatment for esophageal carcinomas, late complications are frequent.



The complication rate is higher when the stent is in a transcatheter position. In our experience, however, stent migrations that are frequently reported occur much less often when a partially covered stent with a large diameter is used, and previous dilation of the tumor stenosis has not been carried out<sup>[10]</sup>. Nevertheless, bleeding, particularly due to mechanical lesions caused by the distal end of the stent which extends into the stomach, and mucosal prolapse with occlusion of the stent, still continue to be major problems. The aim in this case was therefore to take advantage of an individual stent design and provide an optimal solution in the palliative situation for this patient.

The stent described above was designed and manufactured in close collaboration with the producer and distributor of the nitinol stent within 10 d in order to avoid mechanical lesions of the mucosa in this special anatomic situation. The stent was positioned without any special difficulties in conventional way. The early clinical result was good after 24 h. The radiographic, clinical and endoscopic follow-up during a 4-mo period showed that the stent was still correctly positioned and none of usual complications occurred.

We believe that the stent design presented here is particularly suitable for palliative treatment of stenotic distal esophageal carcinomas. The advantage of this stent design is particularly clear when an axial hiatus hernia is present. Further optimization of the design and studies comparing it with conventional stents are required. We believe that individual stent designing could alleviate many of the late complications associated with stent treatment.

## REFERENCES

- 1 **Blot WJ**, Devesa SS, Fraumeni JF Jr. Continuing climb in rates of esophageal adenocarcinoma: an update. *JAMA* 1993; **270**: 1320
- 2 **Devesa SS**, Blot WJ, Fraumeni JF Jr. Changing patterns in the incidence of esophageal and gastric carcinoma in the United States. *Cancer* 1998; **83**: 2049-2053
- 3 **Siersema PD**, Marcon N, Vakil N. Metal stents for tumors of the distal esophagus and gastric cardia. *Endoscopy* 2003; **35**: 79-85
- 4 **Shimi SM**. Self-expanding metallic stents in the management of advanced esophageal cancer: a review. *Semin Laparosc Surg* 2000; **7**: 9-21
- 5 **Siersema PD**, Schrauwen SL, van Blankenstein M, Steyerberg EW, van der Gaast A, Tilanus HW, Dees J. Self-expanding metal stents for complicated and recurrent esophagogastric cancer. *Gastrointest Endosc* 2001; **54**: 579-586
- 6 **Baerlocher MO**, Asch MR, Vellahottam A, Puri G, Andrews K, Myers A. Safety and efficacy of gastrointestinal stents in cancer patients at a community hospital. *Can J Surg* 2008; **51**: 130-134
- 7 **Nathwani RA**, Kowalski T. Endoscopic stenting of esophageal cancer: the clinical impact. *Curr Opin Gastroenterol* 2007; **23**: 535-538
- 8 **Ross WA**, Alkassab F, Lynch PM, Ayers GD, Ajani J, Lee JH, Bismar M. Evolving role of self-expanding metal stents in the treatment of malignant dysphagia and fistulas. *Gastrointest Endosc* 2007; **65**: 70-76
- 9 **Spinelli P**, Cerrai FG, Ciuffi M, Ignomirelli O, Meroni E, Pizzetti P. Endoscopic stent placement for cancer of the lower esophagus and gastric cardia. *Gastrointest Endosc* 1994; **40**: 455-457
- 10 **Dormann AJ**, Eisendrath P, Wiggighaus B, Huchzermeyer H, Deviere J. Palliation of esophageal carcinoma with a new self-expanding plastic stent. *Endoscopy* 2003; **35**: 207-211

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## CASE REPORT

# Subcutaneous cervical emphysema and pneumomediastinum due to a lower gastrointestinal tract perforation

Georg B Schmidt, Maarten W Bronkhorst, Henk H Hartgrink, Lee H Bouwman

Georg B Schmidt, Henk H Hartgrink, Department of Surgery, Leiden University Medical Center, Leiden RC 2300, The Netherlands

Maarten W Bronkhorst, Lee H Bouwman, Department of Surgery, Bronovo Hospital, The Hague AX 2597, The Netherlands

**Author contributions:** Schmidt GB, Bronkhorst MW, Hartgrink HH and Bouwman LH contributed equally to this article.

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**Correspondence to:** Georg B Schmidt, MD, Leiden University Medical Center, Albinusdreef 2, PO-box 9600, Leiden RC 2300, The Netherlands. [g.b.schmidt@lumc.nl](mailto:g.b.schmidt@lumc.nl)

**Telephone:** +31-71-5269111 **Fax:** +31-71-5266750

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geal or chest trauma. It can also occur spontaneously in association with asthma, excessive coughing, or straining. Cervical emphysema occurs when air moves through tissue planes into subcutaneous areas of the face and neck. Subcutaneous neck emphysema, pneumomediastinum, and retroperitoneum have been reported infrequently following colonoscopic perforation. Iatrogenic colonic perforation is a serious but rare complication of colonoscopy. A perforation risk rate of 0.12% has been reported<sup>[1]</sup>.

Subcutaneous emphysema caused by non-traumatic perforations of the colon is extremely rare. However, it should be considered when no obvious case can be found for the origin of subcutaneous emphysema or a pneumomediastinum.

## Abstract

This case report describes a 69-year-old man presenting with an extensive subcutaneous emphysema in his neck and generalized peritonitis caused by a lower gastrointestinal tract perforation. This case emphasizes that subcutaneous emphysema patients with negative thoracic findings should be scrutinized for signs of retroperitoneal hollow viscus perforation.

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**Key words:** Subcutaneous cervical emphysema; Pneumomediastinum; Gastrointestinal tract perforation; Malignancy; Diverticulitis

**Peer reviewer:** Wei Tang, MD, EngD, Assistant Professor, H-B-P Surgery Division, Artificial Organ and Transplantation Division, Department of surgery, Graduate School of Medicine, the University of Tokyo, Tokyo 113-8655, Japan

Schmidt GB, Bronkhorst MW, Hartgrink HH, Bouwman LH. Subcutaneous cervical emphysema and pneumomediastinum due to a lower gastrointestinal tract perforation. *World J Gastroenterol* 2008; 14(24): 3922-3923 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3922.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3922>

## CASE REPORT

A 69-year-old man presented himself at the Accident and Emergency Department with a 6-h history of swelling in the neck, an altered voice and abdominal pain. He was treated for a painful fifth rib on the left which was caused by a metastasis of an unknown primary tumor. Analysis for the primary tumor was ongoing, but not yet concluded. Radiotherapy was started and prednisolone was prescribed.

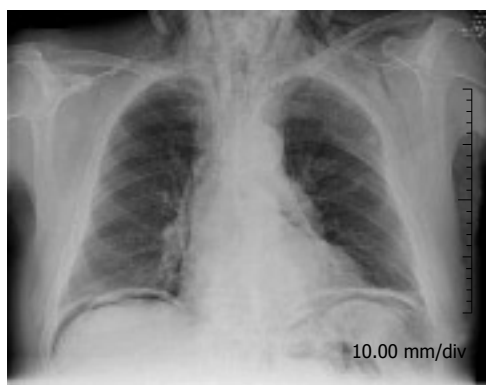
Physical examination revealed signs of extensive subcutaneous emphysema in his neck and generalized peritonitis. Laboratory blood and urine tests were normal apart from an elevated white cell count of  $23 \times 10^9$  cells/L. A plain radiograph of the thorax showed free intraperitoneal air, pneumomediastinum and extensive subcutaneous emphysema, but no sign of pneumothorax (Figure 1).

An explorative laparotomy was performed, a perforation of the small bowel and the caecum was found, which were oversew. There were two perforations of the sigmoid but no palpable tumor. A sigmoid resection was performed with a permanent colostomy. The proximal end of the distal segment was oversew and left in place with a blind rectal pouch. The patient was admitted to the Intensive Care Unit after operation. Pathological examination showed diverticulitis with a perforation, and no tumor was found in the resected sigmoid.

After consultation with the patient and his family, the patient received no further surgical treatment. The patient died of respiratory arrest 12 d after surgery.

## INTRODUCTION

Pneumomediastinum usually occurs following esopha-



**Figure 1** Thorax X-ray examination of the patient showing free air in abdomen, pneumomediastinum and severe subcutaneous cervical emphysema.

## DISCUSSION

Non-traumatic subcutaneous emphysema is a rare presentation of lower gastrointestinal tract perforation due to colorectal cancer or diverticulitis<sup>[2-6]</sup>.

As the rectosigmoid is located in the retroperitoneum, injury can be present in the absence of peritonitis. Mediastinal and cervical emphysema may develop due to dissection of air *via* contiguous tissue planes, which occurs along the perivascular adventitia to the anterior pararenal space, through the diaphragmatic hiatus along the adventitia of great vessels to the mediastinum and pericardium/or pretracheal fascia to the neck<sup>[7,8]</sup>.

Subcutaneous emphysema patients with negative thoracic findings should be scrutinized for signs of retroperitoneal hollow viscus perforation to improve their outcome.

## REFERENCES

- 1 **Lüning TH**, Keemers-Gels ME, Barendregt WB, Tan AC, Rosman C. Colonoscopic perforations: a review of 30,366 patients. *Surg Endosc* 2007; **21**: 994-997
- 2 **Chu S**, Glare P. Subcutaneous emphysema in advanced cancer. *J Pain Symptom Manage* 2000; **19**: 73-77
- 3 **Morita T**, Matsuda T, Tei Y, Takada T. Nontraumatic subcutaneous emphysema from rectal cancer perforation completely resolved after intensive pain control. *J Pain Symptom Manage* 2006; **32**: 3-4
- 4 **Hur T**, Chen Y, Shu GH, Chang JM, Cheng KC. Spontaneous cervical subcutaneous and mediastinal emphysema secondary to occult sigmoid diverticulitis. *Eur Respir J* 1995; **8**: 2188-2190
- 5 **Nedrebo T**. [Subcutaneous emphysema in gastrointestinal tract perforation] *Tidsskr Nor Lægeforen* 1992; **112**: 2855-2856
- 6 **Prete R**, Rohner A. [Pneumomediastinum and subcutaneous cervical emphysema as signs of rectosigmoid perforation] *Gastroenterol Clin Biol* 1992; **16**: 460-462
- 7 **Fitzgerald SD**, Denk A, Flynn M, Longo WE, Vernava AM 3rd. Pneumopericardium and subcutaneous emphysema of the neck. An unusual manifestation of colonoscopic perforation. *Surg Endosc* 1992; **6**: 141-143
- 8 **Ho HC**, Burchell S, Morris P, Yu M. Colon perforation, bilateral pneumothoraces, pneumopericardium, pneumomediastinum, and subcutaneous emphysema complicating endoscopic polypectomy: anatomic and management considerations. *Am Surg* 1996; **62**: 770-774

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## CASE REPORT

# Duplication cyst of the small intestine found by double-balloon endoscopy: A case report

Haruei Ogino, Toshiaki Ochiai, Norimoto Nakamura, Daisuke Yoshimura, Teppei Kabemura, Tetsuya Kusumoto, Hiroshi Matsuura, Akihiko Nakashima, Kuniomi Honda, Kazuhiko Nakamura

Haruei Ogino, Toshiaki Ochiai, Norimoto Nakamura, Daisuke Yoshimura, Teppei Kabemura, Department of Internal Medicine, Saiseikai Fukuoka General Hospital, Higashi-ku Maidashi 3-1-1, Fukuoka-shi, Fukuoka-ken 812-0054, Japan

Tetsuya Kusumoto, Hiroshi Matsuura, Department of Surgery, Saiseikai Fukuoka General Hospital, Higashi-ku Maidashi 3-1-1, Fukuoka-shi, Fukuoka-ken 812-0054, Japan  
Akihiko Nakashima, Department of Pathology, Saiseikai Fukuoka General Hospital, Higashi-ku Maidashi 3-1-1, Fukuoka-shi, Fukuoka-ken 812-0054, Japan

Kuniomi Honda, Kazuhiko Nakamura, Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Higashi-ku Maidashi 3-1-1, Fukuoka-shi, Fukuoka-ken 812-0054, Japan

**Author contributions:** Ogino H and Ochiai T contributed equally to this work; Kusumoto T and Matsuura H performed the surgical operation; Honda K performed the double-balloon endoscopy; Nakashima A did the pathological evaluation; Ogino H and Nakamura K wrote the paper.

**Correspondence to:** Kazuhiko Nakamura, Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Higashi-ku Maidashi 3-1-1, Fukuoka-shi, Fukuoka-ken 812-0054, Japan. [knakamur@intmed3.med.kyushu-u.ac.jp](mailto:knakamur@intmed3.med.kyushu-u.ac.jp)

Telephone: +81-92-6425286 Fax: +81-92-642-5287

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**Key words:** Duplication cyst; Double-balloon endoscopy; Small intestine bleeding

**Peer reviewer:** Nageshwar D Reddy, Professor, Asian Institute of Gastroenterology, 6-3-652, Somajiguda, Hyderabad-500 082, India. [aigindia@yahoo.co.in](mailto:aigindia@yahoo.co.in)

Ogino H, Ochiai T, Nakamura N, Yoshimura D, Kabemura T, Kusumoto T, Matsuura H, Nakashima A, Honda K, Nakamura K. Duplication cyst of the small intestine found by double-balloon endoscopy: A case report. *World J Gastroenterol* 2008; 14(24): 3924-3926 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3924.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3924>

## INTRODUCTION

Double-balloon endoscope (DBE) is a new endoscopic device designed to examine the small intestine. Observation of the entire small intestine can be achieved through a combination of anal and oral approaches. Endoscopic interventions such as mucosal biopsy, clipping, argon plasma and polypectomy can also be performed. We often encounter patients with obscure gastrointestinal bleeding in which bleeding cause cannot be revealed by the usual methods of esophagogastroduodenoscopy (EGD) and colonoscopy. DBE is thus used to find the origin of small intestinal bleeding. Here we report a case in which the origin of the patient's gastrointestinal bleeding was found with DBE and diagnosed as a duplication cyst of the ileum after surgery.

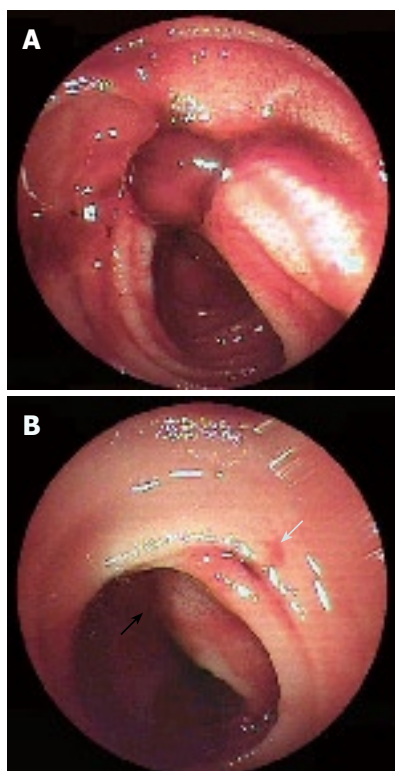
## CASE REPORT

A 35-year-old man was admitted to a hospital with the complaint of right lower abdominal pain in April, 2003. Appendicitis was suspected and appendectomy was performed. However, he continued to suffer from occasional abdominal discomfort and symptoms of subileus. Early in August, bloody stools appeared. Iron deficiency anemia (hemoglobin level, 8.1 g/dL) was also noted. Although EGD, colonoscopy and

## Abstract

A 35-year-old man was admitted due to bloody stool and anemia. The bleeding source could not be detected by esophagogastroduodenoscopy or colonoscopy. Double balloon endoscopy (DBE) revealed a diverticulum-like hole in which coagula stuck in the ileum at 1 meter on the oral side from the ileocecal valve. The adjacent mucosa just to the oral side of the hole was elevated like a submucosal tumor. The lesion was considered the source of bleeding and removed surgically. It was determined to be a cyst with an ileal structure on the mesenteric aspect accompanying gastric mucosa. The diagnosis was a duplication cyst of the ileum, which is a rare entity that can cause gastrointestinal bleeding. In the present case, DBE was used to find the hemorrhagic duplication cyst in the ileum.



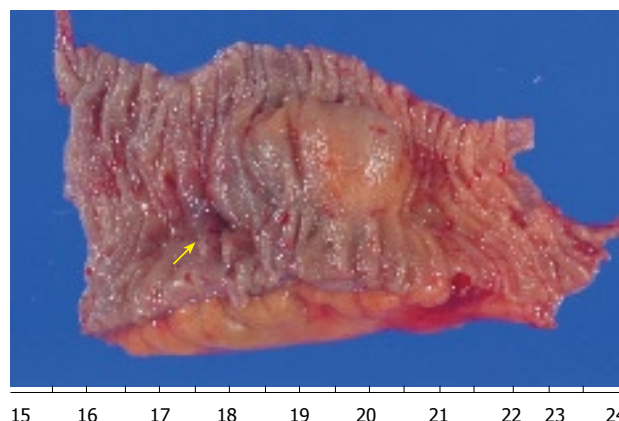


**Figure 1** Double-balloon endoscopy by a perianal approach revealing coagula in the ileum at 1 m on the oral side of the ileocecal valve (A) and a diverticulum-like hole (white arrow) after removal of the coagula with an elevation like a submucosal tumor on the oral side of the hole (black arrow) (B).

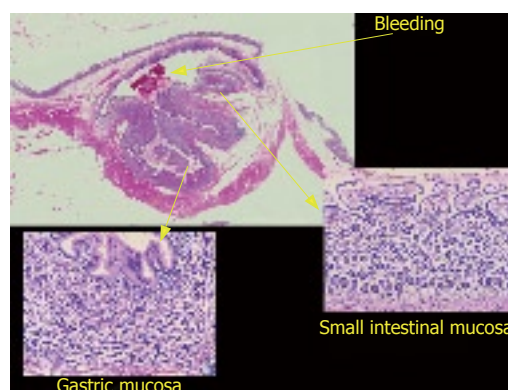
radiological enteroclysis were performed, the origin of the gastrointestinal bleeding remained unknown.

At the end of August 2003, the right lower abdomen pain appeared again after a meal. Inflammatory reactions were also elevated. Thus, he was referred to our hospital for further examination. Physical examination showed local peritonitis. Abdominal CT displayed ascites in the circumference of the liver and thickness of the distal small intestine wall in the pelvis. With appropriate medical treatment and antibiotic therapy, his condition and the inflammatory reaction improved. Colonoscopy and radiological enteroclysis failed to ascertain any lesions responsible for the symptoms. The patient was thus discharged from hospital.

His condition was good until bloody stool suddenly appeared again in July, 2005. He was admitted to our hospital the next day. Anemia gradually progressed. Scintigraphy for hemorrhage revealed a deposit in the ileum end. Colonoscopy, however, failed to detect the source of the bleeding. The patient was then referred to Kyushu University Hospital. DBE performed using a perianal approach revealed coagula adhered to the ileal wall about 1 m on the oral side from the ileocecal valve (Figure 1A). When the coagula were removed, a bleeding diverticulum-like hole appeared and the adjacent mucosa just on the oral side of the hole was elevated like a submucosal tumor (Figure 1B). We injected hypertonic saline with epinephrine and also injected India ink near it as a marker. However, intermittent bleeding continued after the procedure. The patient, therefore, underwent



**Figure 2** Macroscopic appearance of the resected specimen. The cyst was located on the mesenteric aspect and a diverticulum-like hole (arrow) was detected on the anal side.



**Figure 3** HE staining for the resected tissue showing an ileal structure with gastric mucosa and bleeding in the cyst.

an urgent operation. When the inside of the abdominal cavity was observed, a cyst was found on the mesenteric side of the ileum, about 1mm proximal to the ileocecal valve, and connected to the ileocecal valve by an adhesion band. The lesion was considered the bleeding source and thus resected. Histological examination revealed that the cyst was located on the mesenteric aspect and had an ileal structure with gastric mucosa (Figures 2 and 3). A diagnosis of duplication cyst of the ileum was thus made. The patient was discharged from hospital 10 d after surgery and has remained symptom-free since then.

## DISCUSSION

Duplication cysts are congenital malformations that can arise throughout the alimentary tract from the oral cavity to the anus. The majority are diagnosed in infancy and childhood. Duplication cysts of the small intestine constitute about 60% of those in alimentary tract and are located on the mesenteric aspect, in contrast to a Meckel's diverticulum that localizes on the antimesenteric aspect. Duplication cysts are classified into spherical and tubular types. The former is more commonly found in the small intestine<sup>[1]</sup>. The present case was considered a

spherical one.

Hoshi *et al*<sup>[2]</sup> reported that 34% of duplication cyst patients complain of stomachache, 24% vomiting, 17% an abdomen mass and 10.5% bloody stool. In our case, because of the right lower abdominal pain, appendectomy was performed 2 years ago. After the operation, the various symptoms continued. Though these symptoms seemed to be due to a duplication cyst, the relevant diagnosis was not made until 2 years later.

A preoperative diagnosis of duplication cyst is difficult. Indeed, only 11.2% have been correctly diagnosed before operation in Japan. Eighteen point two percent are diagnosed as intussusception, 15.1% as an abdominal mass, 14.4% as ileus, and 26.7% are not able to be diagnosed<sup>[2]</sup>. Cases diagnosed before operation have large lesions that are detectable by abdominal CT and US<sup>[3]</sup>.

Previously, it was relatively difficult to diagnose a small intestinal lesion. However, diagnostic strategy changes with the availability of DBE<sup>[4]</sup> and wireless capsule endoscopy (CE). In fact, Toth *et al*<sup>[5]</sup> reported that they could find a tubular type duplication cyst of the small intestine by CE, suggesting that CE can reveal circumferentially ulcerated stenosis in the ileum. There is no report on an image of a spherical type of duplication cyst in the small intestine detected by endoscopy. Here, for the first time, we showed an image of a spherical

type of duplication cyst obtained by DBE. The cyst was too small to be detected by other modalities such as CT, US and radiological enteroclysis.

Duplication cysts are rare in adults and there are few reports on a diagnosis made before operation. Thus ascertaining the existence of such a lesion is difficult. We presented a case of a duplication cyst of the small intestine found by DBE. We expect that duplication cysts may be more frequently found with the future spread of DBE and CE.

## REFERENCES

- 1 **Yokoyama J.** [Duplications of the alimentary canal] *Nippon Rinsho* 1994; **Suppl 6**: 408-410
- 2 **Hoshi K,** Ohta M, Kanemura E, Koganei K, Takahashi M, Kito F and Fukushima T. A case of ileal duplication presenting with bloody stools. *J Japan Soc Coloproctol.* 2002; **55**:43-46
- 3 **Hocking M,** Young DG. Duplications of the alimentary tract. *Br J Surg* 1981; **68**: 92-96
- 4 **Yamamoto H,** Sekine Y, Sato Y, Higashizawa T, Miyata T, Iino S, Ido K, Sugano K. Total enteroscopy with a nonsurgical steerable double-balloon method. *Gastrointest Endosc* 2001; **53**: 216-220
- 5 **Toth E,** Lillienau J, Ekelund M, Alumets J, Olsson R, Thorlacius H. Ulcerated small-intestine duplication cyst: an unusual source of GI bleeding revealed by wireless capsule endoscopy. *Gastrointest Endosc* 2006; **63**: 192-194

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## Intraperitoneal metastasis of hepatocellular carcinoma after spontaneous rupture: A case report

Min-Chang Hung, Hurng-Sheng Wu, Yueh-Tsung Lee, Chih-Hung Hsu, Dev-Aur Chou, Min-Ho Huang

Min-Chang Hung, Hurng-Sheng Wu, Yueh-Tsung Lee, Chih-Hung Hsu, Dev-Aur Chou, Min-Ho Huang, Department of Surgery, Chang Bing Show-Chwan Memorial Hospital, Changhua 505, Taiwan, China

**Author contributions:** Hung MC wrote the paper; Wu HS revised the paper; Lee YT, Hsu CH, Chou DA, and Huang MH performed the research.

**Correspondence to:** Min-Chang Hung, Department of surgery, Chang Bing Show-Chwan Memorial Hospital, No. 6, Lugang Rd., Lugang Township, Changhua 505, Taiwan, China. [hmjohn@mail2000.com.tw](mailto:hmjohn@mail2000.com.tw)

Telephone: +886-4-7813888-73120 Fax: +886-4-7073226

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### INTRODUCTION

Hepatocellular carcinoma (HCC) is one of the most common malignancies worldwide. It is usually manifested in the 6th and 7th decades of life. Extrahepatic metastases are seen in 64% of patients with HCC. The most frequent sites of extrahepatic metastases are lung, abdominal lymph node and bone, but peritoneal dissemination is unusual<sup>[1,2]</sup>. The incidence of spontaneous rupture of HCC is about 8%-26% in Asia<sup>[3-5]</sup> and the mortality rate of HCC patients is 10%<sup>[6]</sup>. However, peritoneal metastasis of HCC after spontaneous rupture is seldom noted. Here, we report a case of intraperitoneal metastasis of HCC after spontaneous rupture 10 mo ago, which was treated with transarterial embolization.

### Abstract

Rupture of hepatocellular carcinoma (HCC) is a life-threatening complication. Peritoneal metastasis of HCC after spontaneous rupture was seldom noted. We report a case of intraperitoneal metastasis of HCC after spontaneous rupture. A previously asymptomatic 72-year-old man was admitted due to dull abdominal pain with abdominal fullness. He had a history of HCC rupture 10 mo ago and transarterial embolization was performed at that time. Abdominal computer tomography (CT) scan showed a huge peritoneal mass over the right upper quadrant area. Surgical resection was arranged and subsequent microscopic examination confirmed a diagnosis of moderately-differentiated HCC.

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**Key words:** Hepatocellular carcinoma; Spontaneous rupture; Peritoneal metastasis

**Peer reviewers:** Fritz E von Weizsacker, Professor, Department of Medicine, Schlosspark Klinik, Humboldt University, Berlin 14059, Germany; Dr. Paolo Del Poggio, Hepatology Unit, Department of Internal Medicine, Treviglio Hospital, Piazza Ospedale 1, Treviglio Bg 24047, Italy

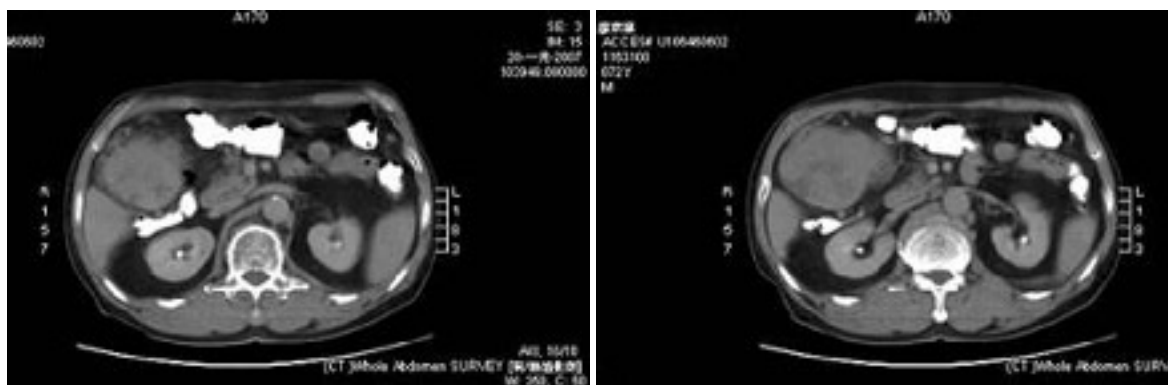
Hung MC, Wu HS, Lee YT, Hsu CH, Chou DA, Huang MH. Intraperitoneal metastasis hepatocellular carcinoma after spontaneous rupture: A case report. *World J Gastroenterol* 2008; 14(24): 3927-3931 Available from: URL: <http://www.wjgnet.com/1007-9327/14/3927.asp> DOI: <http://dx.doi.org/10.3748/wjg.14.3927>

### CASE REPORT

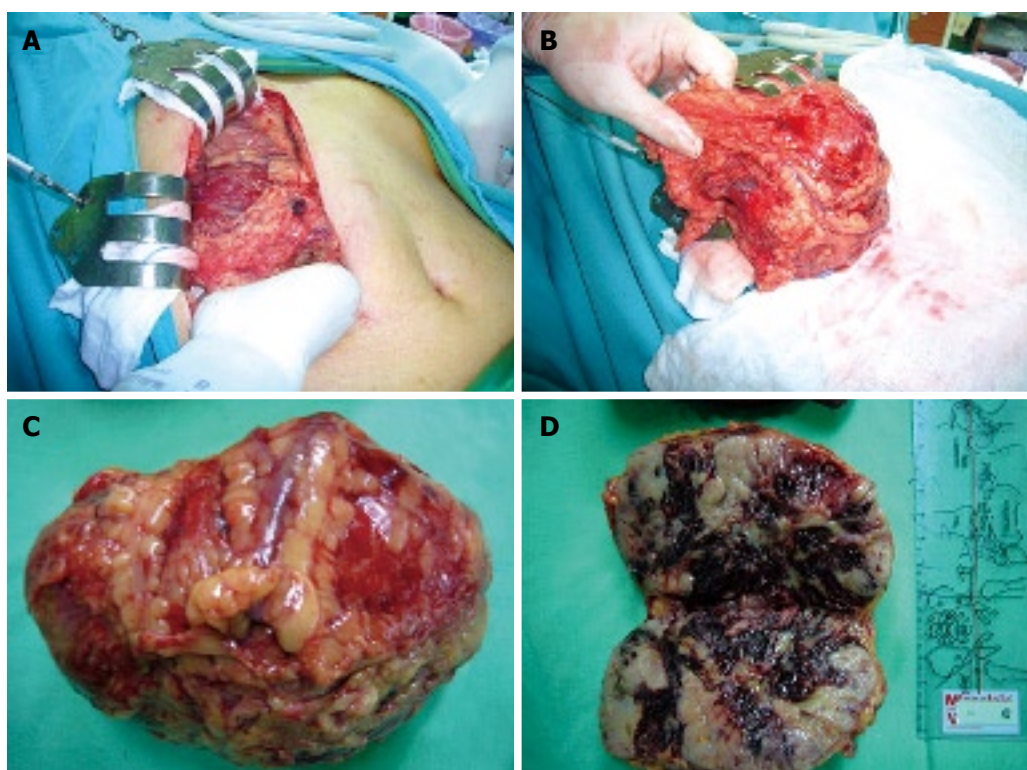
A previously asymptomatic 72-year-old man had a history of chronic hepatitis C-related liver cirrhosis without regular follow-up. Sudden nausea and vomiting with watery diarrhea were noted on January 2006. Then he was sent to Yun-Lin Branch of National Taiwan University Hospital for help. Abdominal computer tomography (CT) scan showed a huge HCC that was suspicious of rupture. Under the request of his family, he was transferred to our hospital and transarterial embolization was performed on January 31, 2006. After discharge, he was regularly followed up at our Gastrointestinal (GI) Outpatient Department (OPD). Dull abdominal pain over the right upper quadrant area, accompanied with fullness sensation, was noted in November 2006. Besides, he also had body weight loss of about ten kilograms in one year. So he visited our hospital again. Abdominal CT scan revealed a peritoneal mass in the right upper quadrant peritoneal area and hepatoma recurrence was considered (Figure 1). Transarterial embolization was arranged again, but failed. After consultation with the surgeon, he was admitted for surgical resection.

Surgical intervention was arranged on January 24, 2007. Operative methods were segmental hepatectomy (S6 and partial S5), excision of extrahepatic tumor, and cholecystectomy. The operation showed a huge tumor (12 cm × 8 cm × 6 cm) over the right upper quadrant area just below liver parenchyma (Figure 2) with its blood supplied from the omentum. Besides, two small mass lesions (3 cm × 2 cm and 2 cm × 1 cm) were found over





**Figure 1** A well-defined mass about 10 cm in diameter in RUQ peritoneal cavity anterior to liver parenchyma.



**Figure 2** A huge tumor (12 cm × 8 cm × 6 cm) over the right upper quadrant area just below liver (A), blood supply of tumor from the omentum (B), and intraperitoneal tumor (C, D).

S5 and S6, respectively (Figure 3). Microscopy showed that the huge extrahepatic tumor and two intrahepatic lesions were moderately differentiated.

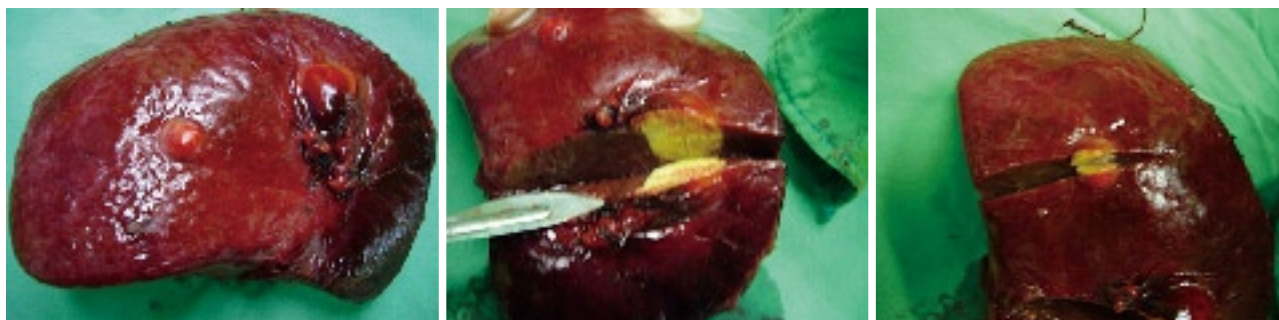
## DISCUSSION

Intraperitoneal metastasis of non-ruptured HCC is rare, but the risk of such a metastasis of ruptured HCC increases<sup>[7]</sup>. The mechanism of spontaneous rupture of HCC is not exactly clear. Hypotheses include rapid expansion of the tumor and central necrosis, venous hypertension caused by venous obstruction due to direct tumor invasion, mild trauma or compression by the diaphragm associated with respiratory movement, coagulopathies such as thrombocytopenia and disturbed prothrombin synthesis, and vascular injury leading to hemorrhage and subsequent rupture<sup>[4,8-10]</sup>. Recently, Zhu *et al* postulated that the poor function of macrophage phagocytosis could result in cumulation of immune

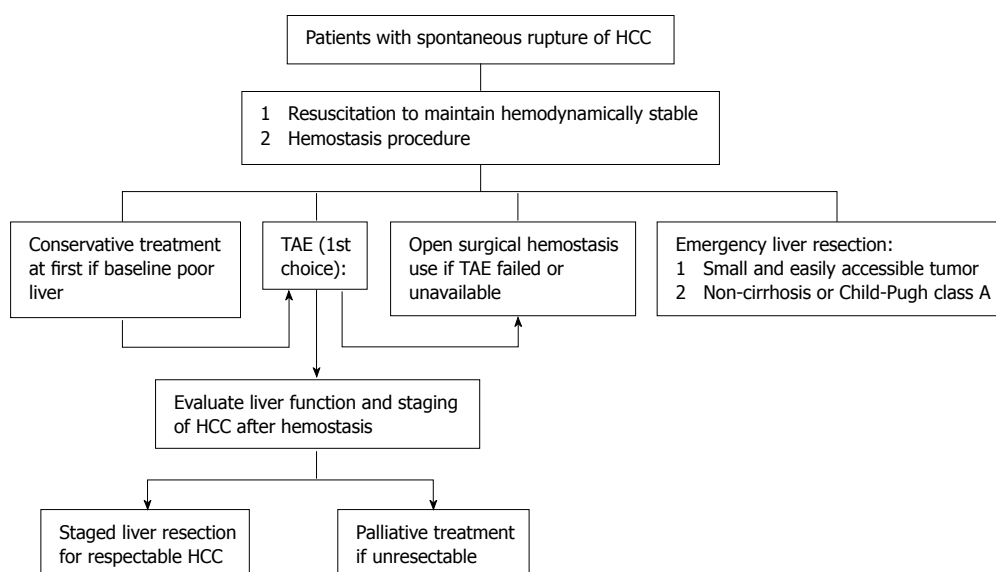
complex and deposition on vascular wall. Then vascular wall could become stiff and weak due to the proliferated fragment elastin and damaged collagen, which would make blood vessels more prone to splitting and result in hemorrhage and rupture of HCC<sup>[11,12]</sup>. Large tumor size, peripheral location and protruding contour are all associated with an increased risk for rupture of HCC<sup>[4,13,14]</sup>.

The diagnosis of HCC rupture is based on blood-stained ascites plus imaging studies and symptoms<sup>[2]</sup>. The most common symptom is sudden onset of abdominal pain (66%-100%)<sup>[6,15-17]</sup>. Yeh *et al* also found that the presence of sudden-onset abdominal pain is the only independent indicator of ruptured HCC<sup>[18]</sup>. Abdominal ultrasonography and computed tomography improve the rate of preoperative diagnosis<sup>[13,14,19,20]</sup>. Peripheral location, protruding contour, discontinuity of hepatic surface, surrounding hematoma and elevated ascitic CT number are helpful signs for the diagnosis of ruptured HCC. In addition, enucleation sign on helical CT could





**Figure 3** Two small mass lesions (3 cm × 2 cm and 2 cm × 1 cm) over S5 and S6, respectively.



**Figure 4** Logarithm about how to approach to the patient with spontaneously ruptured HCC.

be more specific<sup>[14,20,21]</sup>.

Treatment of ruptured HCC is primarily aimed at controlling hemorrhage and preserving the functional liver parenchyma as possible. Open surgical method was the mainstay of treatment during 1960s-1980s. It was reported that various surgical procedures, including perihepatic packing, suture plication of bleeding tumors, injection of alcohol, hepatic artery ligation (HAL), and liver resection, are effective against hemostasis<sup>[10,22-26]</sup>. Besides transarterial embolization (TAE) and transarterial chemoembolization (TACE) for palliative treatment in patients with unresectable HCC, TAE is also gradually used for hemostasis in spontaneous rupture of HCC. In addition, Ng *et al*<sup>[27]</sup> also reported that ruptured HCC could be treated with radiofrequency ablation as a salvage procedure. To our knowledge, no prospective randomized controlled trials have found the best method for hemostasis. There is evidence that TACE is the preferred method to arrest tumor bleeding<sup>[13,28-34]</sup>. Besides, two-stage hepatectomy is advisable because it can prolong the survival of selected patients<sup>[35-40]</sup>. Figure 4 is the logarithm about how to approach the patients with spontaneous ruptured HCC<sup>[35-40]</sup>.

Spontaneous rupture of HCC with intraperitoneal hemorrhage is a life-threatening complication with a high mortality rate. Prognosis is associated with poor liver reserve, advanced disease and severity of hemor-

rhage<sup>[41]</sup>. The median survival time is around 4-5 mo after HCC rupture, and only few patients can have a long-term survival<sup>[42,43]</sup>. Thus, implanted metastases usually do not become clinically apparent. Ong *et al*<sup>[44]</sup> reported the first case of peritoneal metastasis after HCC rupture in 1996 and then only sporadic case reports have been published. Most reported cases of peritoneal metastases are documented 8 mo after rupture<sup>[7,41,45-47]</sup>, but Ryu *et al*<sup>[48]</sup> and Lin *et al*<sup>[49]</sup> reported that peritoneal metastasis can be found 4 and 3 mo respectively after rupture episode. A single metastatic tumor is the most common presentation. Resection is the treatment of choice for peritoneal metastasis if possible and might offer long-term survival benefits<sup>[7,44-48]</sup>.

Peritoneal metastasis after spontaneous rupture of HCC is rare. This is our first experience with such a patient. Our patient developed peritoneal metastasis, which was documented 10 mo after spontaneous HCC rupture. The time from rupture to documentation of peritoneal metastasis is similar to other case reports. Because few cases of peritoneal metastasis after ruptured HCC have been reported, the association between metastatic tumor and viral infection (HBV or HCV), AFP level, or age is lacking. Lin *et al*<sup>[49]</sup> found that most reported cases are males, but the impact of gender is still unclear. The disease-free time is around 7-45 mo according to previous case reports<sup>[40-42,44]</sup>. Our patient had no peritoneal

recurrence until December, 2007 and is now regularly followed up at OPD.

## REFERENCES

- Katyal S, Oliver JH 3rd, Peterson MS, Ferris JV, Carr BS, Baron RL. Extrahepatic metastases of hepatocellular carcinoma. *Radiology* 2000; **216**: 698-703
- Nakashima T, Okuda K, Kojiro M, Jimi A, Yamaguchi R, Sakamoto K, Ikari T. Pathology of hepatocellular carcinoma in Japan. 232 Consecutive cases autopsied in ten years. *Cancer* 1983; **51**: 863-877
- Ong GB, Taw JL. Spontaneous rupture of hepatocellular carcinoma. *Br Med J* 1972; **4**: 146-149
- Chen CY, Lin XZ, Shin JS, Lin CY, Leow TC, Chen CY, Chang TT. Spontaneous rupture of hepatocellular carcinoma. A review of 141 Taiwanese cases and comparison with nonrupture cases. *J Clin Gastroenterol* 1995; **21**: 238-242
- Goel AK, Sinha S, Kumar A, Chattopadhyay TK. Spontaneous hemoperitoneum due to rupture of hepatocellular carcinoma. *Trop Gastroenterol* 1993; **14**: 152-155
- Miyamoto M, Sudo T, Kuyama T. Spontaneous rupture of hepatocellular carcinoma: a review of 172 Japanese cases. *Am J Gastroenterol* 1991; **86**: 67-71
- Sonoda T, Kanematsu T, Takenaka K, Sugimachi K. Ruptured hepatocellular carcinoma evokes risk of implanted metastases. *J Surg Oncol* 1989; **41**: 183-186
- Zhu LX, Wang GS, Fan ST. Spontaneous rupture of hepatocellular carcinoma. *Br J Surg* 1996; **83**: 602-607
- Tanaka T, Yamanaka N, Oriyama T, Furukawa K, Okamoto E. Factors regulating tumor pressure in hepatocellular carcinoma and implications for tumor spread. *Hepatology* 1997; **26**: 283-287
- Chearanai O, Plengvanit U, Asavanich C, Damrongsak D, Sindhvananda K, Boonyapisit S. Spontaneous rupture of primary hepatoma: report of 63 cases with particular reference to the pathogenesis and rationale treatment by hepatic artery ligation. *Cancer* 1983; **51**: 1532-1536
- Zhu LX, Geng XP, Fan SD. [Mechanism of spontaneous rupture of hepatocellular carcinoma.] *Zhonghua Waike Zazhi* 2004; **42**: 1036-1039
- Zhu LX, Geng XP, Fan SD. [Ultrastructure study on patients with spontaneous rupture of hepatocellular carcinoma] *Zhonghua Waike Zazhi* 2006; **44**: 161-164
- Kanematsu M, Imaeda T, Yamawaki Y, Seki M, Goto H, Sone Y, Iinuma G, Mochizuki R, Doi H. Rupture of hepatocellular carcinoma: predictive value of CT findings. *AJR Am J Roentgenol* 1992; **158**: 1247-1250
- Choi BG, Park SH, Byun JY, Jung SE, Choi KH, Han JY. The findings of ruptured hepatocellular carcinoma on helical CT. *Br J Radiol* 2001; **74**: 142-146
- Chen TZ, Wu JC, Chan CY, Sheng WY, Yen FS, Chiang JH, Chau GY, Lui WY, Lee SD. Ruptured hepatocellular carcinoma: treatment strategy and prognostic factor analysis. *Zhonghua Yixue Zazhi (Taipei)* 1996; **57**: 322-328
- Xu HS, Yan JB. Conservative management of spontaneous ruptured hepatocellular carcinoma. *Am Surg* 1994; **60**: 629-633
- Leung KL, Lau WY, Lai PB, Yiu RY, Meng WC, Leow CK. Spontaneous rupture of hepatocellular carcinoma: conservative management and selective intervention. *Arch Surg* 1999; **134**: 1103-1107
- Yeh CN, Lee WC, Jeng LB, Chen MF, Yu MC. Spontaneous tumour rupture and prognosis in patients with hepatocellular carcinoma. *Br J Surg* 2002; **89**: 1125-1129
- Corr P, Chan M, Lau WY, Metreweli C. The role of hepatic arterial embolization in the management of ruptured hepatocellular carcinoma. *Clin Radiol* 1993; **48**: 163-165
- Pombo F, Arrojo L, Perez-Fontan J. Haemoperitoneum secondary to spontaneous rupture of hepatocellular carcinoma: CT diagnosis. *Clin Radiol* 1991; **43**: 321-322
- Ishihara M, Kobayashi H, Ichikawa T, Cho K, Gemma K, Kumazaki T. The value of emergency CT studies in spontaneous rupture of hepatocellular carcinoma. Analysis for tumor protrusion and hemorrhagic ascites. *Nippon Ika Daigaku Zasshi* 1997; **64**: 532-537
- Lai EC, Wu KM, Choi TK, Fan ST, Wong J. Spontaneous ruptured hepatocellular carcinoma. An appraisal of surgical treatment. *Ann Surg* 1989; **210**: 24-28
- Chen MF, Hwang TL, Jeng LB, Jan YY, Wang CS. Clinical experience with hepatic resection for ruptured hepatocellular carcinoma. *Hepatogastroenterology* 1995; **42**: 166-168
- Descottes B, Lachachi F, Valleix D, Durand-Fontanier S, Sodji M, Pech de Laclause B, Maisonnnette F. [Ruptured hepatocarcinoma. Report of 22 cases] *Chirurgie* 1999; **124**: 618-625
- Chiappa A, Zbar A, Audisio RA, Paties C, Bertani E, Staudacher C. Emergency liver resection for ruptured hepatocellular carcinoma complicating cirrhosis. *Hepatogastroenterology* 1999; **46**: 1145-1150
- Vergara V, Muratore A, Bouzari H, Polastri R, Ferrero A, Galatola G, Capussotti L. Spontaneous rupture of hepatocellular carcinoma: surgical resection and long-term survival. *Eur J Surg Oncol* 2000; **26**: 770-772
- Ng KK, Lam CM, Poon RT, Law WL, Seto CL, Fan ST. Radiofrequency ablation as a salvage procedure for ruptured hepatocellular carcinoma. *Hepatogastroenterology* 2003; **50**: 1641-1643
- Sato Y, Fujiwara K, Furui S, Ogata I, Oka Y, Hayashi S, Ohta Y, Iio M, Oka H. Benefit of transcatheter arterial embolization for ruptured hepatocellular carcinoma complicating liver cirrhosis. *Gastroenterology* 1985; **89**: 157-159
- Le Neel JC, De Cervens T, Comy M, Dupas B, Letessier E, Mirallie E. [Ruptured hepatocarcinoma. Report of 20 cases and review of the literature] *Chirurgie* 1994; **120**: 380-384
- Ngan H, Tso WK, Lai CL, Fan ST. The role of hepatic arterial embolization in the treatment of spontaneous rupture of hepatocellular carcinoma. *Clin Radiol* 1998; **53**: 338-341
- Yang Y, Cheng H, Xu A, Chen D, Wang Y, Yao X, Chen H, Wu M. [Transarterial embolization for hemorrhage due to spontaneous rupture in hepatocellular carcinoma] *Zhonghua Zhongliu Zazhi* 2002; **24**: 285-287
- Fujii M, Miyake H, Takamura K, Tashiro S. [Management of spontaneous ruptured hepatocellular carcinoma] *Nippon Geka Gakkai Zasshi* 2004; **105**: 292-295
- Buczkowski AK, Kim PT, Ho SG, Schaeffer DF, Lee SI, Owen DA, Weiss AH, Chung SW, Scudamore CH. Multidisciplinary management of ruptured hepatocellular carcinoma. *J Gastrointest Surg* 2006; **10**: 379-386
- Ribeiro MA Jr, Fonseca AZ, Chaib E, D'Ippolito G, Carnevale FC, Rodrigues JJ, Saad WA. An unusual approach to the spontaneous rupture of hepatocellular carcinoma. *Hepatogastroenterology* 2007; **54**: 1235-1238
- Hirai K, Kawazoe Y, Yamashita K, Kumagai M, Nagata K, Kawaguchi S, Abe M, Tanikawa K. Transcatheter arterial embolization for spontaneous rupture of hepatocellular carcinoma. *Am J Gastroenterol* 1986; **81**: 275-279
- Chen MF, Jan YY, Lee TY. Transcatheter hepatic arterial embolization followed by hepatic resection for the spontaneous rupture of hepatocellular carcinoma. *Cancer* 1986; **58**: 332-335
- Shuto T, Hirohashi K, Kubo S, Tanaka H, Hamba H, Kubota D, Kinoshita H. Delayed hepatic resection for ruptured hepatocellular carcinoma. *Surgery* 1998; **124**: 33-37
- Yoshida H, Onda M, Tajiri T, Umehara M, Mamada Y, Matsumoto S, Yamamoto K, Kaneko M, Kumazaki T. Treatment of spontaneous ruptured hepatocellular carcinoma. *Hepatogastroenterology* 1999; **46**: 2451-2453
- Liu CL, Fan ST, Lo CM, Tso WK, Poon RT, Lam CM, Wong J. Management of spontaneous rupture of hepatocellular

- carcinoma: single-center experience. *J Clin Oncol* 2001; **19**: 3725-3732
- 40 **Mizuno S**, Yamagiwa K, Ogawa T, Tabata M, Yokoi H, Isaji S, Uemoto S. Are the results of surgical treatment of hepatocellular carcinoma poor if the tumor has spontaneously ruptured? *Scand J Gastroenterol* 2004; **39**: 567-570
- 41 **Yunoki Y**, Takeuchi H, Makino Y, Murakami I, Yasui Y, Tanakaya K, Kawaguchi K, Konaga E. Intraperitoneal seeding of ruptured hepatocellular carcinoma: case report. *Abdom Imaging* 1999; **24**: 398-400
- 42 **Tan FL**, Tan YM, Chung AY, Cheow PC, Chow PK, Ooi LL. Factors affecting early mortality in spontaneous rupture of hepatocellular carcinoma. *ANZ J Surg* 2006; **76**: 448-452
- 43 **Al-Mashat FM**, Sibiany AM, Kashgari RH, Maimani AA, Al-Radi AO, Balawy IA, Ahmad JE. Spontaneous rupture of hepatocellular carcinoma. *Saudi Med J* 2002; **23**: 866-870
- 44 **Ong GB**, Chu EP, Yu FY, Lee TC. Spontaneous rupture of hepatocellular carcinoma. *Br J Surg* 1965; **52**: 123-129
- 45 **Shirabe K**, Kitamura M, Tsutsui S, Maeda T, Matsumata T, Sugimachi K. A long-term survivor of ruptured hepatocellular carcinoma after hepatic resection. *J Gastroenterol Hepatol* 1995; **10**: 351-354
- 46 **Kosaka A**, Hayakawa H, Kusagawa M, Takahashi H, Okamura K, Mizumoto R, Katsuta K. Successful surgical treatment for implanted intraperitoneal metastases of ruptured small hepatocellular carcinoma: report of a case. *Surg Today* 1999; **29**: 453-457
- 47 **Kaido T**, Arai S, Shiota M, Imamura M. Repeated resection for extrahepatic recurrences after hepatectomy for ruptured hepatocellular carcinoma. *J Hepatobiliary Pancreat Surg* 2004; **11**: 149-152
- 48 **Ryu JK**, Lee SB, Kim KH, Yoh KT. Surgical treatment in a patient with multiple implanted intraperitoneal metastases after resection of ruptured large hepatocellular carcinoma. *Hepatogastroenterology* 2004; **51**: 239-242
- 49 **Lin CC**, Chen CH, Tsang YM, Jan IS, Sheu JC. Diffuse intraperitoneal metastasis after spontaneous rupture of hepatocellular carcinoma. *J Formos Med Assoc* 2006; **105**: 577-582

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### Dr. Philip Abraham, Professor

Consultant Gastroenterologist & Hepatologist, P. D. Hinduja National Hospital & Medical Research Centre, Veer Savarkar Marg, Mahim, Mumbai 400 016, India

### David Adams, Professor

Liver Research Laboratories, Institute for Biomedical Research, Queen Elizabeth Hospital, University of Birmingham, Birmingham B15 2TT, United Kingdom

### Rakesh Aggarwal Additional, Professor

Department of Gastroenterology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow 226014, India

### Rosemar Joyce Burnett, PhD

Department of Epidemiology National School of Public Health, University of Limpopo, Medunsa Campus PO Box 173, MEDUNSA, Pretoria 0204, South Africa

### Dr. Yogesh K Chawla, Professor

Department of Hepatology, Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India

### Ramsey Chi-man Cheung, MD, Professor

Division of GI & Hepatology, VAPAHCS(154C), 3801 Miranda Ave, Stanford University School of Medicine, Palo Alto, CA 94304, United States

### Dario Conte, Professor

GI Unit-IRCCS Osp. Maggiore, Chair of Gastroenterology, Via F. Sforza, 35, Milano 20122, Italy

### Tsianos Epameinondas, MD, PhD, Professor

1st Division Of Internal Medicine & Hepato-Gastroenterology Unit, Medical school University of Ioannina, PO Box 1186 Ioannina 45110, Greece

### Ikolaus Gassler, Professor

Institute of Pathology, University Hospital RWTH Aachen, Pauwelsstrasse 30, 52074 Aachen, Germany

### Kazuhiro Hanazaki, MD, Professor and Chairman

Department of Surgery, Kochi Medical School, Kochi University, Kohasu, Okochi, Nankoku, Kochi 783-8505, Japan

### Frank Hoentjen, MD, PhD

Department of Gastroenterology, VU Medical Center, Sumatrastraat 16, 2022XL Haarlem, The Netherlands

### Toru Ishikawa, MD

Department of Gastroenterology, Saiseikai Niigata Second Hospital, Teraji 280-7, Niigata, Niigata 950-1104, Japan

### Tsuneo Kitamura, sociate Professor

Department of Gastroenterology, Juntendo University Urayasu Hospital, Juntendo University School of Medicine, 2-1-1 Tomioka, Urayasu-shi, Chiba 279-0021, Japan

### Robert J Korst, MD

Department of Cardiothoracic Surgery, Weill Medical College of Cornell University, Room M404, 525 East 68th Street, New York 10032, United States

### Shiu-Ming Kuo, MD

University at Buffalo, 15 Farber Hall, 3435 Main Street, Buffalo 14214, United States

### Peter L Lakatos, MD, PhD, Assistant Professor

1st Department of Medicine, Semmelweis University, Koranyi S 2A, Budapest H1083, Hungary

### Dr. Yun Ma, MD, PhD

Institute of Liver Studies, King's College Hospital, Denmark Hill, London SE5 9RS, United Kingdom

### Kevin McGrath, MD

Division of Gastroenterology, Hepatology and Nutrition, University of Pittsburgh Medical Center, M2, C wing, PUH, 200 Lothrop St, Pittsburgh, PA 15213, United States

### Ali Mencin, MD, Assistant Professor of Pediatrics

Division of Pediatric Gastroenterology, Morgan Stanley Children's Hospital of New York, CHN-702, 3959 Broadway, New York, NY 10032, United States

### Fanyin Meng, MD, PhD, Assistant Professor

Department of Internal Medicine, Ohio State University, Room 514A Medical Research Facility, 420 West 12th Avenue, Columbus, Ohio 43210, United States

### Sri P Misra, Professor

Gastroenterology, Moti Lal Nehru Medical College, Allahabad 211001, India

### Peter L Moses, MD, FACP, AGAF, Professor

University of Vermont College of Medicine Section of Gastroenterology & Hepatology, 111 Colchester Avenue, Smith 237B, MCHV, Burlington, VT 05401, United States

### Yoshiharu Motoo, MD, PhD, FACP, FACC, Professor and Chairman

Department of Medical Oncology, Kanazawa Medical University, 1-1 Daigaku, Uchinada, Ishikawa 920-0293, Japan

### Atsushi Nakajima, Professor

Division of Gastroenterology, Yokohama City University Graduate School of Medicine, 3-9 Fuku-ura, Kanazawa-ku, Yokohama 236-0004, Japan

### Hiroki Nakamura, MD

Department of Gastroenterology and Hepatology, 1-1-1, Minami Kogushi, Ube, Yamaguchi 755-8505, Japan

### Shotaro Nakamura, MD

Department of Medicine and Clinical Science, Kyushu University, Maidashi 3-1-1, Higashi-ku, Fukuoka 812-8582, Japan

### James Neuberger, Professor

Liver Unit, Queen Elizabeth Hospital, Birmingham B15 2TH, United Kingdom

### Philip Noel Newsome, MBChB, MRCP, PhD

Liver Research Group, 5th Floor, Institute of Biomedical Research, Wolfson Drive, The Medical School, Edgbaston, University of Birmingham, Birmingham B15 2TT, United Kingdom

### Gustav Paumgartner, Professor

University of Munich, Klinikum Grosshadern, Marchioninstr. 15, Munich, D-81377, Germany

### Dr. Bernardino Rampone

Department of General Surgery and Surgical Oncology, University of Siena, viale Bracci, Siena 53100, Italy

### Gerardo Rosati, MD

Medical Oncology Unit, "S. Carlo" Hospita, Via Potito Petrone, 1, Potenza 85100, Italy

### Damian Casadesus Rodriguez, MD, PhD

Calixto Garcia University Hospital, J and University, Vedado, Havana City, Cuba

### Mitsuo Shimada, Professor

Department of Digestive and Pediatric Surgery, Tokushima University, Kuramoto 3-18-15, Tokushima 770-8503, Japan





## Meetings

### Events Calendar 2008-2009

**FALK SYMPOSIA 2008**  
 January 24-25, Frankfurt, Germany  
 Falk Workshop: Perspectives in Liver Transplantation

International Gastroenterological Congresses 2008  
 February 14-16, Paris, France  
 EASL-AASLD-APASL-ALEH-IASL Conference Hepatitis B and C virus resistance to antiviral therapies  
[www.easl.ch/hepatitis-conference](http://www.easl.ch/hepatitis-conference)

February 14-17, Berlin, Germany  
 8<sup>th</sup> International Conference on New Trends in Immunosuppression and Immunotherapy  
[www.kenes.com/immuno](http://www.kenes.com/immuno)

February 28, Lyon, France  
 3<sup>rd</sup> Congress of ECCO - the European Crohn's and Colitis Organisation  
 Inflammatory Bowel Diseases 2008  
[www.ecco-ibd.eu](http://www.ecco-ibd.eu)

February 29, Québec, Canada  
 Canadian Association of Gastroenterology  
 E-mail: [general@cag-acg.org](mailto:general@cag-acg.org)

March 10-13, Birmingham, UK  
 British Society of Gastroenterology Annual Meeting  
 E-mail: [BSG@mailbox.ulcc.ac.uk](mailto:BSG@mailbox.ulcc.ac.uk)

March 14-15, HangZhou, China  
 Falk Symposium 163: Chronic Inflammation of Liver and Gut

March 23-26, Seoul, Korea  
 Asian Pacific Association for the Study of the Liver  
 18<sup>th</sup> Conference of APASL: New Horizons in Hepatology  
[www.apaslseoul2008.org](http://www.apaslseoul2008.org)

March 29-April 1, Shanghai, China  
 Shanghai-Hong Kong International Liver Congress  
[www.livercongress.org](http://www.livercongress.org)

April 05-09, Monte-Carlo (Grimaldi Forum), Monaco  
 OESO 9<sup>th</sup> World Congress, The Gastro-esophageal Reflux Disease: from Reflux to Mucosal Inflammation-Management of Adeno-carcinomas  
 E-mail: [robert.giuli@oeso.org](mailto:robert.giuli@oeso.org)

April 9-12, Los Angeles, USA  
 SAGES 2008 Annual Meeting - part of Surgical Spring Week  
[www.sages.org/08program/html/](http://www.sages.org/08program/html/)

April 18-22, Buenos Aires, Argentina  
 9<sup>th</sup> World Congress of the International Hepato-Pancreato Biliary Association  
 Association for the Study of the Liver  
[www.ca-ihpba.com.ar](http://www.ca-ihpba.com.ar)

April 23-27, Milan, Italy  
 43<sup>rd</sup> Annual Meeting of the European Association for the Study of the Liver  
[www.easl.ch](http://www.easl.ch)

May 2-3, Budapest, Hungary

Falk Symposium 164: Intestinal Disorders

May 18-21, San Diego, California, USA  
 Digestive Disease Week 2008

May 21-22, California, USA  
 ASGE Annual Postgraduate Course  
 Endoscopic Practice 2008: At the Interface of Evidence and Expert Opinion  
 E-mail: [education@asge.org](mailto:education@asge.org)

June 4-7, Helsinki, Finland  
 The 39<sup>th</sup> Nordic Meeting of Gastroenterology  
[www.congex.com/ngc2008](http://www.congex.com/ngc2008)

June 5-8, Sitges (Barcelona), Spain  
 Semana de las Enfermedades Digestivas  
 E-mail: [sepd@sepd.es](mailto:sepd@sepd.es)

June 6-8, Prague, Czech Republic  
 3<sup>rd</sup> Annual European Meeting: Perspectives in Inflammatory Bowel Diseases  
 E-mail: [meetings@imedex.com](mailto:meetings@imedex.com)

June 10-13, Istanbul, Turkey  
 ESGAR 2008 19<sup>th</sup> Annual Meeting and Postgraduate Course  
 E-mail: [fca@netvisao.pt](mailto:fca@netvisao.pt)

June 11-13, Stockholm, Sweden  
 16<sup>th</sup> International Congress of the European Association for Endoscopic Surgery  
 E-mail: [info@aes-eur.org](mailto:info@aes-eur.org)

June 13-14, Amsterdam, Netherlands  
 Falk Symposium 165: XX International Bile Acid Meeting. Bile Acid Biology and Therapeutic Actions

June 13-14, Prague, Czech Republic  
 Central and Eastern European Conference on Colorectal "Cancer" Screening, Prevention and Management  
 E-mail: [idla2008@guarant.cz](mailto:idla2008@guarant.cz)

June 25-28, Barcelona, Spain  
 10<sup>th</sup> World Congress on Gastrointestinal Cancer  
 Imedex and ESMO  
 E-mail: [meetings@imedex.com](mailto:meetings@imedex.com)

June 25-28, Lodz, Poland  
 Joint Meeting of the European Pancreatic Club (EPC) and the International Association of Pancreatologists (IAP)  
 E-mail: [office@epc-iap2008.org](mailto:office@epc-iap2008.org)  
[www.e-p-c.org](http://www.e-p-c.org)  
[www.pancreatology.org](http://www.pancreatology.org)

June 26-28, Bratislava, Slovakia  
 5<sup>th</sup> Central European Gastroenterology Meeting  
[www.ceurgem2008.cz](http://www.ceurgem2008.cz)

July 9-12, Paris, France  
 ILTS 14<sup>th</sup> Annual International Congress  
[www.its.org](http://www.its.org)

September 10-13, Budapest, Hungary  
 11<sup>th</sup> World Congress of the International Society for Diseases of the Esophagus  
 E-mail: [isde@isde.net](mailto:isde@isde.net)

September 13-16, New Delhi, India  
 Asia Pacific Digestive Week  
 E-mail: [apdw@apdw2008.net](mailto:apdw@apdw2008.net)

III FALK GASTRO-CONFERENCE  
 September 17, Mainz, Germany

Falk Workshop: Strategies of Cancer Prevention in Gastroenterology

September 18-19, Mainz, Germany  
 Falk Symposium 166:  
 GI Endoscopy - Standards & Innovations

September 18-20, Prague, Czech Republic  
 Prague Hepatology Meeting 2008  
[www.czech-hepatology.cz/phm2008](http://www.czech-hepatology.cz/phm2008)

September 20-21, Mainz, Germany  
 Falk Symposium 167:  
 Liver Under Constant Attack - From Fat to Viruses

September 24-27, Nantes, France  
 Third Annual Meeting  
 European Society of Coloproctology  
[www.escp.eu.com](http://www.escp.eu.com)



October 8-11, Istanbul, Turkey  
 18<sup>th</sup> World Congress of the International Association of Surgeons, Gastroenterologists and Oncologists  
 E-mail: [orkun.sahin@serenas.com.tr](mailto:orkun.sahin@serenas.com.tr)

October 18-22, Vienna, Austria  
 16<sup>th</sup> United European Gastroenterology Week  
[www.negf.org](http://www.negf.org)  
[www.acv.at](http://www.acv.at)

October 22-25, Minnesota, USA  
 Anstralian Gastroenterology Week 2008  
 E-mail: [gesa@gesa.org.au](mailto:gesa@gesa.org.au)

October 22-25, Brisbane, Australia  
 71<sup>st</sup> Annual Colon and Rectal Surgery Conference  
 E-mail: [info@colonrectalcourse.org](mailto:info@colonrectalcourse.org)

October 31-November 4, Moscone West Convention Center, San Francisco, CA  
 59<sup>th</sup> AASLD Annual Meeting and Postgraduate Course  
 The Liver Meeting  
 Information: [www.aasld.org](http://www.aasld.org)

November 6-9, Lucerne, Switzerland  
 Neurogastroenterology & Motility Joint International Meeting 2008  
 E-mail: [ngm2008@mci-group.com](mailto:ngm2008@mci-group.com)  
[www.ngm2008.com](http://www.ngm2008.com)

November 12, Santiago de Chile, Chile  
 Falk Workshop: Digestive Diseases: State of the Art and Daily Practice

November 28-29, Cairo, Egypt  
 1<sup>st</sup> Hepatology and Gastroenterology Post Graduate Course  
[www.egyptgastrohep.com](http://www.egyptgastrohep.com)

December 7-9, Seoul, Korea  
 6<sup>th</sup> International Meeting  
 Hepatocellular Carcinoma: Eastern and Western Experiences  
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[www.falkfoundation.de](http://www.falkfoundation.de)

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N.O.T.E.S  
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 Laparoscopic Digestive Surgery

June 27-28, November 7-8  
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 E-mail: [bsg@mailbox.ulcc.ac.uk](mailto:bsg@mailbox.ulcc.ac.uk)

May 17-20, Denver, Colorado, USA  
 Digestive Disease Week 2009

November 21-25, London, UK  
 Gastro 2009 UEGW/World Congress of Gastroenterology  
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### Global Collaboration for Gastroenterology

For the first time in the history of gastroenterology, an international conference will take place which joins together the forces of four pre-eminent organisations: Gastro 2009, UEGW/WCOG London. The United European Gastroenterology Federation (UEGF) and the World Gastroenterology Organisation (WGO), together with the World Organisation of Digestive Endoscopy (OMED) and the British Society of Gastroenterology (BSG), are jointly organising a landmark meeting in London from November 21-25, 2009. This collaboration will ensure the perfect balance of basic science and clinical practice, will cover all disciplines in gastroenterology (endoscopy, digestive oncology, nutrition, digestive surgery, hepatology, gastroenterology) and ensure a truly global context; all presented in the exciting setting of the city of London. Attendance is expected to reach record heights as participants are provided with a compact "all-in-one" programme merging the best of several GI meetings. Faculty and participants from all corners of the earth will merge to provide a truly global environment conducive to the exchange of ideas and the forming of friendships and collaborations.



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*In press*

- 3 **Tian D**, Araki H, Stahl E, Bergelson J, Kreitman M. Signature of balancing selection in Arabidopsis. *Proc Natl Acad Sci USA* 2006; In press

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- 4 **Diabetes Prevention Program Research Group**. Hypertension, insulin, and proinsulin in participants with impaired glucose tolerance. *Hypertension* 2002; 40: 679-686 [PMID: 12411462]

*Both personal authors and an organization as author*

- 5 **Vallancien G**, Emberton M, Harving N, van Moorselaar RJ; Alf-One Study Group. Sexual dysfunction in 1, 274 European men suffering from lower urinary tract symptoms. *J Urol* 2003; 169: 2257-2261 [PMID: 12771764]

*No author given*

- 6 21st century heart solution may have a sting in the tail. *BMJ* 2002; 325: 184 [PMID: 12142303]

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- 7 **Geraud G**, Spierings EL, Keywood C. Tolerability and safety of frovatriptan with short- and long-term use for treatment



of migraine and in comparison with sumatriptan. *Headache* 2002; 42 Suppl 2: S93-99 [PMID: 12028325]

*Issue with no volume*

- 8 **Banit DM**, Kaufer H, Hartford JM. Intraoperative frozen section analysis in revision total joint arthroplasty. *Clin Orthop Relat Res* 2002; (401): 230-238 [PMID: 12151900]

*No volume or issue*

- 9 Outreach: Bringing HIV-positive individuals into care. *HRS/A Careaction* 2002; 1-6 [PMID: 12154804]

## Books

*Personal author(s)*

- 10 **Sherlock S**, Dooley J. Diseases of the liver and biliary system. 9th ed. Oxford: Blackwell Sci Pub, 1993: 258-296

*Chapter in a book (list all authors)*

- 11 **Lam SK**. Academic investigator's perspectives of medical treatment for peptic ulcer. In: Swabb EA, Azabo S. Ulcer disease: investigation and basis for therapy. New York: Marcel Dekker, 1991: 431-450

*Author(s) and editor(s)*

- 12 **Breedlove GK**, Schorffheide AM. Adolescent pregnancy. 2nd ed. Wiczorek RR, editor. White Plains (NY): March of Dimes Education Services, 2001: 20-34

*Conference proceedings*

- 13 **Harnden P**, Joffe JK, Jones WG, editors. Germ cell tumours V. Proceedings of the 5th Germ cell tumours Conference; 2001 Sep 13-15; Leeds, UK. New York: Springer, 2002: 30-56

*Conference paper*

- 14 **Christensen S**, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG, editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer, 2002: 182-191

**Electronic journal** (list all authors)

- 15 Morse SS. Factors in the emergence of infectious diseases. *Emerg Infect Dis* serial online, 1995-01-03, cited 1996-06-05; 1(1): 24 screens. Available from: URL: <http://www.cdc.gov/ncidod/EID/eid.htm>

**Patent** (list all authors)

- 16 **Pagedas AC**, inventor; Ancel Surgical R&D Inc., assignee. Flexible endoscopic grasping and cutting device and positioning tool assembly. United States patent US 20020103498. 2002 Aug 1

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